Inhibition of Bacterial, Fungal, and Plant Growth by Testae Extracts of Citrullus Genotypes

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Abstract. Watermelon [Citrullus lanatus var. lanatus (Thunb.) Matsum & Nakai] seed and root exudates inhibit germination and seedling growth of plants and growth of pathogenic fungi and bacteria. This study was conducted to determine if extractable components in the testa (seedcoat) contribute to the inhibition previously reported. Testae of eight genetically diverse Citrullus genotypes were extracted first with dichloromethane to remove less polar components and then with 70% methanol to remove more polar components. The dichloromethane extracts were not inhibitory in a Proso millet radicle growth bioassay; however, they were highly inhibitory to the growth of the fruit blotch bacterial pathogen Acidovorax avenae subsp. citrulli, nectarial fruit blotch, Phytophthora capsici, fruit rot

Materials and Methods

Testa tissue preparation. The genotypes included in this experiment were the watermelon cultivar Charleston Gray, U.S. Vegetable Laboratory experimental watermelon line, 406-1-x 7, a watermelon germplasm accession, PI 167125, three citron melon, C. lanatus var. citroides germplasm accessions, PI 482246, PI 500354, and PI 532738, and two accessions from the related species C. colocynthis. Seeds used in this experiment were obtained from ripe fruit produced on greenhouse-grown plants that were self-pollinated. Seeds were thoroughly rinsed and air-dried after harvest and stored at 5 °C. Seeds were bisected with scissors, and all tissues except the testa were removed with a dissecting needle. Testae were ground to pass through a 60-mesh screen using a Wiley mill and lyophilized. Dried and ground testae were stored under nitrogen at −25 °C until they were extracted.

Testa extraction. Ground testae were extracted first with dichloromethane to remove soluble nonpolar components and then with 70% methanol to extract the more polar soluble components. Extraction was accomplished by placing 3 g of the tissue in a round-bottomed flask with 45 mL solvent. The slurry was placed on a wrist action shaker at 50 rpm in the dark at room temperature for 24 h. Testae were extracted twice with both solvents and the two extractions with the same solvent were combined. Extracts were filtered through nylon 66 filters (0.8 μm), dried on a rotary evaporator, and stored at −25 °C under nitrogen until they were used in bioassay experiments.

Proso millet bioassay. The 70% methanol extracts were re-dissolved in 70% methanol

Received for publication 28 Sept. 2011. Accepted for publication 13 Jan. 2012.

We thank Lance Lawrence, Jennifer Ikeder, and Melanie McMillan for technical assistance.

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to obtain an extract concentration equivalent to 250 mg of testa extracted per mL. Aliquots of the extract plus additional 70% methanol to equal 0.5 mL total volume were pipetted onto filter paper disks in 35-mm plastic petri dishes. The solvent was allowed to evaporate at room temperature. Ten Proso millet seeds and 0.5 mL of distilled water were subsequently added to each dish, and the seeds were incubated in the dark at 24 °C. After 72 h, the petri dishes were placed in a freezer to stop growth. The dishes were removed from the freezer, millet radicle lengths were measured with an electronic caliper, and average radicle length was determined for each dish. For the dichloromethane extract, the extracts were re-dissolved in dichloromethane to obtain a concentration of 250 mg testa extracted per mL. The extracts plus additional dichloromethane to bring the total volume to 1 mL were pipetted onto filter paper in 50-mm glass petri dishes. The solvent was evaporated at room temperature and 1 mL of distilled water and 20 Proso millet seeds were added to each dish. The seeds were incubated, frozen, and radicle lengths measured as described previously. An experiment was conducted as a completely randomized design with five replications and was repeated. Data were analyzed using the PROC GLM procedure of SAS Version 9.1 (SAS Institute, Cary, NC). No treatment-by-experiment interactions were observed; thus, the combined data from two repetitions of the experiments were subjected to analysis of variance, and genotype means within extraction solvents were separated by Tukey’s honestly significant difference (hso) test ($P = 0.05$). A second experiment was conducted to assess the concentration response to the 70% methanol testa extracts. The methods of extraction are described previously. Test concentrations were equivalent to 0, 31, 63, 125, and 250 mg mL$^{-1}$. The experiment was arranged in a completely random design with five replications and was repeated. Data were analyzed using the PROC GLM procedure of SAS Version 9.1. No treatment-by-experiment interactions were observed; thus, data from the two repetitions of the experiment were combined for analysis. Genotype means within extract concentrations were separated using Tukey’s standardized range hso ($P = 0.05$). Nonlinear regression analyses (proc nlin; SAS System; SAS Institute) using the parallel dose–response curves procedure described by Seeffeld et al. (1995) were used to estimate the 70% methanol extract concentration required to cause a 50% reduction in radicle length (GR$_{50}$) for each genotype.

Acidovorax avenae bioassay. The bacterial fruit blotch pathogen, Aae, isolate 531 was obtained under USDA, APHIS permit from Dr. Ron Walcott, University of Georgia, Athens, GA. Isolate 531 was originally isolated from infected watermelon fruit from Georgia and was defined and used as a Group I isolate (Wechter et al., 2011). The bacterium was streaked onto Difco™ nutrient agar (NA) medium (Difco, Detroit, MI; Difco, Detroit, MI) and grown for 24 h at 27 °C. A single colony from the 24-h-old plates was then transferred to an NA plate and grown for an additional 24 h. A 10-μL loop of cells from this plate was inoculated into 100 mL of King’s B broth and place on a gyratory shaker at 150 RPM until midlog phase growth ($OD_{600}$ Sparks, MD) and grown for 24 h at 27 °C. The cells were pelleted by centrifugation at 10,000 RCF. Supernatant was discarded and cell pellet washed twice with sterile 0.01 M phosphate-buffered saline (sPBS), again pelleted by centrifugation, and then resuspended in sPBS to a concentration of $<1 \times 10^8$ colony-forming units (CFU)/mL as determined by optical density at a wavelength 600 nm.

Bacterial assays were performed in 96-well polystyrene microtiter plates. One milliliter aliquots of re-solubilized testa extracts were pipetted into polypropylene microcentrifuge tubes and evaporated to dryness as in a similar fashion. Once the tubes were completely dry of all traces of solvent, 100 μL of a zoospore suspension in sterile distilled water, prepared as described by Keinath and Kousik (2011) was pipetted into the 2-mL microcentrifuge tubes. The tubes were capped and vortexed vigorously until contents were completely suspended. Supernatant was added to each tube, and also enables the zoospores to encyst (Keinath, 2007; Keinath and Kousik, 2011). The tubes were placed in a rack and shuck mildly for 1 h at 26 °C. Then, the suspensions were plated on 1% water agar plates as described previously (Keinath, 2007; Keinath and Kousik, 2011). After 24 h, tubes were transferred back to an NA plate and grown for an additional 24 h. A 10-μL loop of cells was then transferred to another 90 min after which germinated and ungerminated zoospores were counted. Each testa extract was tested on four plates, which served as replications, and two germination counts per replication were recorded. The experiment was repeated once. Data were analyzed transformed and analyzed using the PROC GLM procedure of SAS Version 9.1 (SAS Institute). Significant treatment-by-experiment interactions were observed; thus, the data from each experiment were analyzed separately. Genotype means were separated using Tukey’s standardized range hso ($P = 0.05$).

Results and Discussion

Proso millet bioassay. The dichloromethane extracts contained fatty material that changes the absorptive properties of the filter paper in comparison with the control that was treated with dichloromethane only; however, they did not inhibit radicle growth at 100 mg mL$^{-1}$ (Table 1). Given the high inhibition of the methanol extract of some genotypes, we concluded that the soluble nonpolar components of watermelon testae do not contribute greatly to the inhibition of Proso millet radicle growth observed with seed exudates (Harrison et al., 2010). The 70% methanol extracts of four genotypes, one watermelon genotype (‘Charleston Gray’), one citron melon genotype (PI 532738), and both of the C. colocynthis genotypes inhibited radicle growth at 100 mg mL$^{-1}$ (Table 1). The concentration response experiment also indicated differences between Citrullus genotypes in inhibitory potential of 70% methanol extracts.
Table 1. Response of Proso millet radicle growth to dichloromethane and 70% methanol extracts of testa of eight Citrullus genotypes.

| Genotype     | Species and variety | Dichloromethane extract | 70% methanol extract |
|--------------|---------------------|-------------------------|----------------------|
|              |                     | Radicle length (mm)     | Radicle length (mm)  |
|              |                     | 31 mg mL⁻¹ | 63 mg mL⁻¹ | 125 mg mL⁻¹ | 250 mg mL⁻¹ | 31 mg mL⁻¹ | 63 mg mL⁻¹ | 125 mg mL⁻¹ | 250 mg mL⁻¹ |
| Charleston Gray | lanatus var. lanatus | 25.8 ab  | 24.6 ab  | 16.9 abc  | 7.9 b  | 171 ± 18 |
| 406-1-x 7     | lanatus var. lanatus | 21.9 b   | 21.6 bc  | 16.1 ab   | 10.0 b | 173 ± 18 |
| PI 167125     | lanatus var. lanatus | 24.4 b   | 24.8 ab  | 19.5 ab   | 9.0 b  | 196 ± 20 |
| PI 482246     | lanatus var. citroides | 25.8 ab  | 24.7 ab  | 17.0 abc  | 10.4 b | 191 ± 20 |
| PI 500354     | lanatus var. citroides | 30.7 a   | 29.1 a   | 20.0 ab   | 9.6 b  | 211 ± 21 |
| PI 532738     | lanatus var. citroides | 26.4 a   | 19.8 bc  | 10.0 cd   | 1.5 c  | 102 ± 11 |
| PI 432334     | colocynthis          | 24.7 ab  | 24.0 ab  | 12.9 bcd  | 5.5 bc | 134 ± 14 |
| PI 432337     | colocynthis          | 22.6 b   | 16.6 c   | 7.0 d    | 1.7 c  | 80 ± 9   |
| Control       |                     | 22.3 b   | 22.3 abc  | 22.3 a   | 22.3 a |

P value:a 0.0010 0.0001 <0.0001 <0.0001

aExtracts were tested at a concentration equivalent to 100 mg testa tissue extracted per mL.

bMeans within 70% methanol extracts followed by the same letter are not different by Tukey’s honestly significant difference (P = 0.05) test. Means within the dichloromethane extracts were not different by the test.

cSignificance level of the F test for genotype effect on Proso millet radicle growth.

Table 2. Concentration response of Proso millet radicle growth to 70% methanol extracts of testa from eight Citrullus genotypes.

| Genotype     | Species and variety | Radicle length (mm) | GR50 (mg kg⁻¹) |
|--------------|---------------------|---------------------|----------------|
| Charleston Gray | lanatus var. lanatus | 25.8 ab  | 171 ± 18 |
| 406-1-x 7     | lanatus var. lanatus | 21.9 b   | 173 ± 18 |
| PI 167125     | lanatus var. lanatus | 24.4 b   | 196 ± 20 |
| PI 482246     | lanatus var. citroides | 25.8 ab  | 191 ± 20 |
| PI 500354     | lanatus var. citroides | 30.7 a   | 211 ± 21 |
| PI 532738     | lanatus var. citroides | 26.4 a   | 102 ± 11 |
| PI 432334     | colocynthis          | 24.7 ab  | 134 ± 14 |
| PI 432337     | colocynthis          | 22.6 b   | 80 ± 9   |
| Control       |                     | 22.3 b   | —        |

P value:a 0.0041 0.0001 <0.0001 <0.0001

aConcentrations are expressed as weight of testa extracted per mL water.

bMeans within concentrations followed by the same letter are not different by Tukey’s honestly significant difference (P = 0.05).

cThe concentration of extract that caused a 50% reduction in shoot length (GR50) was estimated for each genotype using the log-logistics analysis described by Seefeldt et al. (1995). GR50 estimates ± the approximate SE of the estimate are presented.

dSignificance level of the F test for genotype effect on Proso millet radicle growth.
Table 3. Growth of Acidovorax avenae subsp. citrulli after incubation in King’s B broth amended with dichloromethane and 70% methanol extracts of testae of eight Citrullus genotypes.

| Accession | Citrullus species | Dichloromethane extract<sup>1</sup> | 70% methanol extract<sup>1</sup> |
|-----------|------------------|----------------------------------|-------------------------------|
|           |                  | Expt. 1     | Expt. 2     | Expt. 1     | Expt. 2     |
| 406-1x7   | lanatus var. lanatus | 205.8 a | 417.5 a | 70.5 de | 26.5 d |
| PI 167125 | lanatus var. lanatus | 1.0 d  | 0.3 b  | 98.9 de | 3.5 d  |
| PI 482246 | lanatus var. citroides | 31.8 d | 49.2 b | 350.7 b | 35.7 b |
| PI 500354 | lanatus var. citroides | 282.0 bc | 450.5 a | 290.5 bc | 185.2 c |
| PI 532738 | lanatus var. citroides | 0.8 d  | 0.7 b  | 0.0 e   | 0.0 d  |
| PI 423334 | colocynthis | 35.2 d | 35.8 b | 296.7 bc | 393.8 b |
| PI 432337 | colocynthis | 44.5 d | 7.3 b  | 396.7 ab | 444.5 ab |
| Charleston Grey | lanatus var. lanatus | 0.0 d  | 0.0 b  | 198.3 dc | 86.2 cd |
| Control<sup>2</sup> | lanatus var. lanatus | 432. a  | 440.0 a | 510.7 a | 562.2 a |
| Solvent control<sup>2</sup> | lanatus var. lanatus | 357.5 ab | 559.7 a | 530.0 a | 370.8 b |
| Negative control<sup>2</sup> | lanatus var. lanatus | 0.0 d  | 0.0 b  | 0.0 e   | 0.0 d  |

<sup>1</sup>Extrahs were tested at a concentration of 100 mg testa tissue extracted per mL.

<sup>2</sup>Means within columns followed by the same letter are not significantly different based on Tukey’s honestly significant difference (P = 0.05).

<sup>3</sup>King’s B broth plus bacterium.

<sup>4</sup>King’s B broth added to evaporated dichloromethane or 70% methanol tube then used in microtitre assay plus bacterium.

<sup>5</sup>King’s B with no added bacterium.

<sup>6</sup>Significance level of the F test for genotype effect on zoospore germination.

CFU = colony-forming unit.

Table 4. Effect of 70% methanol extracts<sup>4</sup> of testae of eight Citrullus genotypes on Phytophthora capsici zoospore germination.

| Genotype       | Citrullus species | Germination (%)<sup>3</sup> |
|----------------|------------------|----------------------------|
|                |                  | Expt. 1     | Expt. 2     |
| PI 482246      | lanatus var. citroides | 88.9 a | 78.8 b |
| PI 432334      | colocynthis | 88.9 a | 74.0 b |
| PI 432337      | colocynthis | 76.1 a | 77.0 b |
| PI 500354      | lanatus var. citroides | 12.2 b | 16.6 d |
| 406-1x7        | lanatus var. lanatus | 5.9 c  | 31.8 c |
| PI 167125      | lanatus var. lanatus | 2.3 bc | 4.6 e |
| PI 532738      | lanatus var. citroides | 0.5 c  | 8.1 e |
| Charleston Grey | lanatus var. lanatus | 0.1 c  | 28.0 c |
| Solvent control | lanatus var. lanatus | 72.8 a | 82.4 ab |
| Water control  | lanatus var. lanatus | 70.5 a  | 88.6 a |

P value<sup>5</sup>

<sup>1</sup>Extracts were tested at a concentration equivalent to 100 mg testa extracted per mL distilled water.

<sup>2</sup>Means followed by the same letter in column are not significantly different based on Tukey’s honestly significant difference (P = 0.05).

<sup>3</sup>Significance level of the F test for genotype effect on zoospore germination.

C. colocynthis genotypes (Boyhan et al., 1994; Levi et al., 2001; Simmons and Levi, 2002; Thies and Levi, 2007). Further research is warranted to identify biologically active compounds in testae and other tissues of watermelon and related species and investigates their role in pest resistance and allelopathy.

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