University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Publications from USDA-ARS / UNL Faculty

U.S. Department of Agriculture: Agricultural
Research Service, Lincoln, Nebraska

2012

Effects of piperidine and piperidine alkaloids from the venom of red imported fire ants, *Solenopsis invicta* Buren, on *Pythium ultimum* Trow growth *in vitro* and the application of piperidine alkaloids to control cucumber damping-off in the greenhouse

Shezeng Li

Hebei Academy of Agricultural and Forestry Sciences

Xixuan Jin

USDA-ARS-MSA, xixuan.jin@ars.usda.gov

Jian Chen

USDA-ARS-MSA, jian.chen@ars.usda.gov

Follow this and additional works at: <https://digitalcommons.unl.edu/usdaarsfacpub>

Li, Shezeng; Jin, Xixuan; and Chen, Jian, "Effects of piperidine and piperidine alkaloids from the venom of red imported fire ants, *Solenopsis invicta* Buren, on *Pythium ultimum* Trow growth *in vitro* and the application of piperidine alkaloids to control cucumber damping-off in the greenhouse" (2012).

Publications from USDA-ARS / UNL Faculty. 1109.

<https://digitalcommons.unl.edu/usdaarsfacpub/1109>

This Article is brought to you for free and open access by the U.S. Department of Agriculture: Agricultural Research Service, Lincoln, Nebraska at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Publications from USDA-ARS / UNL Faculty by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Effects of piperidine and piperidine alkaloids from the venom of red imported fire ants, *Solenopsis invicta* Buren, on *Pythium ultimum* Trow growth *in vitro* and the application of piperidine alkaloids to control cucumber damping-off in the greenhouse

Shezeng Li,^a Xixuan Jin^{b*} and Jian Chen^b

Abstract

BACKGROUND: *Pythium ultimum* is a plant pathogen that causes significant yield losses on many economically important crops. Chemical treatment has been used for disease control. In searching for alternatives, venom piperidine and piperidine alkaloids from red imported fire ants were tested against *P. ultimum in vitro*, and piperidines were employed to control cucumber damping-off in the greenhouse as drench treatments.

RESULTS: Piperidine and piperidine alkaloids of the red imported fire ant significantly inhibited mycelium growth of *P. ultimum*. Piperidine alkaloids were stable at both room and elevated temperatures. The inhibitory activity positively correlated with the concentrations of piperidine alkaloids in the medium, and the $EC_{50} = 17.0 \mu\text{g ml}^{-1}$. Germination of sporangia of *P. ultimum* was negatively correlated with the concentrations of piperidine alkaloids in the medium, and the $EC_{50} = 12.3 \mu\text{g ml}^{-1}$. The piperidine alkaloid drenching treatment significantly improved seedling emergence and seedling height of cucumber.

CONCLUSION: This is the first report describing the use of venom alkaloids from the red imported fire ant to inhibit *P. ultimum* in the laboratory and the application of piperidine alkaloids to control damping-off disease caused by *P. ultimum* in the greenhouse. These findings may lead to the development of a new group of fungicides.

© 2012 Society of Chemical Industry

Supporting information may be found in the online version of this article.

Keywords: piperidines; piperidines; venom alkaloids; *Pythium ultimum*; red imported fire ant

1 INTRODUCTION

Several *Pythium* species are ubiquitous soilborne plant pathogens that attack a wide range of economically important host plant species, including forestry and horticultural nursery plants.¹ *Pythium* infection causes pre- and post-emergence damping-off or poor growth of mature plants, resulting in significant yield losses. Control measures for reducing damage caused by *Pythium* spp. usually consist of modifications to cultural practices and application of chemical pesticides.² Seed treatment with chemicals is employed to protect seeds or seedlings from *Pythium* infection, whereas modifications of cultural practice, such as controlling seed planting depth, are used to promote germination and reduce root infections of seedlings. Drench treatments with chemicals and biologicals have also been used by growers to manage plant diseases caused by *Pythium* spp. Dichlorone, captan and cycloheximide were the first group of fungicides used to control plant diseases caused by *Pythium* spp.; however, they provided only limited protection. In 1979, metalaxyl was registered in the

United States specifically for the control of *Pythium* spp. Long-term application of chemical fungicides has resulted in the development of pathogen resistance and failure of disease control.³ Chemicals used to control *Pythium* infections have shown hazardous effects on the environment and humans. It has been documented that captan is not only hazardous to fish but also a mutagenic and carcinogenic agent.^{4,5} Concerns over the hazardous effects of chemical pesticides on the environment and humans have led to

* Correspondence to: Xixuan Jin, Biological Control of Pests Research Unit, The National Biological Control Laboratory, USDA-ARS-MSA, 59 Lee Road, PO Box 67, Stoneville, MS 38776, USA. E-mail: xixuan.jin@ars.usda.gov

a Institute of Plant Protection, Hebei Academy of Agricultural and Forestry Sciences, Baoding, Hebei Province, China

b Biological Control of Pests Research Unit, The National Biological Control Laboratory, USDA-ARS-MSA, Stoneville, MS, USA

the search for alternatives in the control of plant diseases caused by *Pythium* spp.

Efforts have been made in the search for biological and other natural means for integrated pest management (IPM) programs to manage *Pythium* diseases efficiently. Biological control efforts mainly focus either on utilizing a single strain of a microorganism or on introducing a group of microorganisms through composts.^{6–9} It has been proven that *Trichoderma* spp. and other microorganisms can be used to control or suppress soilborne plant pathogens, and to promote plant growth.^{9–16} Natural products can also be used to control plant diseases. These products include organic compounds and a variety of plant extracts.¹⁷ Natural bioactive products can be used as leads for the synthesis of a new generation of safe pesticides. The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), is an invasive ant species introduced into the United States in the late 1930s. According to recent reports, *S. invicta* is distributed in many countries and areas and affects humans, wildlife, crops and livestock, causing billions of dollars in losses.^{18–20} In spite of the damage and economical loss, the alkaloid-rich venom produced by the red imported fire ant might be used against plant pathogens such as *P. ultimum*. The venom chemistry of the red imported fire ant has been previously characterized and described by various authors.^{21–24} The alkaloid fraction of the venom of *S. invicta* contains mainly 2-methyl-6-alkyl or -alkenyl piperidines, known as solenopsins. Although piperidine alkaloids had long been believed to be the only alkaloids in *S. invicta*, Chen *et al.*^{25,26} recently identified six piperidine alkaloids from *S. invicta*, its sister species, *S. richteri*, and their hybrid, including 2-methyl-6-tridecyl-6-piperidine, 2-methyl-6-tridecyl-6-piperidine, 2-methyl-6-pentadecyl-6-piperidine, 2-methyl-6-pentadecyl-6-piperidine, 2-methyl-6-heptadecyl-6-piperidine and 2-methyl-6-heptadecyl-6-piperidine. Therefore, it is clear that the venom alkaloids extracted from the red imported fire ant consist of two groups of alkaloids: piperidine and piperidine alkaloids.

The insecticidal and antibiotic properties of the raw venom were discovered in the late 1950s.²⁷ The venom was highly toxic to the fruit fly, the housefly, a termite species, the boll weevil, the rice weevil and two species of mites. Using a paper disc method, Blum *et al.*²⁷ demonstrated that a 1/50 dilution of the venom effectively inhibited the growth of *Micrococcus pyogenes*, *Streptococcus pyogenes*, *Escherichia coli*, *Lactobacillus casei* and a variety of molds. Gram-positive bacteria were more sensitive to piperidine alkaloids than gram-negative ones.²⁸ *Solenopsis invicta* venom alkaloids also inhibited the germination and hyphal development of the entomopathogenic fungi *Beauveria bassiana* AF-4, *B. bassiana* 447, monosporal isolates of *Metarhizium anisopliae* and *Paecilomyces fumosoroseus*.²⁹ No research efforts have been made to study the activities of venom alkaloids against plant pathogenic oomycetes such as *Pythium* spp. or the potential application of the piperidine and piperidine alkaloids to plant disease control. This study is the first to report on the use of purified piperidine and piperidine alkaloids from the red imported fire ant against plant pathogenic *P. ultimum* in the laboratory and the application of piperidine alkaloids in the control of pre- and post-emergence damping-off of cucumber caused by *P. ultimum* in the greenhouse.

2 MATERIALS AND METHODS

2.1 Extraction and purification of piperidine and piperidine alkaloids

Workers (2.5 g) of the imported fire ant were extracted in 15 mL of hexane in a 50 mL beaker 3 times. The pooled extract was then concentrated to about 0.5 mL under air flow. A flash chromatography system (Isolera Four; Biotage, LLC, Charlotte, NC) with a SNAP silica gel cartridge (10 g) was used to isolate and purify the piperidine and piperidine alkaloids. The UV detector wavelengths for the flash chromatography were 254 nm for channel 1 and 280 nm for channel 2. The cartridge was first equilibrated with 45 mL of hexane before the sample was loaded. A gradient of hexane and acetone was used as a mobile phase at a flow rate of 12 mL min⁻¹. Acetone was increased from 0.0 to 100% within 19 min. The fraction collection interval was 6 mL. Fractions 17 to 22 were pooled and found to contain piperidine alkaloids, and fractions 38 to 70 contained piperidine alkaloids. Chromatograms and mass spectra of each alkaloid component are shown in the supporting information (Figs S1 to S7 for piperidine alkaloids and Figs S8 to S16 for piperidine alkaloids). The purity was 99.8% for piperidine alkaloids and 87.8% for piperidine alkaloids

2.2 Inhibitory activity of piperidine and piperidine alkaloids against mycelium growth of *P. ultimum*

Plant pathogenic *P. ultimum* 214-04 was kindly provided by Dr David Ingram, Central Mississippi Research and Extension Center, Mississippi State University. This strain was isolated from tomato (variety 'Trust') in Newton County, Mississippi. This pathogen was maintained on potato dextrose agar (PDA) (Becton Dickinson & Company, Sparks, MD) plates (each contained 20 mL of PDA). After 30 h incubation at 28 °C, *P. ultimum* culture discs (5 mm in diameter) were cut from the growing edge of the colonies. Each culture disc was placed at the center of a freshly made PDA plate.

Fisherbrand P8 filter paper (Fisher Scientific, Pittsburgh, PA) was used to make 5 mm discs for carrying venom piperidine or piperidine alkaloids in acetone solutions. The paper discs were sterilized by autoclave at 121 °C for 30 min. The sterile filter paper discs were placed in a glass petri dish, and 5 µL of acetone solution of piperidine (3.8 µg µL⁻¹) or piperidine (3.8 µg µL⁻¹) alkaloids was added to each disc. Each filter paper disc had 19.0 µg of either piperidine or piperidine alkaloids. After the acetone was evaporated under a laminar flow hood, four filter paper discs were placed on a PDA plate about 2.0 cm away from a *P. ultimum* culture disc at the 12, 3, 6, and 9 o'clock positions respectively. The *P. ultimum* culture disc was placed in the center of the plate. Filter paper discs with only 5 µL of acetone were used as controls. Thus, there were two treatments (piperidine or piperidine alkaloids) and one control (acetone); each plate was one treatment. All plates were incubated at 28 °C with no light for 48 h, and the inhibitory zones, the linear distance between the growth edge of a *P. ultimum* colony and the edge of a filter paper disc, were measured. This experiment was repeated 4 times.

2.3 Stability of inhibitory activity of piperidine alkaloids stored at different temperature levels against mycelium growth of *P. ultimum*

Only piperidine alkaloids were used in the stability study. A quantity of 500 µL of the acetone solution (3.8 µg µL⁻¹ of piperidine alkaloids) was transferred into a 2 mL amber bottle, and the cap was screwed on tightly. Three bottles wrapped with aluminum foil comprised one storage package. Three packages were kept at

4, 22 and 54 °C respectively. Each storage temperature was one treatment. The solution was sampled 6 times at 2 week intervals for the packages stored at 4 and 22 °C to conduct inhibitory assays. The packages stored at 54 °C were sampled twice at 2 and 4 weeks of storage. The paper disc method described in Section 2.2 was employed to study the stability of inhibitory activities of piperidine alkaloids against the mycelium growth of *P. ultimum*. This experiment was repeated 4 times.

2.4 Inhibitory efficacy of piperidine alkaloid concentrations against mycelium growth of *P. ultimum*

The acetone solution containing piperidine alkaloids was blended in sterile deionized water (SDW) containing 0.1% Tween-80 (Fisher Scientific, Fairlawn, NJ), and the mixture was then incorporated with PDA at 46 °C to achieve 1.6, 3.2, 6.4, 12.8 and 25.6 µg piperidine alkaloids mL⁻¹ PDA medium. A quantity of 20 mL of medium was poured into one petri plate. Each piperidine alkaloid concentration was one treatment. The control had an equal amount of acetone–0.1% Tween-80 SDW. *Pythium ultimum* culture discs (5 mm in diameter, as described in Section 2.2) were then placed at the center of the plates with different concentrations of piperidine alkaloids. All plates were incubated at 28 °C in the dark for 48 h. The diameters of the *P. ultimum* colonies were measured. The inhibitory efficacy was calculated using the formula

$$\text{Inhibitory efficacy} = \frac{\text{Colony diameter of control} - \text{colony diameter of treatment}}{\text{Colony diameter of control}} \times 100$$

This experiment was repeated 6 times.

2.5 Inhibitory activity of piperidine alkaloids on the germination of sporangia of *P. ultimum*

The method for the production of *P. ultimum* sporangia published by Kageyama and Nelson³⁰ was employed in this study with slight modifications. A *P. ultimum* culture disc (5 mm in diameter) was transferred to a defined mineral salt medium containing 0.1% soy lecithin (SM + L). After 5 days of incubation at 28 °C, the entire agar plate was leached for two consecutive 10 min intervals in a buffer (pH 5.8) containing 10 mM of Ca(NO₃)₂·4H₂O, 4 mM of MgSO₄·7H₂O and 5 mM of KNO₃, followed by a final 3 h interval in darkness. This procedure was conducted in darkness, and the buffer was replaced after each leaching period. After the final leaching period, leachate was removed, and the agar plates were rinsed twice with SDW and kept at 24 °C in darkness for 2 days. Sporangia were harvested by adding 500 µL of SDW per plate and scraping sporangia from the surface of the culture. Sporangia suspensions were filtered through sterile cheesecloth, centrifuged 3 times at 6000 rpm and washed twice with SDW. Potato dextrose broth (PDB) (Becton Dickinson) was added after the final centrifugation to adjust the sporangia density to 1 × 10⁵ sporangia mL⁻¹ PDB using a Neubauer Brightline hemacytometer (Hausser Scientific Co., Horsham, PA).

The acetone solution of piperidine alkaloids (3.8 µg µL⁻¹) was first adjusted to 256 µg mL⁻¹ by mixing with 0.1% Tween-80 SDW. Serial dilutions were made to dilute the 256 µg mL⁻¹ suspension to achieve concentrations of 8.0, 16.0, 32.0, 64.0 and 128.0 µg piperidine alkaloids mL⁻¹ by incorporating with 0.1% Tween-80 SDW. A quantity of 1 mL of each concentration of piperidine alkaloids and the control was then mixed with 4.0 mL of PDB containing 1 × 10⁵ sporangia mL⁻¹ in a 50 mL centrifuge tube. The final concentrations of piperidine alkaloids in the PDB were

0.0, 1.6, 3.2, 6.4, 12.8, 25.6 and 51.2 µg mL⁻¹ respectively. Each piperidine alkaloid concentration was one treatment. The control was acetone–0.1% Tween-80 SDW only. All centrifuge tubes were incubated at 28 °C for 48 h, and samples were taken to count the germination of sporangia under a light microscope. A total of 100–200 sporangia were counted for each piperidine alkaloid concentration, and sporangia were considered to have germinated when a developing germ tube was visible. Then the germination percentage was calculated for data analysis. Three samples were taken from each piperidine alkaloid concentration, and this experiment was repeated twice.

2.6 Control of cucumber damping-off caused by *P. ultimum* using piperidine alkaloids in the greenhouse

Redi-Earth plug and seedling mix (Sun Gro Horticulture Distribution Inc., Bellevue, WA) was used as growth medium. This mix was specially designed for small-type propagation in small containers, and was formulated with fine Canadian Sphagnum peat moss, vermiculite, starter nutrient charge (with gypsum), dolomitic limestone and a long-lasting wetting agent. The growth mix was autoclaved twice at 121 °C for 1 h at 24 h intervals.

Certified organic cucumber (*Cucumis sativus* L.) seeds (Straight 8; Bar Farm Service Inc., Bay, MI) were used in this experiment. Germination of cucumber seeds was 100% on water agar plates at 22 ± 1 °C. Cucumber seeds were surface disinfected by soaking in 0.5 mL NaOCl L⁻¹ for 5 min and then rinsed with SDW.

Preliminary studies showed that soaking seeds with different concentrations of piperidine alkaloids did not provide any control, but drench treatment with piperidine alkaloids significantly improved the seedling emergence and seedling heights. Therefore, drench treatments were conducted with piperidine alkaloids in a greenhouse with two treatments and four controls. *Pythium ultimum* was grown on the PDA plates for 5 days at 28 °C in the dark. Two mycelium discs (5 mm in diameter) were excised from the growing edge of a colony, transferred to the surface of the sterile growth mix (2 cm in depth) in a small growth container (7.5 × 5.5 × 5.5 cm³) and covered with a layer of sterile growth mix (1 cm). Cucumber seeds were placed on the surface of the inoculated growth mix and covered with 1 cm of growth mix. Ten seeds were sowed in each growth container, which had 3 cm growth mix in depth, and covered with 1 cm of growth mix as described above. Subsequently, 3 mL of piperidine alkaloids in acetone–0.1% Tween-80 SDW suspension at a concentration of 18.8 µg mL⁻¹ (total of 56.5 µg of piperidine alkaloids) and 188.4 µg mL⁻¹ (total of 565.2 µg of piperidine alkaloids) was applied to the surface of the growth mix in each container. The two treatments and four controls were as follows:

1. Control 1 had no *P. ultimum*, and only 3 mL of acetone–0.1% Tween-80 SDW containing no piperidine alkaloids was applied to the surface of the growth mix in each container after sowing.
2. Control 2 had *P. ultimum* inoculum, and 3 mL of acetone–0.1% Tween-80 SDW containing no piperidine alkaloids was applied to the surface of the growth mix in each container after sowing.
3. Control 3 had no *P. ultimum*, and 3 mL of acetone–0.1% Tween-80 SDW containing 56.5 µg of piperidine alkaloids was applied to the surface of the growth mix in each container after sowing.
4. Control 4 had no *P. ultimum*, and 3 mL of acetone–0.1% Tween-80 containing 565.2 µg of piperidine alkaloids was applied to the surface of the growth mix in each container after sowing.

5. Treatment 1 had *P. ultimum* inoculum, and 3 mL of acetone–0.1% Tween-80 containing 56.5 µg of piperidine alkaloids was applied to the surface of the growth mix in each container after sowing.
6. Treatment 2 had *P. ultimum* inoculum, and 3 mL of acetone–0.1% Tween-80 containing 565.2 µg of piperidine alkaloids was applied to the surface of the growth mix in each container after sowing.

The growth containers were then placed in trays on greenhouse benches, and water was added to the trays as needed. The temperature range in the greenhouse was 18–25 °C, and the relative humidity (RH) was maintained at 60–90%. The seedling stands were counted, and seedling heights were measured 20 days after sowing using the methods described by Chen and Nelson.⁹ The photoperiod was not controlled. Each treatment had one container with ten seeds, and this study was repeated 3 times.

2.7 Statistical analysis

Experimental design was a randomized complete block (RCB) for all experiments. Data were subjected to analysis of variance (ANOVA) and regression analysis using general linear mixed model procedures in SAS.³¹ Values were presented as means of replicated experiments and subjected to the least-squares *post hoc* test to identify significant differences among treatments at the 5% probability level. Analysis performed on each experiment was as follows:

- 2.2 – ANOVA followed by mean comparisons was performed for RCB with two treatments and four replications.
- 2.3 – ANOVA followed by mean comparisons was performed for a split-plot arrangement where the main unit was an RCB with three temperature treatments and the subunit treatment was a repeated measure over time.
- 2.4 and 2.5 – regression analysis was performed for inhibitory effects against mycelium growth (2.4) or sporangium germination (2.5) as a function of concentration of piperidine alkaloids.
- 2.6 – ANOVA followed by mean comparisons was performed for RCB with six treatments and three replications.

3 RESULTS

3.1 Inhibitory activity of piperidine and piperidine alkaloids against mycelium growth of *P. ultimum*

Acetone had no inhibitory effect against mycelium growth of *P. ultimum*; therefore, results from the acetone treatment were omitted from the statistical analysis. Both piperidine and piperidine alkaloids significantly inhibited mycelium growth of *P. ultimum* (Fig. 1). There was no significant difference in inhibitory effects between these two alkaloids ($P > 0.05$).

3.2 Stability of inhibitory activity of piperidine alkaloids stored at different temperatures against mycelium growth of *P. ultimum*

Results indicated that storage temperature levels had no significant effects on the inhibitory activity of piperidine alkaloids ($P > 0.05$) (Fig. 2). The inhibitory activity was significantly improved in the first weeks of storage ($df_{\text{num}} = 6$, $df_{\text{den}} = 59$, $F = 3.71$, $P < 0.05$). The inhibitory activity of piperidine alkaloids was quite stable from 2 to 12 weeks of storage at 4 and 22 °C. There was a decrease in inhibitory activity of piperidine alkaloids after 4 weeks of storage at 54 °C. However, this decrease was not statistically significant ($P > 0.05$).

Figure 1. Inhibitory effects of piperidine and piperidine alkaloids from the venom of the red imported fire ant (*Solenopsis invicta*) on mycelium growth of *Pythium ultimum* on potato dextrose agar (PDA) plates incubated at 28 °C for 48 h; a paper disc method was used; each disc had 19.0 µg of either piperidine or piperidine alkaloids. The purity was 99.8% for piperidine alkaloids and 87.8% for piperidine alkaloids. Values are the means of four replicates. Because acetone had no inhibitory effect against mycelium growth of *P. ultimum*, acetone treatment was omitted from the statistical analysis. Means with different letters are significantly different ($P < 0.05$).

Figure 2. Effects of storage temperatures on the inhibitory activity of piperidine alkaloids (purity = 99.8%) from the venom alkaloids of the red imported fire ant (*Solenopsis invicta*) against mycelium growth of *Pythium ultimum* on potato dextrose agar (PDA) plates over time; the paper disc method was utilized. Values are the means of four replicates. Means with different letters across the sampling time are significantly different ($P < 0.05$).

3.3 Inhibitory efficacy of piperidine alkaloid concentrations against mycelium growth of *P. ultimum*

The inhibitory efficacy against mycelium growth of *P. ultimum* was positively correlated with the concentrations of piperidine alkaloids in the PDA medium ($R^2 = 0.8869$, $y = 2.4822x + 7.7279$) (Fig. 3). In the tested concentration range, higher concentrations demonstrated higher inhibitory efficacy. Based on the positive linear equation, the inhibitory $EC_{50} = 17.0 \mu\text{g ml}^{-1}$.

3.4 Inhibitory activity of piperidine alkaloids against sporangium germination of *P. ultimum*

Germination of sporangia of *P. ultimum* was negatively correlated with the concentration increase of piperidine alkaloids in PDB ($R^2 = 0.8458$, $y = -1.2871x + 65.775$) (Fig. 4). Sporangia of *P. ultimum* were sensitive to the presence of piperidine alkaloids. Based on the negative linear equation, the inhibitory activity

Figure 3. Inhibitory efficacy against mycelium growth of *Pythium ultimum* on potato dextrose agar (PDA) plates containing different concentrations of piperidine alkaloids (purity = 99.8%) from the red imported fire ant *Solenopsis invicta*. The acetone solution containing piperidine alkaloids was mixed with sterile deionized water (SDW) containing 0.1% Tween-80, and the mixture was then incorporated into PDA at 46 °C to achieve different concentrations of piperidine alkaloids mL⁻¹ PDA medium. Values are the means of six replicates, and bars represent standard errors.

Figure 4. Germination of sporangia of *Pythium ultimum* in potato dextrose broth (PDB) containing different concentrations of piperidine alkaloids (purity = 99.8%) from the red imported fire ant *Solenopsis invicta*. The acetone solution of piperidine alkaloids was diluted with 0.1% Tween-80 in sterile deionized water, and was then mixed with PDB to achieve different concentrations of piperidine. The incubation was conducted at 28 °C for 48 h. Values are the means of six samples of two replicates, and bars represent standard errors.

EC₅₀ = 12.3 µg mL⁻¹. Germination of sporangia was less than 10% when the piperidine alkaloid concentration reached 51.2 µg mL⁻¹.

3.5 Control of cucumber damping-off caused by *P. ultimum* using piperideine alkaloids in the greenhouse

Results indicated that drenching treatment with both concentrations of piperideine alkaloids (18.8 and 188.5 µg mL⁻¹) significantly improved the seedling emergence ($df_{\text{num}} = 5$, $df_{\text{den}} = 10$, $F = 61.1$, $P < 0.05$) and seedling heights ($df_{\text{num}} = 5$, $df_{\text{den}} = 10$, $F = 27.27$, $P < 0.05$) (Table 1). These results were comparable with the controls. There was no significant difference in disease control effects between these two concentrations of piperideine alkaloids ($P > 0.05$). Acetone–0.1% Tween-80 SDW with or without piperideine alkaloids at both concentrations had no adverse effects on the seedling emergence and seedling heights.

4 DISCUSSION

This study is the first report to show the inhibitory activity of both groups of venom alkaloids, piperidines and piperideines, against the soilborne plant pathogen *P. ultimum*. Although it was believed that control of *Pythium* diseases would eventually be achieved by the development of resistant or tolerant crop varieties,² these efforts have not been fruitful owing to the wide host range of *Pythium* spp. and the unavailability of strong resistance. Damping-off disease has been mainly controlled by seed treatment with chemical fungicides and cultural practices. Commercialization of *Trichoderma* spp.-based products to control damping-off caused by *Pythium* spp. has made considerable progress over the past two decades in the United States and in the developing countries.^{32,33} However, the commercialization of biological control products has been hindered by the difficulties existing in mass production, stabilization, formulation development and shelf life, as well as the registration process.

Natural products can be important in the search for alternatives in insect pest and plant disease management because of their enormous diversity.¹⁷ Dayan *et al.*³⁴ reviewed natural products in detail, including plant extracts, antibiotics from microbes and plant disease resistance elicitors for the management of plant pathogens. The present laboratory results showed that both piperidine and piperideine alkaloids extracted from the red imported fire ant inhibited the mycelium growth of *P. ultimum* on PDA plates. Although statistically the inhibitory effects were significant, the inhibitory zones were small because the PDA medium used in the present studies might have absorbed certain amounts of either piperidine or piperideine alkaloids. Water agar should be used in future studies to avoid medium absorbency. It is the authors' understanding that piperidine alkaloids are more stable than piperideine alkaloids because piperideines have a double bond on their piperidine rings. It was also discovered that piperidine alkaloids were stable at room temperatures for 12 weeks. The inhibitory effects against mycelium growth of *P. ultimum* were positive in accordance with the concentrations of piperidine alkaloids. Higher concentrations resulted in better inhibitory effects, and the inhibitory EC₅₀ = 17.0 µg mL⁻¹.

Pythium ultimum is a ubiquitous plant pathogen and one of the most pathogenic *Pythium* spp. on crop species,² and it can survive in air-dried muck soil for 12 years.³⁵ The ability of sporangia to persist under adverse conditions, coupled with their extraordinary capacity to avoid competition by rapid germination and rapid germ tube extension in response to low exogenous nutrients and by producing secondary sporangia and retracting and conserving protoplasm under starvation conditions, enables *P. ultimum* to occupy its soil ecological niche. Consequently, sporangia of *P. ultimum* function as one of the major survival structures in cultivated soils to provide inoculum.³⁶ The present results indicated that sporangia were more sensitive than mycelium to the presence of piperidine alkaloids, and the inhibitory EC₅₀ = 12.3 µg mL⁻¹. In the preliminary study it was found that drench treatment, 56.5 µg of piperidine alkaloids per container, significantly improved seedling emergence and heights (data not shown). Use was made of 56.5 or 565.2 µg of piperideine alkaloids per container as drench treatment in this study. This amount of piperideine alkaloids inhibited both mycelium growth and sporangium germination. Therefore, seedling emergence and seedling heights of cucumber treated with piperideine alkaloids were comparable with those of the controls (Table 1). There were no significant differences in inhibitory activities between these two piperideine alkaloid concentrations; therefore, the

Table 1. Control of cucumber (*Cucumis sativus*) damping-off caused by *Pythium ultimum* using drench treatments with piperidine alkaloids (purity = 87.8%) extracted from the red imported fire ant (*Solenopsis invicta*). The relative humidity in the 25–28 °C greenhouse was maintained at 60–90%. The seedling stands were counted and seedling heights were measured 20 days after sowing^{a,b}

Treatment	Description	Seedling emergence ^c (%) (mean ± SE)	Seedling height ^d (cm) (mean ± SE)
Control 1	– <i>P. ultimum</i>	93.33 ± 3.33 a	11.54 ± 0.54 a
Control 2	–piperidine + <i>P. ultimum</i>	33.3 ± 3.33 b	6.74 ± 0.27 b
Control 3	– <i>P. ultimum</i>	96.67 ± 3.33 a	11.26 ± 0.35 a
Control 4	+piperidine 56.5 µg – <i>P. ultimum</i>	96.67 ± 3.33 a	11.48 ± 0.45 a
Treatment 1	+ <i>P. ultimum</i> +piperidine 56.5 µg	96.67 ± 3.33 a	11.60 ± 0.22 a
Treatment 2	+ <i>P. ultimum</i> +piperidine 565.2 µg	96.67 ± 3.33 a	11.76 ± 0.20 a

^a Numbers are the means of three replicates and are presented with standard errors (SE).

^b Different letters in each column indicate a significant difference at the $P < 0.05$ level.

^c ANOVA followed by mean comparison showed that treatments had significant effects on seedling emergence ($df_{\text{num}} = 5$, $df_{\text{den}} = 10$, $F = 61.10$, $P < 0.05$).

^d ANOVA followed by mean comparison showed that treatments had significant effects on the seedling heights ($df_{\text{num}} = 5$, $df_{\text{den}} = 10$, $F = 27.27$, $P < 0.05$).

lower concentration of piperidine alkaloids could be used to control damping-off in the greenhouse. Drench treatment can be applied to hydroponic systems in greenhouse production to control damping-off caused by *Pythium* spp.

The present findings involving piperidine and piperidine alkaloids extracted from the red imported fire ant to control the most destructive damping-off diseases caused by *P. ultimum* may lead to the development of a new group of fungicidal chemicals. Further studies must focus on the synthesis of piperidine and piperidine alkaloids and on formulation development that may provide satisfactory control of damping-off caused by *P. ultimum*.

ACKNOWLEDGEMENTS

The technical assistance of Robert B Elliott and Alfred Martin is gratefully acknowledged. The authors thank Debbie Boykin, USDA-ARS-MSA, Stoneville, MS, for providing statistical assistance. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

- Hendrix FF and Campbell WA, Pythiums as plant pathogens. *Annu Rev Phytopathol* **11**:77–98 (1973).
- Martin FN and Loper JE, Soilborne plant diseases caused by *Pythium* spp.: ecology, epidemiology, and prospects for biological control. *Crit Rev Plant Sci* **18**:111–181 (1999).
- Sander PL, Failure of metalaxyl to control *Pythium* blight on turfgrass in Pennsylvania. *Plant Dis* **68**:776–777 (1984).
- Ruiz MJ and Marzin D, Genotoxicity of six pesticides by Salmonella mutagenicity test and SOS chromotest. *Mutat Res* **390**:245–255 (1997).
- Battershill JM and Fielder RJ, Mouse-specific carcinogens: an assessment of hazard and significance for validation of short-term carcinogenicity bioassays in transgenic mice. *Hum Exp Toxicol* **17**:193–205 (1998).
- Benhamou N, Gagné S, Le Quéré D and Dehbi L, Bacterial-mediated induced resistance in cucumber: beneficial effect of the endophytic bacterium *Serratia plymuthica* on the protection against infection by *Pythium ultimum*. *Phytopathology* **90**:45–56 (2000).
- Fatemeh M, Björkman T and Harman GE, Seed treatment with *Trichoderma harzianum* alleviates biotic, abiotic and physiological stresses in germinating seeds and seedlings. *Phytopathology* **100**:1213–1221 (2010).
- Scheuerell SJ and Mahaffee WF, Compost tea as a container medium drench for suppressing seedling damping-off caused by *Pythium ultimum*. *Phytopathology* **94**:1156–1163 (2004).
- Chen MH and Nelson EB, Seed-colonizing microbes from municipal biosolids compost suppress *Pythium ultimum* damping-off on different plant species. *Phytopathology* **98**:1012–1018 (2008).
- Xue AG, Biological control of pathogens causing root rot complex in field pea using *Clonostachys rosea* strain ACM941. *Phytopathology* **93**:329–335 (2003).
- Harman GE, Petzoldt R, Comis A and Chen J, Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and effects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. *Phytopathology* **94**:147–153 (2004).
- Harman GE, Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* **96**:190–194 (2006).
- Howell CR, Cotton seedling preemergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with *Trichoderma* spp. *Phytopathology* **92**:177–180 (2002).
- Howell CR, Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis* **87**:4–10 (2003).
- Howell CR, Effect of seed quality and combination fungicide–*Trichoderma* spp. seed treatments on pre- and postemergence damping-off in cotton. *Phytopathology* **97**:66–71 (2007).
- Postma J, Geraats BPJ, Pastoor R and van Elsas JD, Characterization of the microbial community involved in the suppression of *Pythium aphanidermatum* in cucumber grown on rockwool. *Phytopathology* **95**:808–818 (2005).
- Jaspers ABK and De Waard MA, Natural products in plant protection. *Eur J Plant Pathol* **99**(Suppl. 3): 109–117 (1993).

- 18 Wojcik DP, Allen CR, Brenner RJ, Forsy EA, Jouvenaz DP and Lutz RS, Red imported fire ants: impact on biodiversity. *Am Entomol* **47**:16–23 (2001).
- 19 Sánchez-Peña SR, Patrock RJW and Gilbert LA, The red imported fire ant is now in Mexico: documentation of its wide distribution along the Texas–Mexico border. *Entomol News* **116**:363–366 (2005).
- 20 Gutrich JJ, VanGelder E and Loope L, Potential economic impact of introduction and spread of the red imported fire ant, *Solenopsis invicta*, in Hawaii. *Environ Sci Policy* **10**:685–696 (2007).
- 21 MacConnell JG, Blum MS and Fales HM, Alkaloid from fire ant venom: identification and synthesis. *Science* **168**:840–841 (1970).
- 22 MacConnell JG, Blum MS and Fales HM, The chemistry of fire ant venom. *Tetrahedron* **27**:1129–1139 (1971).
- 23 Jones TH, Blum MS and Fales HM, Ant venom alkaloids from *Solenopsis* and *Monomorium* species, recent developments. *Tetrahedron* **38**:1949–1958 (1982).
- 24 Leclercq S, Thirionet I, Broeders F, Daloze D, Vander Meer R and Braekman JC, Absolute configuration of Solenopsins, venom alkaloids of the fire ants. *Tetrahedron* **50**:8465–8478 (1994).
- 25 Chen J, Cantrell CL, Shang HW and Rojas MG, Piperidine alkaloids from the poison gland of the red imported fire ant (Hymenoptera: Formicidae). *J Agric Food Chem* **57**:3128–3133 (2009).
- 26 Chen J, Shang HW and Jin X, Interspecific variation of $\Delta^{1,6}$ -piperidines in imported fire ants. *Toxicon* **55**:1181–1187 (2010).
- 27 Blum MS, Walker JR, Callahan PS and Novak AF, Chemical, insecticidal, and antibiotic properties of fire ant venom. *Science* **128**:306–307 (1958).
- 28 Jouvenaz DP, Blum MS and MacConnell JG, Antibacterial activity of venom alkaloids from the imported fire ant, *Solenopsis invicta* Buren. *Antimicrob Agents Chemother* **2**:291–293 (1972).
- 29 Storey GK, Vander Meer RK, Boucias DG and McCoy CW, Effect of fire ant (*Solenopsis invicta*) venom alkaloids on the *in vitro* germination and development of selected entomogenous fungi. *J Invertebr Pathol* **58**:88–95 (1991).
- 30 Kageyama K and Nelson EB, Differential inactivation of seed exudate stimulation of *Pythium ultimum* sporangium germination by *Enterobacter cloacae* influences biological control efficacy on different plant species. *Appl Environ Microbiol* **69**:1114–1120 (2003).
- 31 Littell RC, Milliken GA, Stroup WW, Wolfinger RD and Schabenberger O, *SAS for Mixed Models*, 2nd edition. SAS Institute, Cary, NC (2006).
- 32 Jin X, Hayes CK and Harman GE, Principles in the development of biological control system employing *Trichoderma* species against soil-borne plant pathogenic fungi, in *Frontiers in Industry Mycology*, ed. by Leatham GF. Chapman and Hall, Inc., New York, NY, pp. 174–195 (1992).
- 33 Harman GE, Obregón MA, Samuels GJ and Matteo L, Changing models for commercialization and implementation of biocontrol in the developing and the developed world. *Plant Dis* **94**:928–939 (2010).
- 34 Dayan FE, Cantrell CL and Duke SO, Natural products in crop protection. *Bioorg Med Chem* **17**:4022–4034.
- 35 Hoppe PE, *Pythium* species still viable after 12 years in air-dried muck soil. *Phytopathology* **56**:1411 (1966).
- 36 Stanghellini ME and Hancock JG, The sporangium of *Pythium ultimum* as a survival structure in soil. *Phytopathology* **61**:157–164 (1971).

GC-MS Analyses: A Varian GC-MS system was used. It consisted of a CP-3800 gas chromatograph and a Saturn 2000 mass selective detector, which was controlled by a Mass Spectrometry WorkStation Version 6.4.1 (Varian, Walnut Creek, CA). A 30 m × 0.25 mm i.d., 0.25 µm film thickness, DB-1 capillary column was used (J & W Scientific, Folsom, CA). The GC temperature program was as follows: The initial temperature was set at 50 °C, held for 1 min and increased to 250 °C at a rate of 20 °C/ min, and held for 40 min. A 2 µL sample was injected at 250 °C with 10:1 split ratio. Helium was used as the carrier gas at a flow rate of 1.0 mL/min, and the transfer line temperature was set at 200 °C. The mass spectrometer was operated at 70 eV in the electron impact mode.

Figure S1. GC-MS-EI TIC of piperidine alkaloids (fractions 17-22 from flash chromatography)

Peak assignment:

1. 2-methyl-6-tridecenyl-6-piperidine (0.23%)
2. 2-methyl-6-tridecyl-6-piperidine (4.30%)
3. 2-methyl-6-pentadecenyl-6-piperidine (21.66%)
4. 2-methyl-6-pentadecyl-6-piperidine (44.83%)
5. 2-methyl-6-heptadecenyl-6-piperidine (24.94%)
6. 2-methyl-6-heptadecyl-6-piperidine (4.03%)

Figure S2. Mass spectrum of 2-methyl-6-tridecenyl-6-piperidine.

Figure S3. Mass spectrum of 2-methyl-6-tridecyl-6-piperidine.

Figure S4. Mass spectrum of 2-methyl-6-pentadecenyl-6-piperidine

Figure S5. Mass spectrum of 2-methyl-6-pentadecyl-6-piperidine

Figure S6. Mass spectrum of 2-methyl-6-heptadecenyl-6-piperidine

Figure S7. Mass spectrum of 2-methyl-6-heptadecyl-6-piperidine

Figure S8. GC-MS-EI TIC of piperidine alkaloids (fractions 38-70 from flash chromatography)

Peak assignment:

1. *cis*-2-methyl-6-tridecenylpiperidine (0.36%)
2. *cis*-2-methyl-6-tridecylpiperidine (0.19%)
3. *trans*-2-methyl-6-tridecenylpiperidine (26.04%)
4. *tran*-2-methyl-6-tridecylpiperidine (16.48%)
5. *cis*-2-methyl-6-pentadecenylpiperidine (0.33%)
6. *cis*-2-methyl-6-pentadecylpiperidine (0.053%)
7. *trans*-2-methyl-6-pentadecenylpiperidine (36.10%)
8. *tran*-2-methyl-6-pentadecylpiperideine (20.44%)

Figure S9. Mass spectrum of *cis*-2-methyl-6-tridecenylpiperidine

Figure S10. Mass spectrum of *cis*-2-methyl-6-tridecylpiperidine

Figure S11. Mass spectrum of *trans*-2-methyl-6-tridecenylpiperidine

Figure S12. Mass spectrum of *trans*-2-methyl-6-tridecylpiperidine

Figure S13. Mass spectrum of *cis*-2-methyl-6-pentadecenylpiperidine

Figure S14. Mass spectrum of cis-2-methyl-6-pentadecylpiperidine

Figure S15. Mass spectrum of *trans*-2-methyl-6-pentadecenylpiperidine

Figure S16. Mass spectrum of *trans*-2-methyl-6-pentadecylpiperidine