

Diketopiperazines from *Alternaria dauci*

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Abstract. *Alternaria dauci* is the causal agent of Alternaria leaf blight (ALB), a foliar disease of carrot crops (*Daucus carota*) around the world. In terms of phytotoxic metabolites production, *A. dauci* has received limited attention. Previous studies carried out on the secondary metabolites involved in the pathogenicity of this fungus have only reported the isolation of a ubiquitous non-selective phytotoxin known as zinniol. Because of this, the aim of this research is directed towards the isolation and identification of secondary metabolites involved in the plant-pathogen interaction process. *A. dauci* was cultured in the Czapek-Dox medium, and the culture filtrate was extracted with ethyl acetate. The leaf-spot assay of fractions resulting from the partition process showed a phytotoxic effect in the ethyl acetate fraction. The chromatographic separation of ethyl acetate fraction allowed the isolation of seven diketopiperazines, identified as cyclo-(pro-val) (**1**), cyclo-(pro-leu) (**2**), cyclo-(pro-phe) (**3**), cyclo-(val-leu) (**4**), cyclo-(val-phe) (**5**), cyclo-(leu-phe) (**6**) and cyclo-(leu-tyr) (**7**). The structures of the different metabolites were established by comparing their spectroscopic (¹H NMR) and spectrometric (GC-MS) data with those reported in the literature.

Keywords: *Daucus carota*; *Alternaria dauci*; phytopathogen; phytotoxicity; diketopiperazine.

Resumen. *Alternaria dauci* es el agente causal del tizón de la hoja (ALB), una enfermedad foliar que afecta los cultivos de zanahoria (*Daucus carota*) alrededor del mundo. En términos de producción de metabolitos fitotóxicos, *A. dauci* ha recibido una atención muy limitada. Estudios previos llevados a cabo sobre los metabolitos secundarios involucrados en la patogenicidad de este hongo, solo han reportado el aislamiento de una fitotoxina no selectiva y ubicua conocida como zinniol. Debido a lo anterior, el objetivo de esta investigación se dirige al aislamiento e identificación de metabolitos secundarios implicados en la interacción planta-patógeno. Para esto el fitopatógeno se cultivó en medio Czapek-Dox y el filtrado del cultivo se extrajo con acetato de etilo. La evaluación de las fracciones resultantes de la partición, en el ensayo de manchas foliares en hojas, mostró un efecto fitotóxico en la fracción de acetato de etilo. La separación cromatográfica de la fracción de acetato de etilo permitió el aislamiento de siete diketopiperazinas identificadas como ciclo-(pro-val) (**1**), ciclo-(pro-leu) (**2**), ciclo-(pro-phe) (**3**), ciclo-(val-leu) (**4**), ciclo-(val-phe) (**5**), ciclo-(leu-phe) (**6**) y ciclo-(leu-tyr) (**7**). Las estructuras de los diferentes metabolitos se establecieron comparando sus datos espectroscópicos (1H RMN) y espectrométricos (CG-EM) con los reportados en la literatura.

Palabras clave: *Daucus carota*; *Alternaria dauci*; fitopatógeno; fitotoxicidad; diketopiperazina.

Introduction

The *Alternaria* genus, widely distributed and commonly found in the soil and organic matter is known for its pathogenicity against many important crops worldwide [1,2]. To the date, a wide variety of different metabolites have been reported from *Alternaria* fungal cultures, and these include terpenoids, pyranones, quinones, phenolics, and nitrogen-containing metabolites [1]. The isolated metabolites are reported to exhibit several biological activities, including phytotoxic, cytotoxic, and antimicrobial, which suggest their potential application in different areas such as chemistry, pharmacy, and agriculture.

Many secondary metabolites produced by *Alternaria* spp. are known to be responsible for their pathogenicity, these metabolites are known as phytotoxins. Examples of these phytotoxins include the cyclodepsipeptides destruxins A and B produced by *A. linicola* [3,4]; tenuazonic acid, a nitrogen-containing metabolite, isolated from *A. alternata* [5]; zinniol, a phenolic phytotoxin commonly identified in extracts from many *Alternaria* spp., including *A. carthami*, *A. cichorii*, *A. cirsinioxia*, *A. dauci*, *A. macrospora*, *A. porri*, *A. solani*, *A. tagetica*, and *A. zinniae* [1, 6–10]; and maculosin, a diketopiperazine reported from *A. alternata* [11].

One *Alternaria* species that has received limited attention, in terms of its secondary metabolites production, is *Alternaria dauci*, the causal agent of *Alternaria* leaf blight (ALB), a foliar disease of carrot crops (*Daucus carota*) around the world [12,13]. The disease appears initially in the leaves as small greenish-brown lesions, becoming larger and turning dark-brown, often surrounded by a chlorotic halo [14]. The main effects associated with the disease are the reduction of leaf photosynthetic activity and carbohydrate production, followed by separation of necrotic foliage from the taproot. These effects contribute to a significant reduction in harvesting efficiency [12]. Previous studies carried out on the metabolites produced by *A. dauci* have reported the identification of alternariol and alternariol monomethyl ether, together with the ubiquitous non-host selective phytotoxin zinniol [15, 16]. However, Lecomte *et al.* [13] demonstrated that zinniol is not responsible for the phytotoxic effect caused by the organic extract of *A. dauci*, since the pure phytotoxin proved to be not toxic towards carrot embryogenic cultures and the UHPLC-MS analysis of the fungal organic extract did not show significant amounts of this phytotoxin in the chromatographic profile. Here, as part of a project directed towards understanding the role of natural products in plant-pathogen interactions, we wish to report the isolation and identification of seven diketopiperazines from the culture filtrate of *A. dauci*, when cultured in Czapek-Dox medium.

Experimental

General Experimental Procedures

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian/Agilent (AR Premium COMPACT) at 600 (1H) and 150 MHz (13C); chemical shifts were recorded as δ values. Gas Chromatography-Mass Spectrometry (GC-MS) were analyzed by an Agilent (Santa Clara, CA, USA) 6890N gas chromatograph coupled to an Agilent 5975C INERT mass spectrometer detector. High-Performance Liquid Chromatography (HPLC) was carried out using a Waters Alliance® HPLC system equipped with a 2998-photodiode array detector (PDA). Control of the equipment, data acquisition, processing, and management of chromatography was performed by the Empower 2 software program (Waters) (version 3). UV spectra were measured with a 2998-photodiode array UV detector (PDA). Analytical HPLC was performed using a Phenomenex–Luna RP–C18 column (150 × 4.6 mm, 5 μ m particle size) used as the stationary phase at a 1.0 mL/min flow rate. Semi-preparative HPLC was performed using a Phenomenex-Luna RP–C18 column (250 × 10.0 mm, 5 μ m particle size) with a 2.3 mL/min flow rate. Column chromatography (CC) was carried out on a Silica gel 60 Å (70–230 mesh ASTM) (Merck KGaA, Darmstadt, Germany). Thin-layer chromatography (TLC) analyses were carried out on silica gel 60 F254 plates (Merck KGaA, Darmstadt, Germany) using phosphomolybdic acid as a color reagent.

Fungal Material

The reference strain of *A. dauci* (FRA017) used in this investigation was isolated in 2000 from naturally infected carrot leaves collected in Gironde, France; fungal structures isolated from lesions were confirmed as *A. dauci* based on conidial morphology and molecular analysis using the elongation factor 1α (EF-1 α) and ITS sequences [12]. The fungal strain FRA017 has been deposited in the Institut de Recherche en Horticulture et Semences (IRHS) in Angers, France. For the production of conidia, *A. dauci* (FRA017) was grown on V8 juice agar medium [200 mL V8 vegetable juice (HERDEZ[®]), 3 g CaCO₃, 15 g agar L⁻¹, pH 6.8], and incubated at 24 ± 2 °C for 20 days under natural light conditions.

Fungal Growth Conditions

Fernbach flasks containing 1 L of Czapek-Dox liquid medium [1.5 g of potassium nitrate (Sigma-Aldrich[®]), 0.5 g of potassium chloride (Fermont), 0.5 g of magnesium sulfate (Sigma-Aldrich[®]), 0.01 g of ferric sulfate (Sigma-Aldrich[®]), 1.0 g of monobasic potassium phosphate (J.T. Baker[®]), 6 g of sucrose (commercial brand), and 2.0 g of casein (Sigma Aldrich[®]) in 1 L of distilled water, with final pH 6.0] were inoculated with a conidial suspension adjusted to 1 × 10⁴ conidia mL⁻¹ of *A. dauci*. Fungal cultures were grown at room temperature (25 °C), under shake (100 rpm) conditions, for 96 hours.

Extraction, Isolation and Purification

The mycelia and culture filtrates from a 15 L culture of *A. dauci* were separated by filtration through cheesecloth. The aqueous culture filtrate was extracted with ethyl acetate (three times, 2:1, v/v), and the organic layer was evaporated *in vacuo*, yielding 432.5 mg of extract (ca. 45 mg L⁻¹). A portion of the organic crude extract (ca. 420 mg) was suspended in 50 mL of a 95:5 mixture of water/methanol, and the resulting suspension was partitioned successively between hexane (3 × 50 mL) and ethyl acetate (3 × 50 mL), to produce three fractions: hexane (**A**; 43.8 mg), ethyl acetate (**B**; 202.7 mg) and aqueous (**C**; 132.6 mg). The ethyl acetate fraction (**B**) was subjected to column chromatography eluting with mixtures of hexane-dichloromethane-methanol to yield sixteen semipurified fractions (**B1-B16**). Reverse-phase High-Performance Liquid Chromatography (HPLC) purification (Phenomenex-Luna RP-C18 250 × 10.0 mm column; 2.3 mL min⁻¹) of fractions **B6** to **B9**, and **B12**, using gradient elution from 10 % to 90 % aqueous acetonitrile for 20 minutes, resulted in the isolation of the seven diketopiperazines.

cyclo-(pro-val) (**1**): white powder (1.1 mg, 0.25%); UV (PDA, CH₃CN/H₂O) λ_{\max} 203 nm; ¹H NMR (600 MHz, CD₃OD) δ 4.20 (1H, dd, *J* = 8.4, 6.6 Hz, H-6), 4.04 (1H, s, H-3), 3.56 (1H, m, H-9), 3.51 (1H, m, H-9), 2.49 (1H, m, H-7), 2.32 (1H, m, H-10), 2.02 (1H, m, H-8), 1.94 (1H, m, H-7 and H-8), 1.09 (3H, d, *J* = 7.2 Hz, CH₃-11), 0.94 (3H, d, *J* = 6.6 Hz, CH₃-11'); ¹³C NMR (150 MHz, CD₃OD) δ 172.6 (C-2), 167.6 (C-5), 61.5 (C-6), 60.0 (C-3), 46.2 (C-9), 29.9 (C-10), 29.5 (C-7), 23.3 (C-8), 18.8 (C-11), 16.7 (C-11'); EIMS *m/z* 196 [M]⁺ (5), 154 (C₇H₁₀N₂O₂, 100), 125 (C₆H₇NO₂, 44), 70 (C₄H₈N⁺, 82).

cyclo-(pro-leu) (**2**): white powder (0.6 mg, 0.14%); UV (PDA, CH₃CN/H₂O) λ_{\max} 200 nm; ¹H NMR (400 MHz, CD₃OD) δ 4.26 (1H, t, *J* = 7.6 Hz, H-6), 4.13 (1H, m, H-3), 3.51 (2H, dd, *J* = 8.6, 4.8 Hz, H-9), 2.31 (1H, m, H-7), 2.01 (2H, m, H-8 and H-10), 1.90 (3H, m, H-7, H-8 and H-10), 1.52 (1H, t, *J* = 8.0 Hz, H-11), 0.97 (3H, d, *J* = 6.4 Hz, CH₃-12), 0.96 (3H, d, *J* = 6.4 Hz, CH₃-12'); ¹³C NMR (100 MHz, CD₃OD) δ 172.8 (C-2), 168.9 (C-5), 60.3 (C-6), 54.7 (C-3), 46.4 (C-9), 39.4 (C-10), 29.1 (C-7), 25.8 (C-11), 23.7 (C-8), 23.3 (C-12), 22.2 (C-12'); EIMS *m/z* 210 [M]⁺ (5), 154 (C₇H₁₀N₂O₂, 100), 125 (C₆H₉N₂O⁺, 18), 86 (C₅H₁₂N⁺, 71), 70 (C₄H₈N⁺, 69).

cyclo-(pro-phe) (**3**): white powder (1.1 mg, 0.25%); UV (PDA, CH₃CN/H₂O) λ_{\max} 203 and 257 nm; ¹H NMR (600 MHz, CD₃OD) δ 7.27 (5H, m, H-2'-H-6'), 4.45 (1H, dd, *J* = 5.4, 4.8 Hz, H-6), 4.07 (1H, dd, *J* = 10.8, 6.6 Hz, H-3), 3.54 (1H, m, H-9), 3.37 (1H, m, H-9), 3.17 (2H, t, *J* = 4.8, 3.6 Hz, H-10), 2.10 (1H, m, H-7), 1.80 (2H, m, H-7 and H-8), 1.23 (1H, m, H-8); EIMS *m/z* 244 [M]⁺ (40), 153 (C₇H₉N₂O₂, 33), 125 (C₅H₄N₂O₂, 100), 91 (C₇H₇, 51), 70 (C₄H₈N⁺, 40).

cyclo-(val-leu) (**4**): white powder (1.2 mg, 0.28%); UV (PDA, CH₃CN/H₂O) λ_{\max} 203 nm; ¹H NMR (600 MHz, CD₃OD) δ 3.95 (1H, dd, *J* = 4.8, 4.2 Hz, H-6), 3.77 (1H, dd, *J* = 4.2, 1.2 Hz, H-3), 2.23 (1H, m, H-7), 1.87 (1H, m, H-9), 1.75 (1H-m, H-10), 1.61 (1H, m H-9), 1.05 (3H, dd, *J* = 6.6, 1.8 Hz, CH₃-8), 0.97 (3H, dd, *J* = 7.2, 1.8 Hz, CH₃-8'), 0.96 (3H, dd, *J* = 7.2, 1.8 Hz, CH₃-11), 0.96 (3H, dd, *J* = 6.6, 1.8 Hz, CH₃-11'); EIMS *m/z* 170 (C₈H₁₄N₂O₂, 27), 156 (C₇H₁₂N₂O₂, 100), 113 (C₆H₁₁NO, 59), 72 (C₅H₉, 50).

cyclo-(val-phe) (5): white powder (1.2 mg, 0.28%); UV (PDA, CH₃CN/H₂O) λ_{\max} 203 and 258 nm; ¹H NMR (600 MHz, CD₃OD) δ 7.25 (5H, m, H-2'~H-6'), 4.31 (1H, t, J = 5.4, 4.8 Hz, H-3), 3.65 (1H, d, J = 4.8 Hz, H-6), 3.23 (1H, dd, J = 13.8, 5.4 Hz, H-9), 3.04 (1H, dd, J = 13.8, 4.8 Hz, H-9), 1.65 (1H, m, H-7), 0.80 (3H, dd, J = 7.2, 1.8 Hz, H-8), 0.44 (3H, dd, J = 7.2, 1.8 Hz, H-8'); EIMS m/z 246 [M]⁺ (42), 127 (C₅H₇N₂O₂, 58), 91 (46), 91 (C₇H₇, 100).

cyclo-(leu-phe) (6): white powder (1.3 mg, 0.30 %); UV (PDA, CH₃CN/H₂O) λ_{\max} 203 and 257 nm; ¹H NMR (600 MHz, CD₃OD) δ 7.25 (5H, m, H-2'~H-6'), 4.31 (1H, t, J = 4.8, 3.6 Hz, H-3), 3.66 (1H, dd, J = 4.8, 4.2 Hz, H-6), 3.29 (1H, dd, J = 3.6 Hz, H-10), 2.95 (1H, dd, J = 4.8 Hz, H-10), 1.43 (1H, m, H-8), 0.87 (1H, m, H-7), 0.73 (3H, dd, J = 7.2, 0.6 Hz, H-9), 0.69 (3H, dd, J = 7.8, 0.6 Hz, H-10), 0.08 (1H, m, H-7); EIMS m/z 260 [M]⁺ (22), 204 (C₁₁H₁₂N₂O₂, 40), 141 (C₈H₁₅NO, 52), 113 (C₄H₅N₂O₂, 44), 91 (C₇H₇, 100).

cyclo-(leu-tyr) (7): white powder (0.8 mg, 0.19%); UV (PDA, CH₃CN/H₂O) λ_{\max} 203, 225 and 275 nm; ¹H NMR (600 MHz, CD₃OD) δ 7.00 (2H, dd, J = 8.3, 1.8 Hz, H-2' and 6'), 6.71 (2H, dd, J = 8.3, 1.8 Hz, H-3' and 5'), 4.23 (1H, t, J = 3.6 Hz, H-3), 3.66 (1H, dd, J = 9.6, 4.2 Hz, H-6), 3.20 (1H, dd, J = 13.8, 3.6 Hz, H-10), 2.83 (1H, dd, J = 13.8, 3.6 Hz, H-10), 1.44 (1H, m, H-8), 0.89 (1H, m, H-7), 0.76 (3H, d, J = 6.0 Hz, CH₃-9), 0.74 (3H, d, J = 6.6 Hz, CH₃-9'), 0.13 (1H, m, H-7); EIMS m/z 276 [M]⁺ (8), 170 (C₈H₁₄N₂O₂, 96), 107 (C₇H₇O, 100), 70 (C₅H₉, 90).

Leaf-spot assay

Parsley (*Petroselinum crispum*) leaves were excised and washed with water. A 20 μ L drop of the sample (5.0 mg/mL for extracts and fractions), non-inoculated medium (negative control), or sterile distilled water (blank), was placed over a lightly scratched area made with a sterile, trimmed paintbrush on the adaxial face of the leaf. The leaves were kept in a high humidity chamber under natural light. Three leaves, with two applications each, were used for each treatment [16].

Results and discussion

The culture filtrate of *A. dauci* was separated from the mycelial mat and extracted with ethyl acetate, and the resulting crude extract was partitioned with solvents of increasing polarity to produce three fractions that were subjected to biological evaluation. Evaluation of the phytotoxic activity of the extract and the different fractions using the leaf-spot assay, which has been shown to be an effective method to identify phytotoxic bioactive fractions and pure metabolites, showed strong activity in the medium polarity (ethyl acetate, **B**) fraction. Column chromatography purification of the phytotoxic fraction **B**, followed by semipreparative HPLC purification of the resulting phytotoxic fractions **B6** and **B7**, yielded three diketopiperazines in pure form: cyclo-(pro-val) (**1**), cyclo-(pro-leu) (**2**) and cyclo-(pro-phe) (**3**). Similar semipreparative HPLC purification of fractions **B8**, **B9**, and **B12** allowed the purification of four additional diketopiperazines: cyclo-(val-leu) (**4**), cyclo-(val-phe) (**5**), cyclo-(leu-phe) (**6**), and cyclo-(leu-tyr) (**7**) (Fig. 1). The GC-MS analysis of **1** showed a single component with a parent ion peak at m/z 196, corresponding to a molecular formula of C₁₀H₁₆N₂O₂. The nature of the two nitrogen atoms in the structure **1** was established from its ¹³C NMR spectrum which showed the characteristic amide carbonyls of a piperazine-2,5-dione at δ_C 172.6 and 167.6 [17], together with two methine signals at δ_C 61.5 and 60.0 ppm corresponding to the sp³ carbons bonded to nitrogen [18]. The low field chemical shift of the methine proton signals (δ_H 4.20 and 4.04) in the ¹H NMR spectrum of **1** further confirmed them being on a nitrogen-bonded carbon, *a* to a carbonyl group. Comparing this data, including additional signals in the ¹H and ¹³C NMR spectra corresponding to three methylenes (δ_C/δ_H 46.2, 29.5 and 23.3 / 3.54, 2.49 and 2.02) and two methyl groups (δ_C/δ_H 18.8 and 16.7 / 1.09 and 0.94), with those reported in the literature allowed the identification of **1** as cyclo-(pro-val) [19,20], a bicyclic diketopiperazine isolated from *Aspergillus ochraceus* and *Oospra destructor* [19] and recently reported from marine sponge-associated bacteria [21].

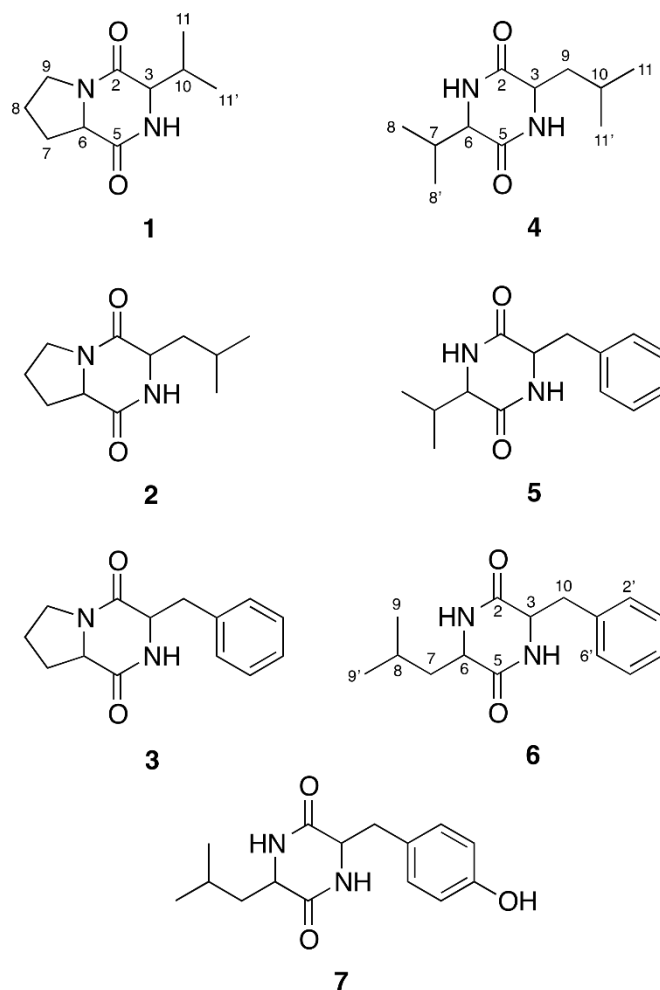


Fig. 1. Diketopiperazines identified from the crude extract of *A. dauci*

The ^1H and ^{13}C NMR spectra of **2** proved to be very similar to that of **1**, the only difference being the presence of a two-proton signal at δ_{H} 2.01 and 1.90 in ^1H NMR spectrum of **2**, indicating the presence of an extra methylene group in the side chain. This was confirmed by the parent ion peak at m/z 210 observed in the GC-MS analysis of **2**, allowing its identification as cyclo-(pro-leu); final confirmation came from comparing the spectroscopic data of **2** with those reported in the literature [20,22]. The ^1H NMR spectrum of **3** showed the characteristic signals of a diketopiperazine, together with signals for a mono-substituted aromatic ring and a two-proton signal corresponding to benzylic methylene; comparing this data with those reported in the literature allowed the identification of **3** as cyclo-(pro-phe) [20,23]. Diketopiperazines **5–7** showed similar spectroscopic data to those of **1–3** and were identified based on their MS and ^1H NMR data as cyclo-(val-leu) (**4**), cyclo-(val-phe) (**5**), cyclo-(leu-phe) (**6**) and cyclo-(leu-tyr) (**7**) [23–25].

The seven diketopiperazines isolated from the phytotoxic ethyl acetate fraction of the culture filtrate of *A. dauci* have been previously reported from different microorganisms. Diketopiperazines **1–4** have recently been reported from culture supernatants of *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida*, *P. alcaligenes*, *Proteus mirabilis*, *Enterobacter agglomerans*, *Vibrio vulnificus*, and *Citrobacter freundii* [26]. While, cyclo-(val-phe) (**5**) was isolated from a culture of a marine bacteria found in marine sediments and is reported to enhance the plasticity of various axonal populations, suggesting that it may stimulate spinal cord repair by

promoting compensatory sprouting of spinally projecting axons [27]. Similarly, diketopiperazines 1–3 were isolated from the co-culture of sponge-associated Actinomycetes, *Actinokineospora* sp., and *Nocardioopsis* sp. Cyclo-(leu-phe) (6) was isolated from roasted coffee [24]; diketopiperazines 3 and 7 have been reported from *Callyspongia* sp., and cyclo-(leu-tyr) (7) has been reported to stimulate MCP-1 (monocyte chemoattractant protein-1) [21,28].

Diketopiperazines are recognized as an important class of biologically active natural products, with a potential application in drug discovery and development because of their diverse pharmaceutical effects, including antibacterial, antifungal and antiviral [29–31] as well as different types of biological activities such as cytotoxic [32], free radical scavenging [33,34], quorum-sensing signaling regulation [23,26], and plant-growth promotion [35,36]. Most importantly, several diketopiperazines have been shown to be phytotoxic, including (L-trp-L-val) isolated from the blackleg fungus *Phoma lingam* [37]; and exserohilone and dihydroexserohilone produced by *Exserohilum holm* [38]; the thaxtomins, a group of phytotoxins characterized as 4-nitroindol-3-yl-containing 2,5-dioxopiperazines, produced by plant-pathogenic bacteria of the genus *Streptomyces* that cause the common scab disease of potato [37,39], and have been shown to cause cell swelling, reduce seedling growth, and inhibit cellulose synthesis in plants [40]; and maculosin [cyclo(L-Tyr-L-Pro)], the first host-specific fungal phytotoxin produced by a weed pathogen, causing necrotic lesions on spotted knapweed (*Centaurea maculosa*) [11,38]. Even though the limited amounts of material prevented evaluation of the isolated diketopiperazines 1–7 in the leaf spot assay, three of the diketopiperazines isolated during this investigation, namely cyclo-(pro-val) (1), cyclo-(pro-leu) (2), and cyclo-(pro-phe) (3), have been previously reported as phytotoxic metabolites [9, 22, 41]. Finally, considering the fact that previously reported phytotoxic diketopiperazines have been identified as having the L,L configuration, it is possible that metabolites 1, 2, and 3, obtained from fractions having phytotoxic activity, might correspond to the same isomers.

Conclusion

This is the first report of diketopiperazines, a completely different class of metabolites produced by *Alternaria dauci*, which could be involved in the plant-pathogen interaction between carrot plants and the phytopathogen.

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