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Massarilactones D and H, phytotoxins produced by *Kalmusia variispora*, associated with grapevine trunk diseases (GTDs) in Iran

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ABSTRACT

A strain of *Kalmusia variispora* associated with grapevine trunk diseases (GTDs) was identified in Iran and induced disease symptoms on the host in greenhouse conditions. The grapevine pathogens are able to produce a plethora of toxic metabolites belonging to different classes of naturally occurring compounds. Two homogeneous compounds were isolated from the organic extract of *K. variispora* culture filtrates. They were identified by physic (specific optical rotation), and spectroscopic (essentially 1D ^{1}H and ^{13}C NMR and HR ESIMS) methods as the fungal polyketides massarilactones D and H (**1** and **2**). The unassigned absolute configuration of massarilactone D was unambiguously determined by X-ray diffractometric analysis. Massarilactones D and H showed phytotoxic activity on *Vitis vinifera* L. at two concentrations used and depending from the days of inoculation. Phytotoxicity is also increased when the 3,4,7-*O*,*O'*,*O''*-triacetyl derivative of massarilactone D (**3**) was assayed on the host plant. This is the first report on the investigation of phytotoxic metabolites produced by *K. variispora* isolated from infected grapevine in Iran and they seem to be involved in the development of disease symptoms.

KEYWORDS

Grapevine trunk diseases (GTDs); *Kalmusia variispora;* phytotoxins; massarilactone D and H; absolute configuration of massarilactone D

1. Introduction

Grapevine is now cultivated and distributed all around the world and its economic impact is essentially due to the vine production. Thus, the management of abiotic and biotic stresses that strongly affected the specific organoleptic feature and vine production industry has received increased attention. Among biotic stresses the phytopathogenic fungi play a significant role for the negative effects on the quantity and quality of harvested grapevine. Grapevine trunk diseases (GTDs) are among the most important fungal diseases of grape. Botryosphaeria dieback, esca and eutypa dieback are well-known and the most significant grapevine trunk diseases which lead to grape decline and threaten the grape cultivation and wine industry (Bertsch et al., 2013). Esca disease complex the most important grapevine truck disease with five syndromes including brown wood streaking, Petri disease, young esca or grapevine leaf stripe disease (GLSD), esca and esca proper is associated with the tracheomycotic fungal species belong to ascomycetes genera (i.e. *Phaeomoniella* and *Phaeoacremonium*) and basidiomycetes fungi such as *Fomitiporia* and other genera (Mugnai et al., 1999; Wagschal et al., 2008; Andolfi et al., 2011; Bertsch et al. 2013; Fisher et al., 2019). *Eutypa lata* and its relatives are associated with eutypa dieback (Fallot et al., 1997; Jiménez-Teja et al., 2006; Wagschal et al., 2008; Andolfi et al., 2011; Bertsch et al. 2013), and several species of *Botryosphaeriaceae* are involved in botryosphaeria dieback (Andolfi et al. 2011; Bertsch et al. 2013; Fontaine et al., 2016; Billones-Baaijens and Savocchia 2019). Apart from these well-known and common fungi several different fungal species have been reported in association with grapevine trunk diseases (Jayawardena et al. 2018; Abed-Ashtiani et al. 2019).

These fungi produce a plethora of phytotoxic secondary metabolites the play an important role in the induction of the disease symptoms (Bruno et al., 2007; Evidente et al., 2010; Andolfi et al., 2011; Bertsch et al. 2013). They belong to several different class of natural compounds such as chromanones, cyclohexen epoxide, dihydrofuranes, jasmonic acid ester, naphthalenones, and related compounds and a recent perspective review has been reported on the advanced results on this topic (Masi et al., 2018).

During August to November 2016 in a survey on grapevine trunk diseases in vineyards located in Ilam, Kermanshah, Lorestan and Markazi provinces 19 fungal species belong to different group of fungi were isolated from grapes showing general decline symptoms and vascular discoloration and necrosis related to grapevine trunk diseases. Of which, *Kalmusia variispora* (syn. = *Dendrothyrium variisporum*) isolated from infected wood tissues (Figure 1A) was identified based on ITS phylogeny using maximum parsimony analysis and confirmed as pathogen on 2-year old grapevines in greenhouse condition (Figure 1B). Thus far, this species has been reported from Iran and Syria on *Vitis vinifera*, *Vitis* sp., *Rosa hybrid* and *Pinus eldarica* (Farr and Rossman, 2020), but no studies were carried out on the phytotoxic metabolites produced in vitro by *K. variispora*.

This manuscript reports the isolation and the identification of the two main phytotoxic metabolites produced *in vitro* by a strain of *K. variispora* isolated from infected grapevine in Iran. The phytotoxic activity of these compounds on host plant is also reported for the first time as well as the unambiguously assignment of absolute configuration of massarilactone D.

2. Results and discussion

The organic extract of the culture filtrates of *K. variispora* was purified, by combination of column and TLC as detailed described in the Experimental section, to yield two homogeneous compounds identified as massarilactones D and H (**1** and **2**, Figure 2). In particular, **1** was identified by its optical (specific optical rotation) and spectroscopic properties (essentially ^{1}H NMR and HR ESIMS, see Figures S6 and S7 and Tables S1). However, the absolute configuration (AC) of **1** remained undetermined considering that Kock et al. (2007) assigned to **1** the same AC of massarilactone B (**4**, Figure 1) only on the basis of their specific optical rotation similarity. The AC of **4** was previously assigned by X-ray of its *bis*-(4-bromobenzoate) ester (**5**, Figure 1) when it was isolated together to massarilactone A as an antibiotic metabolite from the culture of the freshwater aquatic fungus *Massarina tunicate* (Oh et al. 2001). **1** gave optical active single crystal by slow evaporation from *i*-PrOH-H_2O (99:1) solution at room temperature, and its absolute configuration was unambiguously assigned by X-ray diffractometric analysis. Massarilactone D (**1**) crystallizes in the monoclinic P

$2_12_12_1$ space group with one molecule of **1** and one H_2O solvent molecule contained in the asymmetric unit. Bond lengths and angles are in the normal range and clearly indicate the presence of sp^2 hybrid carbon atoms at C5, C4a and C7a (Tables S2–S4 of Supplementary Materials). The molecule consists of one six- and one five-membered rings condensed through a double bond at C4a-C7a junction and correspond to a α,β unsaturated lactone. The ring core assumes an almost planar conformation with a degree of flexibility in the six-membered ring that adopts a half-chair conformation with C2 and C3 atoms up and down the mean plane C4/C4a/C7a/O1 (Figures S2 and S3). The hydroxy groups occupy axial positions and are involved in a 3D hydrogen bonding pattern that includes the solvent H_2O molecules in the crystal packing (Figure S4). Four chiral centres are present at C2,C3,C4,C7 and the 2*S*,3*R*,4*S*,7*S* AC was assigned by X-ray analysis accordingly to the literature methods reported to assign the AC in light-atom structures when MoKα radiation is used (Escudero-Adan et al. 2014; Parsons et al. 2013; Parsons 2017). The crystal data are reported in Tables S2–S4.

Massarilactone D (**1**) was also converted into the 3,4,7-*O*,*O*′,*O*″-triacetyl derivative (**3**) by usual acetylation carried out with pyridine and acetic anhydride. The ^{1}H NMR of **3** (Table S1, Figures S10 and S11) differed from that of **2** for the downfield shifts (Δδ 0.84 and 0.87) of H-4 and H-3, resonating as two broad singlets at δ 5.17 and 4.69, respectively, and for the presence of a broad singlet at δ 2.1 due to the overlapped signals of the three acetyl groups. Its ESI MS showed the sodiated dimer $[2M + Na]^+$ and sodiated $[M + Na]^+$ adducts at *m/z* 759 and 391 and the significant fragmentation ions due to successive losses of two acetic acid moieties $[M + H - AcOH]^+$ and $[M + H - 2xAcOH]^+$ and a ketene residue $[M + H - 2xAcOH - CH_2CO]^+$ at *m/z* 309, 249, 207.

Massarilactone H (**2**), obtained as an amorphous solid was identified by comparing its optical (specific optical rotation) and spectroscopic (^{1}H NMR and ESI MS, see Figures S8 and S9) data with those reported by Zhang et al. (2012).

Massarilactone D (**1**) was previously isolated from the endophyitic fungus *Coniothyrium* sp. obtained from *Carpobrotus edulis*, together with massarilactone C, massarigenin E, coniothyrenol, graphislactone A and massarilactone A (Kock et al. 2007). When tested at 5 mg/mL for antifungal, antibacterial and antialgal activities, **1** as well as all the other metabolites were inactive against the gram-positive bacterium, *Bacillus megaterium*, the fungus, *Microbotryum violaceum*, and the green alga, *Chlorella fusca* (Kock et al. 2007).

Massarilactone H (**2**) was previously isolated, together with the new polyketide arthropsadiol C and the already known massarilactones B, E and G, massarigenins C and D, and enalin A, from the culture filtrates of the marine-derived fungus *Phoma herbarum*. Its structure was determined by

single-crystal X-ray analysis of its 3,4-*O*,*O'*-dibenzoate derivative. **2** showed moderate neuraminidase inhibitory activity compared to the positive control oseltamivir (Zhang et al. 2012).

Successively, both massarilactones D and H, were isolated together with the two new furanone derivatives (5*S*)-*cis*-gregatin B and graminin D from the culture filtrates extract of *Dendrothyrium variisporum*, a fungus isolated from the roots of the Algerian plant *Globularia alypum*. Massarilactone D, three new and two known anthranilic acid analogues, and three cyclopeptides were biosynthesized from the same fungus when fermented in large scale (Teponno et al. 2017). Massarilactones D and H when tested for antimicrobial and cytotoxic activities against various bacteria, fungi, and two mammalian cell lines did not showed any biological activity (Teponno et al. 2017). Some pyranfuranones close related to massarilacones D and H, named isoaigialones A, B, and C were recently isolated from the organic extract of a *Phaeoacremonium* sp. culture filtrates, an endophytic fungus obtained from the leaves of *Senna spectabilis*. The already known and related aigialone was also isolated from the same organic extract and showed antifungal activity against the phytopathogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum* (Silva et al. 2017).

Assayed on *Vitis vinifera* L. (Table S5, Figure S5), massarilactones D and H showed phytotoxic effects at two concentrations used (10^{-3} and 10^{-4} M) and depending from the days of inoculation. Phytotoxicity is also increased when the 3,4,7-*O*,*O'*,*O''*-triacetyl derivative of massarilactone D (**3**) was assayed on the same host plant. In particular, 72 h after inoculation **3** induced severe necrosis and shriveling of the leaves at both concentrations while a marked shriveling and moderate wilting was observed for both massarilactones D and H depending from concentration. The highest phytotoxicity of **3** in respect to **1** and **2** could be explained by the known lethal metabolism (Hassal 1990). In fact, due to its higher liphofilicity, **3** could more easily translocated across cell membrane and then at physiological pH be hydrolyzed in massarilactone D (**1**).

3. Experimental

The general experimental procedures, the identification data and the ^{1}H NMR and HRESI MS spectra of compounds **1** and **2**, the preparation of compound **3** and its spectroscopic data, X-ray crystallographic analysis of **1** and the phytotoxic bioassays of **1**–**3** are available as supplementary materials.

3.1. Fungal isolates and culture conditions

The strain of *K. variispora* (BMM-K1) used in this study was obtained from grapevine shoot of a vineyard located in Ostorinan, Lorestan Province, Iran showing decline symptoms and vascular discoloration and necrosis related to grapevine trunk diseases. DNA extraction and identification of the strain BMN-K1 based on ITS sequence data were carried out as described by Abdollahzadeh et al. (2009). The strain BMN-K1 was stored on potato dextrose agar (PDA) at 4 °C in fungal collection of the Department of Plant Protection, University of Kurdistan, Iran. For phytotoxin production it was inoculated and grown in stationary culture of potato dextrose broth (PDB) for 21 days at 25 °C in the dark. The mycelium was removed, and the liquid cultures were lyophilized prior to the extraction procedure.

3.2. Extraction and purification of K. variispora phytotoxins

The culture filtrates (4 L) of *K. variispora* were dissolved in 1/10 of the initial volume (pH 6) and extracted with EtOAc (3 x 400 mL). The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure giving a corresponding oily residue (370 mg). The resultant aqueous phase was acidified (pH 2.5) with 1M formic acid and extracted as reported above yielding a corresponding oily residue (330 mg). The neutral organic extract was purified by silica gel column chromatography, eluted with $CHCl_3$-*i*-PrOH (9:1) to (7:3) yielding seven homogeneous fraction groups. The residue of fraction 2 (70 mg) was further purified by TLC eluted with $CHCl_3$-*i*-PrOH (98:2), resulting in one amorphous solid identified as massarilactone H (**2**, Figure 1, 50 mg). The residue of fraction 3, crystallized from slow evaporation of a *i*-PrOH-H_2O (99:1) solution at room temperature and the crystals were identified by as massarilactone D (**1**, Figure 1, 200 mg). The acidic organic extract was purified by silica gel column chromatography, as reported above yielding a further amount of massarilactone D (90 mg).

4. Conclusions

This manuscript reports for the first time the isolation and identification of the known fungal polyketides massarilactones D and H as the two main phytotoxic metabolites produced *in vitro* by a strain of *K. variispora* isolated from infected grapevine tissues in Iran. Furthermore, the unambiguously assignment of absolute configuration of massarilactone D, determined by X-ray diffractometric analysis, is reported. This is the first report on the investigation of the phytotoxic secondary metabolites produced by *K. variispora* isolated from infected grapevine in Iran.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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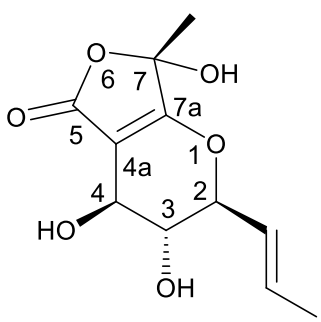
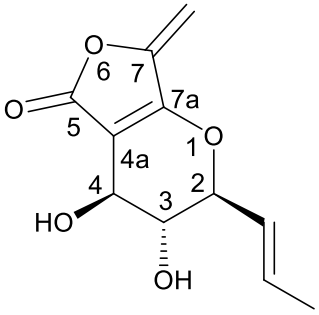


1, R=H
3, R=***p***-Br-PhCO
4, R=Ac



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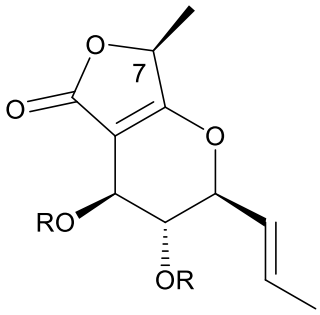

5, R=H
6, R=***p***-Br-PhCO

Figure 1. The structure of compounds **1**–**5**.