Phytotoxins produced by *Didymella glomerata* and *Truncatella angustata*, associated with grapevine trunk diseases (GTDs) in Iran

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**ABSTRACT**

*Didymella glomerata* and *Truncatella angustata* associated with grapevine trunk diseases (GTDs) in Iran, were grown *in vitro* to evaluate the production of phytotoxic metabolites as potential pathogenicity determinants. 2,5-Dihydroxymethylfuran and (+)-6-hydroxyramulosin were isolated from the culture filtrates of *D. glomerata* and *T. angustata*, respectively. They were identified by physical and spectroscopic (essentially 1D and 2D 1H and 13C NMR and ESIMS) methods and X ray analysis. Both compounds induced significant necrosis and curling on the leaves of the host plant *Vitis vinifera* L. and the effects were concentration dependent. No effect was observed on the leaves of the non-host *Solanum lycopersicum* L. plant.

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1. Introduction

Grapevine cultivation and related industries are worldwide distributed and have important economical aspects in countries of both hemispheres. As the other plants, grapevine is affected by various microbial pathogens, essentially fungi, which cause considerable damage to the quantity and quality of harvested grapevine. *Botryosphaeria* dieback, *esca* and eutypa dieback are the most significant and serious grapevine trunk diseases (GTDs) which have heavy effects on grape production (Masi et al. 2018). *Esca*, which is a complex disease, is mainly associated with tracheomycotic...
fungi as *Phaeomoniella clamydospora* and *Phaeoacremonium aleophilum* (re-classified as *P. minimum*) and *Fomitiporia mediterranea*. Eutypa dieback is induced by *Diatrypaceae* members especially *Eutypa lata*, while Botryosphaeria dieback is caused by several *Botryosphaeriaceae* species (Masi et al. 2018; Billones-Baaijens and Savocchia 2019). Recently, several other fungi have been identified in association with grapevine trunk diseases (Jayawardena et al. 2018; Abed-Ashtiani et al. 2019).

These fungi produce various phytotoxins belong to different classes of natural compounds such as chromanones, cyclohexene epoxides, dihydrofurans, jasmonic acid esters, naphthalenones, naphthoquinones, phenols and related compounds (Masi et al. 2018; Xu et al. 2021). Several researchers have reported some of these metabolites as pathogenicity determinants and confirmed their role in induction of the disease symptoms (Masi et al. 2018).

Recently, massarilactones D and H, which are two polyketides, were isolated from a strain of *Kalmusia variispora* associated with grapevine trunk diseases (GTDs) in Iran. They showed phytotoxic activity on *Vitis vinifera* L. at both concentrations used (10^{-3} and 10^{-4} M) and the effect was time dependent (Cimmino et al. 2020).

*Didymella glomerata* (tub2 phylogeny) and *Truncatella angustata* (ITS phylogeny), which were identified on phylogenetic analyses of DNA sequence data, were isolated as fungi associated with grapevine trunk diseases in vineyards. The grapevine showed esca and decline symptoms in aerial part and vascular tissue necrosis in cross-sections of cordon and trunks (Figure 1A, C). The generated sequences of the two strains were submitted to GenBank (ITS = MZ350092; tub2 = MZ351888).

*D. glomerata* (Basionym: *Coniothyrium glomeratum*), also known as *Aposphaeria glomerata*, *Phoma glomerata* and *Peyronellaea glomerata* (Chen et al. 2015a), has been
reported as an endophyte or pathogenic species associated with various woody plants such as *V. vinifera* (Deb et al. 2020; Farr and Rossman 2021). Recently, *D. glomerata* was isolated as an endophytic fungus from *Saussurea laniceps*, a rare plant in Himalaya, South-West China and Northern Birmania. It produced a new naphthalenone, named didymelol A, and three new naphthols, named didymelols B-D, together with three known naphthalenones, which were assayed for cytotoxic activity against non-small cell lung cancer A549 cells breast carcinoma MDA-MB-435 cells (Luo et al. 2020).

*T. angustata* is a well-known pestalotioid species belong to the *Sporocadaceae* family, which has also been isolated from different trees as endophyte or pathogenic species in association with various fungal diseases (e.g., fruit rot, canker, dieback and decline), especially grapevine trunk diseases (Farr and Rossman 2021). As a sponge-associated fungus this species produced fourteen new isoprenylated cyclohexanols, namely truncateols A-N, and truncateol M which showed potent inhibitory effect against influenza A (Zhao et al. 2015). Furthermore, the same strain of *T. angustata*, grown in solid culture, produced eight new isoprenylated cyclohexanols, namely truncateols O-V, together with 14 known analogues, with truncateols O and P exhibiting inhibition toward both HIV-1 and H1N1 virus and HIV-1 virus, respectively (Zhao et al. 2018). It also synthesises new α-pyrone, namely angupyrones A-E, which showed a moderate antioxidant activation in HepG2C8 cells (Zhao et al. 2017).

To our knowledge, this paper reports for the first time *D. glomerata* as pathogenic fungus associated with grapevine trunk diseases in the world, the phytotoxic secondary metabolites produced by *D. glomerata* and *T. angustata* in vitro and evaluation of their phytotoxicity on host and non-host plants.

2. Results and discussion

Pathogenicity of both identified species *D. glomerata* and *T. angustata* was confirmed under greenhouse conditions. Disease symptoms including leaf yellowing and necrosis and vascular tissues necrosis on cross-sections of inoculated stems were observed (Figure 1B, D).

The organic extracts of the culture filtrates of *D. glomerata* and *T. angustata* were purified, by combination of column and TLC as detailed described in the Experimental section. The metabolite isolated from *D. glomerata* was identified as the 2,5-dihydroxymethylfuran (1, Figure 2) comparing its spectroscopic (1H, 13C, HSQC and HMBC NMR

![Figure 2. The structure of compounds 1 and 2.](image-url)
spectra, Figures S1-S4) and X-ray properties with those recently reported (Di Lecce et al. 2020).

(+)-6-hydroxyramulosin (2, Figure 2) was isolated from _T. angustata_ culture filtrates and was identified comparing its spectroscopic and physical data with those previously reported (Islam et al. 2007). In particular, its structure was confirmed by 1D and 2D 1H and 13C NMR spectroscopy (1H, 13C COSY, HSQC, HMBC NMR and ESIMS spectra) as reported in SI (Figures S5-S7, S10-S12). The relative configuration was determined by NOESY spectrum (Figures S8-S9). In fact, in addition to the expected correlation between H-3 and its geminal methyl (Me-C-3), that between the two protons at C-7, between H-6 with both protons of the adjacent methylene groups (H2C-5 and H2C-6) and Me-C-3 and H-7A, the correlation between H-4A and both H 2-5 and the weak one between H-6 and Me-C-3 and H-3 and H-4a were observed (Figure S9).

Compound 2, as well as its related metabolites, are well known as fungal phytotoxins showing a broad range of biological activity as cytotoxic, antigermination and antimicrobial properties. Several syntheses of these isocoumarins were also realised (Chen et al. 2015b). Compound 2 was isolated together with two new derivatives, namely, 7α-hydroxy-8-dihydro- and 7-keto-ramulosin, and the two already known compounds identified as ramulosin and 8-dihydro-ramulosin from _T. angustata_ obtained as an entomogeneous fungus associated to the _Septobasidium_-infected insect _Aspidiotus_ sp. All the compounds isolated were tested for their cytotoxic activity against four human carcinoma cells (HeLa, A459, MCF-7 and T24) and only (+)-6-hydroxyramulosin showed cytotoxic effects against A459 and T24 cells (Chen et al. 2015b).

When assayed on _Vitis vinifera_ L. 2,5-dihydroxymethylfuran (1) induced the highest phytotoxicity on the host plant. In fact, marked necrosis and shriveling of the leaves were observed already after 24 h of inoculation at 10^{-3} and 10^{-4} M. Necrosis and moderate wilting were also observed for (+)-6-hydroxyramulosin (2) after 48 h. However, both compounds showed phytotoxicity concentration dependent (Table 1, Figure 3). No effects were observed on the leaves of the non-host _Solanum lycopersicum_ L. plant.

### Table 1. Phytotoxicity of compounds.a

| Plant                  | 1   | 2   | Controlb |
|------------------------|-----|-----|----------|
| _Vitis vinifera_ L.    | 4   | 0   | 0        |
| _Solanum lycopersicum_ L. | 0   | 0   | 0        |

a Observations were made 48 h post treatment. Intensity of symptoms in cutting assay: 4, severe necrosis and wilting; 3, necrosis and wilting; 2, intermediate symptoms; 1, slight symptoms; 0, no symptoms.

b 4% MeOH in distilled water.

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### 3. Experimental

The general experimental procedures, the identification data and the 1D and 2D 1H and 13C NMR and HRESI MS spectra of compounds 1 and 2, are available as supplementary materials.
3.1. Fungal isolates and culture conditions

The strains of *D. glomerata* (CJAZBCMMK1) and *T. angustata* (CJAZBSRK1) used in this study were obtained from vineyards showing grapevine trunk diseases symptoms including decline and vascular discoloration and necrosis (Figure 1A, C) in Lorestan Province (Kamalabad) and Kermanshah Province (Dinavar), respectively. DNA extraction, PCR and maximum parsimony analysis were carried out as described by Abdollahzadeh et al. (2009). The identification of *D. glomerata* and *T. angustata* were identified based on a part of β-tubulin gene (*tub2*) and ITS region of ribosomal DNA, respectively. To confirm their pathogenicity under greenhouse conditions (22–28°C), Koch’s postulate was verified. Both fungal strains CJAZBCMMK1 and CJAZBSRK1 were stored on potato dextrose agar (PDA) at 4–8°C in fungal collection of the Department of Plant Protection, University of Kurdistan, Iran. For phytotoxin production they were 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 lyophilised prior to the extraction procedure.

3.2. Extraction and purification of *D. glomerata* and *T. angustata* phytotoxins

The lyophilised culture filtrates (5 L) of *D. glomerata* were dissolved in 1/10 of the initial volume (pH 5.5) and extracted with EtOAc (3 x 400 mL). The organic extracts were combined, dried (Na₂SO₄), and evaporated under reduced pressure giving a corresponding residue of 370.0 mg. This latter was purified by silica gel column...
chromatography, eluted with CHCl₃-i-PrOH (9:1) yielding seven homogeneous fraction groups. The residue of fraction 5 (10.0 mg) was further purified by TLC eluted with CHCl₃-i-PrOH (95:5), resulting in one amorphous solid. This compound crystallised from slow evaporation of a i-PrOH-H₂O (95:5) solution at room temperature and was identified by ¹H NMR and HR ESIMS and X-ray analysis as 2,5-dihydroxymethylfuran (1, Figure 2, 4.0 mg). The lyophilised culture filtrates (5 L) of T. angustata were dissolved in 1/10 of the initial volume (pH 4.5) and extracted with EtOAc (3 x 400 mL). The organic extracts were combined, dried (Na₂SO₄), and evaporated under reduced pressure giving a corresponding residue of 90.0 mg. This latter was purified by silica gel column chromatography, eluted with CHCl₃-i-PrOH (9:1) yielding seven homogeneous fraction groups. The residue of fraction 3 (9.0 mg) was further purified by TLC eluted with CHCl₃-i-PrOH (9:1), resulting in one amorphous solid identified as (+)-6-hydroxyramulosin (2, Figure 2, 5.0 mg).

3.3. Phytotoxic assays

Grapevine leaves cutting assay. Compounds 1 and 2 were assayed under the same conditions as previously reported (Cimmino et al. 2020). The compounds were dissolved in 40 µL of MeOH, and the volume was adjusted to 1 mL in sterile distilled water (SDW). The bioassays were conducted at 1 × 10⁻³, 1 × 10⁻⁴ M and 1 × 10⁻⁵ M. Disease-free grapevine leaves were harvested from the fifth node of glasshouse grown Vitis vinifera L., and the petiole of each leaf was immersed in a vial containing 1 mL of each the solutions for 24 h. SDW with 4% MeOH was used as the negative control. The leaves were then transferred to a new vial with 2 mL of SDW, placed in a growth chamber with a 12 h light/12 h darkness period at 28 °C, and maintained for an additional 24 h period. Lesions on the leaf surface were visually recorded after 48 h. Each experiment was conducted in triplicate. The same procedure was applied to test the compounds on tomato cutting.

Tomato cutting assay. Tomato (Solanum lycopersicum L.) cuttings were taken from 21-day-old seedlings, and compounds 1 and 2 assayed at 1 × 10⁻³, 1 × 10⁻⁴ M and 1 × 10⁻⁵ M. Cuttings were placed in the test solutions (2 mL) for 24 h and then transferred to distilled water. Symptoms were visually evaluated up to 7 days. MeOH in distilled water (4%) was used as negative control.

4. Conclusions

This manuscript reports for the first time the isolation and identification of the known fungal phytotoxic metabolites, namely 2,5-dihydroxymethylfuran and (+)-6-hydroxyramulosin, produced in vitro by D. glomerata and T. angustata isolated from vineyards showing grapevine trunk diseases symptoms in Iran. Considering the phytotoxicity showed on host plant and the lack of toxic effect on non-host plant (tomato), their function as the pathogenicity determinants was confirmed. The phytotoxicity of both characterised metabolites was concentration dependent. This is the first attempt to characterise the phytotoxic secondary metabolites of both D. glomerata and T.
angustata. The valuable findings contribute to increase the knowledge on the fungus-host interaction and management of grapevine trunk diseases.

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