FULL PAPER

Lasiolactols A and B Produced by the Grapevine Fungal Pathogen *Lasiodiplodia mediterranea*

by **Anna Andolfi***[a]), **Sara Basso**[a]), **Selene Giambra**[b]), **Gaetano Conigliaro**[b]), **Sandra Lo Piccolo**[b]), **Artur Alves**[c]), and **Santella Burruano**[b])

[a]) Dipartimento di Scienze Chimiche, Università di Napoli Federico II, Complesso Universitario Monte Sant'Angelo, Via Cintia 4, IT-80126 Napoli (phone: +39-081-2539179; fax: +39-081-674330; e-mail: andolfi@unina.it)
[b]) Dipartimento di Scienze Agrarie e Forestali, Università degli Studi di Palermo, Viale delle Scienze 4, IT-90128 Palermo
[c]) Departamento de Biologia, CESAM, Universidade de Aveiro, Campus Universitário de Santiago, PT-3810-193 Aveiro

A strain of *Lasiodiplodia mediterranea*, a fungus associated with grapevine decline in Sicily, produced several metabolites in liquid medium. Two new dimeric γ-lactols, lasiolactols A and B (**1** and **2**), were characterized as (2*S**,3*S**,4*R**,5*R**,2′*S**,3′*S**,4′*R**,5′*R**)- and (2*R**,3*S**,4*R**,5*R**,2′*R**,3′*S**,4′*R**,5′*R**)-(5-(4-hydroxymethyl-3,5-dimethyl-tetrahydro-furan-2-yloxy)-2,4-dimethyl-tetrahydro-furan-3-yl]-methanols by IR, 1D- and 2D-NMR, and HR-ESI-MS. Other four metabolites were identified as botryosphaeriodiplodin, (5*R*)-5-hydroxylasiodiplodin, (–)-(1*R*,2*R*)-jasmonic acid, and (–)-(3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone (**3** – **6**, resp.). The absolute configuration (*R*) at hydroxylated secondary C-atom C(7) was also established for compound **3**. The compounds **1** – **3**, **5,** and **6**, tested for their phytotoxic activities to grapevine cv. Inzolia leaves at different concentrations (0.125, 0.25, 0.5, and 1 mg/ml) were phytotoxic and compound **5** showed the highest toxicity. All metabolites did not show *in vitro* antifungal activity against four plant pathogens.

Keywords: *Lasiodiplodia mediterranea*, Botryosphaeria dieback, Phytotoxins, Lasiolactols A and B, Jasmonic acid

Introduction

In the last decade, a large number of species belonging to different genera in the fungal family Botryosphaeriaceae have been associated with Botryosphaeria dieback of grapevines (*Vitis vinifera* L.) causing sunken cankers associated with wedge-shaped lesions to vascular tissues and other disease symptoms [1 – 13]. Among the species in the genus *Lasiodiplodia* known to be pathogenic to grapevines, *L. theobromae* (Pat.) Griffon & Maubl. is the predominant one [1]. Moreover, investigations based on DNA sequence analyses of the ITS and EF-1α regions allowed the identification of cryptic species within the *L. theobromae* species complex [2-6-9].

In particular, *L. crassispora* T. I. Burgess & Barber, *L. exigua* and *L. mediterranea* Linaldeddu, Deidda & Berraf-Tebbal, *L. missouriana* Úrbez-Torres, Peduto & Gubler, *L. parva* A. J. L. Phillips, A. Alves & Crous, *L. pseudotheobromae* A. J. L. Phillips, A. Alves & Crous, *L. theobromae* and *L. viticola* Úrbez-Torres, Peduto & Gubler [1][7 – 12] have been associated with declining grapevines.

In this regard, a recent study on Botryosphaeria dieback in Sicily shows, unlike the first report [10], four Botryosphaeriaceae species associated with the syndrome [11]. The comparison of sequence data of both EF1-α and β-tubulin with those in GenBank confirmed the identification for *Diplodia seriata* De Not., *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A. J. L. Phillips and *N. vitifusiforme* (Van Niekerk & Crous) Crous, Slippers & A. J. L. Phillips, with the exception of *L. theobromae*, then named *Lasiodiplodia* sp.

Furthermore, these botryosphaeriaceous fungi can occasionally produce foliar chlorosis, characterized by fluctuating occurrence depending on the pathogen–host environment combination. Since they have never been detected in leaves, it was hypothesized that leaf symptoms could be due to fungal extracellular compounds, being produced in the woody tissues and translocated to the leaves through the transpiration stream [13]. Moreover, some Botryosphaeriaceae isolated from declining grapevines produce phytotoxic metabolites belonging to different classes of natural compounds [14][15]. So far, literature has been lacking about secondary metabolite production of *Lasiodiplodia* isolates from grapevines, except for *L. theobromae* which biosynthesizes several lipophilic and hydrophilic metabolites showing biological activities [16 – 27]. Recently, three new jasmonates, lasiojasmonates A – C, and the two natural 16-*O*-acetylbotryosphaeriolactones A and C produced by *L. mediterranea* have been investigated, but

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only the main metabolite jasmonic acid proved to be phytotoxic [28].

Since we observed a morphological analogy between the *Lasiodiplodia* sp. strain B6 and *L. mediterranea*, this research was planned with the following goals: *i*) identifying the fungal strain and confirming its pathogenicity; *ii*) isolating and characterizing the main secondary metabolites produced *in vitro* by strain B6; and *iii*) evaluating its phytotoxic and antifungal activities.

Result and Discussion

Fungal Identification

A BLASTn search of the ITS and EF1-α sequence data of strain B6 against the GenBank database retrieved 100% similarity of both regions with sequences of *L. mediterranea* (ITS: KJ170150; EF1-α: KJ638331) thus confirming the identity of the strain.

Pathogenicity Testing

L. mediterranea B6 showed pathogenic activity on the inoculated grapevines. Vascular discolorations were found upward and downward starting from the point of inoculation and were observed in all inoculated plants 6 months after inoculation, except for control (*Fig. 1*). Wedge-shaped cankers were observed in cane transversal sections of all plants inoculated. The vascular discolorations caused by *L. mediterranea* strain B6 measured 8.0 ± 0.5 cm (mean ± S.E.). Statistical analysis showed significant differences between control and inoculated canes in vascular discoloration dimensions. The inoculated

Fig. 1. Brown necrosis caused by *L. mediterranea* extending along a large part of the trunk in longitudinal section *a*) and cross section *c*). Absence of xylematic symptoms in the control in longitudinal section *b*) and cross section *d*).

fungi were always re-isolated from the inoculated canes, but never from the control.

Metabolite Identification

The fungal culture filtrates were exhaustively extracted with AcOEt at acid pH, the corresponding organic extracts were purified by combining column and TLC chromatography, as detailed in the experimental section, and accordingly six metabolites were yielded (**1** – **6**, *Fig. 2*). The structures of the known compounds were confirmed by physical and spectroscopic methods (Optical rotations, IR, UV, ^{1}H- and ^{13}C-NMR, and ESI-MS) and by comparing the data obtained with those reported in literature for botryosphaeriodiplodin [29], (5*R*)-5-hydroxylasiodiplodin [19], (1*R*,2*R*)-jasmonic acid [17][30], (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone (**3** – **6**, resp.) [28], and with an authentic sample of (±)-jasmonic acid. The preliminary ^{1}H- and ^{13}C-NMR investigation of **1** and **2** showed that they were closely structurally related to botryosphaeriolactones A and C [29], and these new compounds (described below) were named lasiolactols A and B.

Structure Elucidation of New Compounds

Compounds **1** and **2** were obtained as oil and as an inseparable mixture of two epimeric lactols (**1**/**2**, 75:25), as shown by TLC and HPLC analysis. Their molecular formulas were $C_{14}H_{26}O_5$ with two degrees of unsaturation, as deduced from HR-ESI-MS (*m*/*z* 313.1456 ($[M + K]^+$) and 297.1649 ($[M + Na]^+$)). The IR spectrum exhibited absorption band of OH groups (3300 cm^{-1}) [31]. The UV spectrum showed the absence of chromophores [32]. The ^{1}H-NMR spectrum (*Table 1*) showed the presence of a broad *singlet* (δ(H) 5.06) and of a *doublet* (δ(H) 5.24, *J* = 4.1 Hz) typical for acetalic H-atoms H–C(2) of lactol rings in **1** and **2**, respectively [29]. The same spectrum displayed a double *quartet*s at δ(H) 4.20 (*dq*, *J* = 7.0, 6.1, H–C(5)), a *doublet* of *doublet quartet* at δ(H) 2.10 (*ddq*, *J* = 8.5, 7.2, 6.0, H–C(3)), and a *multiplet* at δ(H) 1.54 1.57 (*m*, H–C(4)) attributed to **1** (*Table 1*). Moreover, two secondary Me groups were inferred from two H(3) *doublet*s at δ(H) 1.14 (*d*, *J* = 7.2, Me(7)), and 1.33 (*d*, *J* = 6.1, Me(6)) (*Table 1*). The ^{13}C-NMR spectrum for **1** exhibited seven C-atom resonances of four CH (one of acetalic H-atom) at δ(C) 103.8 (C(2)), 43.9 (C(3)), 54.9 (C(4)), and 76.5 (C(5)), one CH_2 at δ(C) 62.7 (C(8)) O-bearing, and two Me at δ(C) 21.4 (C(6)), and 19.2 (C(7)) groups (*Table 1*). Comparative analysis of ^{1}H- and ^{13}C-NMR data with those of botryosphaeriolactones A and C suggested the presence of a γ-lactol moiety [28] in **1**. These data allowed to assign a partial structure of 4-hydroxymethyl-3,5-dimethyl-tetrahydrofuran-2-ol to **1**. The 2D-NMR spectra data supported this hypothesis. In fact ^{1}H,^{1}H-COSY spectrum exhibited correlations of the signal at δ(H) 5.06 (H–C(2) with that at δ(H) 2.10 (H–C(3); of the latter with those at δ(H) 1.54 – 1.57 (H–C(4)) and 1.14 (Me(7)); of the signal at δ(H) 1.54 – 1.57 (H–C(4)) with those at δ(H) 3.75 – 3.71 (CH_2(8)) and 4.20 (H–C(5)); and finally of the latter with that at δ(H) 1.33 (Me(6)). The key HMBCs δ(C) 103.8 (C(2)) with those at δ(H) 2.10 (H–C(3)) and 1.14 (H–C(7)), δ(C) 43.9 (C(3)) with those at δ(H) 1.54 – 1.57 (H–C(4)) and 4.20 (H–C(5)), δ(C) 54.9 (H–C(4)) with those at δ(H) 3.75 and 3.71 (CH_2(8)), and δ(C) 76.5 (C(5)) with those at δ(H) 2.10 (H–C(3)), 1.54 – 1.57 (H–C(4)) and 1.33 (H–C(6)) further supported this assignment.

The ^{1}H- and ^{13}C-NMR spectra, in addition to the signals of the acetalic CH previously mentioned, showed the presence of signals attributed to **2**, as report in *Table 1*. Its structure was assigned on the basis of 2D-NMR spectra and in comparison with that of **1**.

The structure of dimer γ-lactol was formulated for **1** and **2**, considering HR-ESI-MS data (reported below), which gave a molecular formula containing twice the number of C- and H-atoms observed in the NMR spectra. Moreover, the same spectrum exhibited ions resulting from an asymmetric fragmentation of ethers [33]. In particular, the cleavage of the acetalic bond O–C(2), as previously report for 16-*O*-acetylbotryosphaerilactones A and C [28], gave 4-hydroxymethyl-3,5-dimethyl-tetrahydrofuran-2-ol (*m*/*z* 169.0818) corresponding to a molecular formula $C_7H_{14}NaO_3$ and (2,4-dimethyl-tetrahydro-furan-3-yl)-methanol (*m*/*z* 129.0897 ($[M - C_7H_{13}O_3]^+$)) corresponding at molecular $C_7H_{13}O_2$.

The relative configuration of **1**, as depicted in *Fig. 2*, was deduced by comparing the coupling constants measured in its ^{1}H-NMR spectrum with those reported for

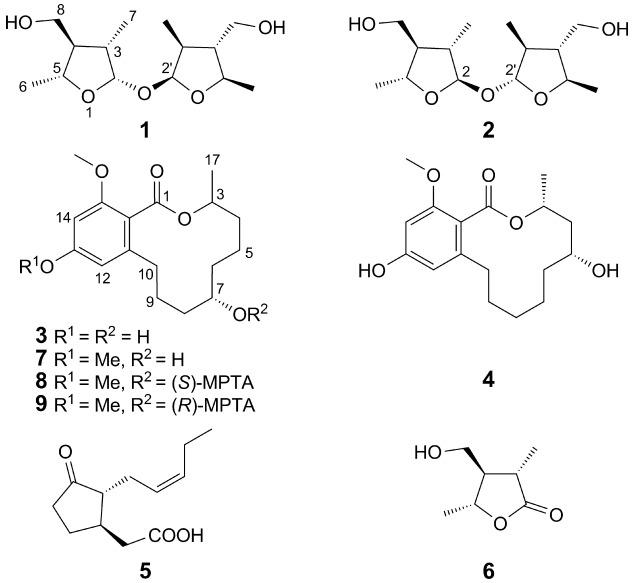

Fig. 2. Structures of lasiolactols A and B, botryosphaeriodiplodin, (5*R*)-5-hydroxylasiodiplodin, (–)-jasmonic acid, 4-hydroxymethyl-3,5-dimethyldihydro-2-furanone, 13-*O*-methylbotryosphaeriodiplodin, and its 7-*O*-*S* and 7-*O*-*R*-MPTA esters (**1** – **9**) produced by *Lasiodiplodia mediterranea*.

Table 1. ^{1}H- and ^{13}C-NMR data, and HMBCs of **1** and **2**[a]). Atom numbering as indicated in *Fig. 2*. δ in ppm, J in Hz.

Position	**1**		**2**		HMBC
	δ(C)[b])	δ(H)	δ(C)[b])	δ(H)	
2	103.8 (*d*)	5.06 (br. *s*, 1 H)	99.6 (*d*)	5.24 (*d*, *J* = 4.1, 1 H)	H(3), H(7)
3	43.9 (*d*)	2.10 (*ddq*, *J* = 8.5, 7.2, 6.0, 1 H)	41.5 (*d*)	2.04 (*ddq*, *J* = 6.8, 5.0, 4.1, 1 H)	H(2), H(4), H(5), H(7)
4	54.9 (*d*)	1.54 – 1.57 (*m*, 1 H)	52.2 (*d*)	1.77 – 1.83 (*m*, 1 H)	H(2), H(5), H(3), H_2(8)
5	76.5 (*d*)	4.20 (*dq*, *J* = 7.0, 6.1, 1 H)	76.5 (*d*)	4.02 (*dq*, *J* = 8.2, 6.1, 1 H)	H(3), H(4), H(6)
6	21.4 (*q*)	1.33 (*d*, *J* = 6.1, 3 H)	23.2 (*q*)	1.39 (*d*, *J* = 6.1, 3 H)	H(4), H(5)
7	19.2 (*q*)	1.14 (*d*, *J* = 7.2, 3 H)	11.9 (*q*)	1.07 (*d*, *J* = 6.8, 3 H)	H(2), H(3), H(4)
8	62.7 (*t*)	3.75 (*dd*, *J* = 10.4, 4.6, 1 H)	62.5 (*t*)	3.75 (*dd*, *J* = 10.4, 4.6, 1 H)	H(4)
		3.71 (*dd*, *J* = 10.4, 4.0, 1 H)		3.71 (*dd*, *J* = 10.4, 4.0, 1 H)	
OH		3.60 (br. *s*, 1 H)		2.38 (br. *s*, 1 H)	

[a]) ^{1}H,^{1}H-COSY, HSQC, ^{1}H-, and ^{13}C-NMR experiments delineated the correlations of all the H-atoms and the corresponding C-atoms. [b]) Multiplicities were assigned by DEPT spectrum.

same moiety in the botryosphaeriolactone A [29]. This configuration was also confirmed by the correlations observed in the NOESY spectrum [34]. In fact, this spectrum showed the correlations between H–C(2) and H–C(3), so that a (2*S*,3*S*,4*R*,5*R*,2′*S*,3′*S*,4′*R*,5′*R*)-**1** relative configuration was established.

The relative configuration of **2** was also confirmed by the correlations observed in the NOESY spectrum [34]. In this case, NOESY spectrum did not show the correlations between H-atoms H–C(2) and H–C(3). Therefore, a (2*R*,3*S*,4*R*,5*R*,2′*R*,3′*S*,4′*R*,5′*R*)-**2** relative configuration was established.

Compound **3** was identified as botryosphaeriodiplodin by comparing its spectroscopic and optical proprieties with those reported by [29]. This compound was recently isolated from mycelia extract of the endophytic fungus *Botryosphaeria rhodina* (= *Lasiodiplodia theobromae*) PSU-M35 together with other known lasiodiplodins [29]. However, the authors could not assign the configuration at C(7) since NOEDIFF data were not conclusive and the compound was obtained in low amount [29].

The absolute configuration of **3** at C(7) was established for its 13-*O*-methyl derivative (**7**) by an advanced *Mosher* method [35]. This derivative was obtained by the usual methylation of the phenolic group with ethereal solution of CH_2N_2. Its ^{1}H-NMR spectrum (*Table 2*) essentially differed from the one of **3** only for the presence of another MeO group in **7**, resonating as singlet at δ(H) 3.78. ESI-MS spectrum of **3**, recorded in positive mode, showed the potassiated and sodiated clusters and

Table 2. ^{1}H-NMR data of 13-*O*-methylbotryosphaeriodoplodin, and its 7-*O*-(*S*)-, and 7-*O*-(*R*)-MTPA esters (**7** – **9** resp.). Atom numbering as indicated in *Fig. 2*. δ in ppm, J in Hz.

Position	**7**	**8**	**9**
3	5.23 (*dq*, *J* = 6.4, 3.1)	5.222 (*dq*, *J* = 6.0, 3.2, 1 H)	5.260 (*dq*, *J* = 6.1, 3.0, 1 H)
4	1.95 – 2.02 (*m*, H_a)	1.981 – 2.010 (*m*, H_a)	2.024 – 2.033 (*m*, H_a)
	1.59 – 1.64 (*m*, H_b)	1.668 – 1.695 (*m*, H_b)	1.758 – 1.770 (*m*, H_b)
5	1.63 – 1.69 (*m*, H_a)	1.754 – 1.788 (*m*, H_a)	1.786 – 1.803 (*m*, H_a)
	1.49 – 1.51 (*m*, H_b)	1.563 – 1.583 (*m*, H_b)	1.581 – 1.596 (*m*, H_b)
6	1.55 – 1.62 (*m*, H_a)	1.611 – 1.645 (*m*, H_a)	1.682 – 1.691 (*m*, H_a)
	1.59 – 1.51 (*m*, H_b)	1.592 – 1.612(*m*, H_b)	1.611 – 1.623 (*m*, H_b)
7	3.80 – 3.83 (*m*)	5.348 – 5.338 (*m*)	5.326 – 5.337 (*m*)
8	1.45 – 1.51 (*m*, H_a)	1.501 – 1.521 (*m*, H_a)	1.489 – 1.512 (*m*, H_a)
	1.30 – 1.35 (*m*, H_b)	1.377 – 1.401 (*m*, H_b)	1.364 – 1.388 (*m*, H_b)
9	1.86 – 1.92 (*m*, H_a)	1.975 – 1.992 (*m*, H_a)	1.975 – 1.992 (*m*, H_a)
	1.49 – 1.53 (*m*, H_b)	1.463 – 1.495 (*m*, H_b)	1.442 – 1.433 (*m*, H_b)
10	2.73 (*ddd*, *J* = 14.0, 9.3, 6.2, H_a)	2.813 (*ddd*, *J* = 13.8, 9.0, 6.0)	2.714 (*ddd*, *J* = 13.8, 9.0, 6.0)
	2.57 (*ddd*, *J* = 14.0, 6.2, H_b)	2.587 (*ddd*, *J* = 13.8, 6.0, 1 H)	2.485 (*ddd*, *J* = 13.8, 6.0, 1 H)
12	6.32 (*d*, *J* = 2.0)	6.308 (br. *s*, 1 H)	6.302 (*d*, *J* = 1.9, 1 H)
14	6.30 (*d*, *J* = 2.0)	6.308 (br. *s*, 1 H)	6.269 (*d*, *J* = 1.9, 1 H)
17	1.33 (*d*, *J* = 6.4)	1.334 (*d*, *J* = 6.0, 3 H)	1.356 (*d*, *J* = 6.1, 3 H)
15-MeO	3.78 (*s*)	3.798 (*s*, 3 H)	3.783 (*s*, 3 H)
13-MeO	3.80 (*s*)	3.785 (*s*, 3 H)	3.783 (*s*, 3 H)
Ph	–	7.535 – 7.404 (*m*, 5 H)	7.548 – 7.407 (*m*, 5 H)
MeO	–	3.549 (*s*, 3 H)	3.575 (*s*, 3 H)

pseudomolecular ions $[M + K]^+$, $[M + Na]^+$, and $[M + H]^+$ at *m*/*z* 361, 345, and 323, respectively. Compound **7** was converted to (*S*)- and (*R*)-MPTA esters (**8** and **9**) by reaction with (–)-*R*-α-methoxy-α-trifluoromethylphenylacetyl (MPTA) and (+)-*S*-MPTA chlorides, respectively. The chemical shift differences between **8** and **9** ($\Delta\delta(H) = \delta(S) - \delta(R)$, *Table 2* and *Fig. 3*) indicated a (*R*) configuration at C(7). The relative configuration at C(3) was not assigned.

Biological Activities

Culture filtrates, corresponding extracts, and chromatographic fractions showed phytotoxic activity in the different assays. The toxicity of compounds **1** – **3**, **5**, and **6** used in the leaf-puncture assay on grapevine leaves showed different values, depending on the kind of compound and its concentration (*Fig. 4*). The phytotoxicity of all compounds increased with increasing concentrations; moreover, compound **5** was the most active compound and produced a necrotic area of 5.3 mm^2. Differences between toxicity values were statistically significant (*Fig. 4*). The compounds **1** – **3**, **5**, and **6** did not show *in vitro* antifungal and antioomycete activity against four assayed plant pathogens.

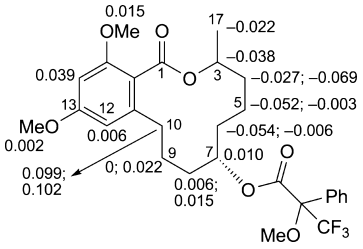

Fig. 3. Structures of 7-*O*-*S*- and 7-*O*-*R*-MPTA esters of 13-*O*-methylbotryosphaeriodiplodin (**8** and **9**), and delta–delta values ($\Delta\delta((S) - (R))$ in ppm measured for each H-atom.

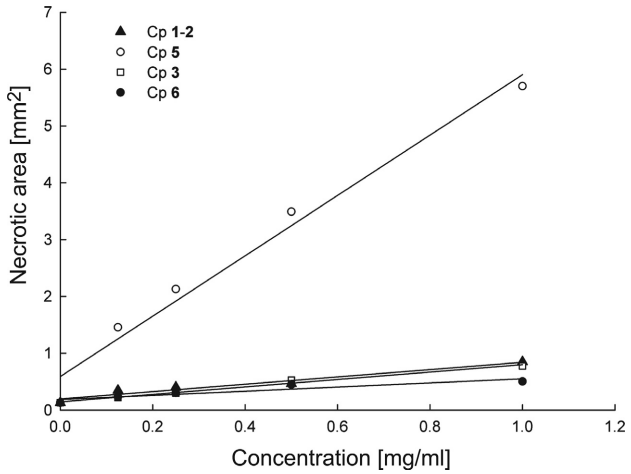

Fig. 4. Linear association between necrotic area (NA) and concentration (Conc) of assayed compounds **1** and **2**: NA = 0.198 + 0.644 Conc $R^2 = 0.947$ $P < 0.001$; **5**: NA = 0.590 + 5.312Conc $R^2 = 0.978$ $P < 0.001$; **3**: NA = 0.147 + 0.653Conc $R^2 = 0.985$ $P < 0.001$; **6**: NA = 0.189 + 0.362Conc $R^2 = 0.8709$ $P < 0.001$. Standard deviation values: **1** – **2** Conc. 0: 0.07; Conc. 0.125: 0.13; Conc. 0.25: 0.17; Conc. 0.5: 0.28; Conc. 1: 0.46; **5** Conc. 0: 0.07; Conc. 0.125: 0.29; Conc. 0.25: 0.78; Conc. 0.5: 0.21; Conc. 1: 1.40; **3** Conc. 0: 0.07; Conc. 0.125: 0.05; Conc. 0.25: 0.12; Conc. 0.5: 0.34; Conc. 1: 0.51; **6** Conc. 0: 0.07; Conc. 0.125: 0.12; Conc. 0.25: 0.16; Conc. 0.5: 0.17; Conc. 1: 0.28.

Conclusions

Pathogenicity testing, although with low number of replicates, allowed to confirm the pathogenic role of *L. mediterranea* on grapevine cultivar Inzolia. From its culture filtrates, two new dimeric γ-lactols, **1** and **2**, were isolated. Four known metabolites, **3** – **6**, were also identified. The absolute configuration was established at C(7) for **3**. The crude extract, fractions, and pure compounds obtained from *L. mediterranea* were screened for phytotoxicity activity against host and non-host plants. Compound **5** was the most phytotoxic. This result confirmed phytotoxic effect of jasmonic acid on grapevine leaf [28]. Moreover, the antifungal activity of compounds **1** – **3**, **5,** and **6** against some *Botryosphaeriaceae* agents of grapevine trunk diseases was evaluated for the first time. Finally, the metabolite profile of this strain was determined in the perspective of a chemotaxonomic classification of *Lasiodiplodia* species.

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Experimental Part

Fungal Strain

Strain B6 was isolated in 2007 from a declining grapevine cv. Inzolia grafted on 140R rootstock of 13-year-old in a Western Sicilian vineyard (Marsala, TP). The B6 colony was obtained from fragment of cane and trunk showing both the subcortical discolourations and xylematic sectorial necrosis [10]. Strain B6 was grown and maintained on potato-dextrose-agar (*PDA*; *Oxoid*, Milan, Italy) and stored at 4 °C in the collection of the Dipartimento di Scienze Agrarie e Forestali, Università degli Studi di Palermo, Italy. Genomic DNA was extracted from mycelium as described by [36]. The ITS region of the rDNA was amplified with primers ITS5 [37] and NL4 [38], while part of the translation elongation factor 1-alpha (EF1-α) gene was amplified with primers EF1-688F and EF1-1251R [36]. The PCR reactions and DNA sequencing were carried out as described by [36]. Sequences of both ITS and EF1-α regions were deposited in GenBank (ITS: accession number KP178596; EF1-α: accession number

KP178599). Identification of the fungal strain was done through a BLASTn search of the ITS and EF1-α sequences against the NCBI GenBank nucleotide database.

Pathogenicity Testing

In July 2013, the pathogenicity of strain B6 was tested on 1-year-old grapevine plants of cv. Inzolia. Bark surface of each grapevine trunk was sterilized with EtOH 70%, and wounded between the first and second internode using a cork borer with 5-mm-diameter. Mycelial plugs (5-mm-diameter) of 1-week-old cultures were inoculated into each wound and covered with *Parafilm® M* (*Pechiney*, Chicago, IL, USA). Three grapevines were inoculated with colonized agar plugs and three with non-colonized agar plugs as negative control. All plants were kept in natural environmental conditions. The block randomized as experimental design was used and the experiment was repeated twice. To monitor the occurrence of symptoms, the plants were checked monthly. In January 2014, the grapevines were longitudinally sectioned to visualize probable vascular discoloration. Total length of xylematic discolorations was measured upward and downward, starting from the inoculation point. Moreover, in order to fulfill *Koch*'s postulates, surface of each grapevine trunk was disinfected using EtOH 70% for 3 min and some small pieces of necrotic tissue from the edge of each lesion were cut and inoculated on PDA.

Data of lesion length, including the noninoculated control, were compared using the *Student*'s t test at $P < 0.05$ using SAS version 9.0 (*SAS Institute*, Cary, NC, USA). Data obtained were expressed as mean ± standard error (S.E.).

General Chemical Procedures

Column chromatography (CC): silica gel (SiO_2, 0.063–0.200 mm; *Merck*, Darmstadt, Germany). Prep. and anal. TLC: *Kieselgel 60, F_{254}* (0.25 and 0.5 mm resp.; *Merck*), and on reversed phase *DC Kieselgel 60 RP-18*, F_{254} (0.20 mm; *Merck*); detection: UV radiation (253 nm) and/or 10% H_2SO_4 in MeOH, followed by heating at 110 °C for 10 min. HPLC: *Shimatzu*, *LC-10Ad$_{VP}$* (*Shimatzu*, Tokyo, Japan) liquid chromatograph with a *Macherey-Nagel* (Duren, Germany) column (*Nucleosil 100-5 C_{18} HD*, 250 × 4.6 mm i.d; 5 μm), *SPD-10AV$_{VP}$* (*Shimatzu*, Tokyo, Japan) spectrophotometric detector; detection was performed at 200 nm, eluent: H_2O/MeCN 95% to 5% in 40 min, flow rate 0.7 ml/min. Optical rotations: *JASCO P-1010* (*JASCO*, Tokyo, Japan) digital polarimeter ($CHCl_3$). UV Spectra: *JASCO V-530* (*JASCO*, Tokyo, Japan) UV/VIS spectrophotometer (MeCN); λ_{max} (log ε) in nm. IR Spectra: *Thermo Electron Corporation Nicolet 5700 FT-IR* spectrometer; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR, COSY-45, HSQC, HMBC, and NOESY Spectra: *Bruker AV-400* (Karlsruhe, Germany) spectrometer, at 400 (^1H) and 100 (^{13}C) MHz ($CDCl_3$), δ in ppm rel. to the solvent peak, J in Hz. HR-ESI-MS (+) and ESI-MS (+): *Thermo* (*Scientific*, Waltham, MA, USA) LTQ Velos, and *Agilent Technologies* (Santa Clara, CA, USA) *6120 Quadrupole LC/MS* instruments, in *m/z*.

Fermentation, Extraction, and Isolation of Metabolites

L. mediterranea strain B6 was grown in 1 l *Roux* flasks containing 250 ml of *Czapek* medium (*Oxoid*) amended with 2% corn meal (pH 5.7). Each *Roux* (25) was inoculated with about 5 ml of a mycelial suspension and incubated at 25 °C for 21 days in darkness.

The culture filtrates were obtained by sterile filtering the culture in a vacuum on a 500 ml Stericup (0.45 μm *HV Durapore* membrane; *Millipore Corp.*, Billerica, MA, USA) and stored at −20 °C. The culture filtrates (5.0 l) were acidified to pH 4 with 2M HCl and extracted exhaustively with AcOEt. The org. extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure to give a brown-red oil residue (989.8 mg). The residue was submitted to a bioassay-guided fractionation through CC (SiO_2; $CHCl_3$/^{i}PrOH 95:5 eluates) to furnish seven fractions that after removal solvent were screened for their phytotoxic activity, *Frs. I – VII*. The major bioactive *Fr. II* (603.5 mg) was submitted to CC (SiO_2, $CHCl_3$/^{i}PrOH 95:5 eluates) which afforded six fractions, *Frs. II.1 – II.6*. *Fr. II.3* (323.0 mg) was further purified by CC (SiO_2, hexane/AcOEt 1:1, eluates) to furnish four fractions, *Frs. II.3.1 – II.3.4*. *Fr. II.3.2* (150.3 mg) was purified by prep. TLC (SiO_2, $CHCl_3$/^{i}PrOH 93:7 eluates), to furnish three fractions *Frs. A – C*. *Fr. A* (21.3 mg) was purified by TLC (SiO_2, hexane/acetone 7:3, three times) yielding **3** (17.4 mg, R_f (hexane/acetone 7:3) 0.24, and R_f ($CHCl_3$/^{i}PrOH 92:8) 0.41, three times) and **4** (2.4 mg, R_f (hexane/acetone 7:3) 0.20, and R_f ($CHCl_3$/^{i}PrOH 92:8) 0.35, three times).

Fr. C (65.3 mg) was purified by prep. TLC (SiO_2, $CHCl_3$/^{i}PrOH 92:8 eluate) yielding **6** a homogeneous amorphous solid (19.2 mg R_f 0.62 in the same eluent; R_f 0.76, TLC on reversed phase, EtOH/H_2O 6:4, eluate). *Fr. IV* (130.5 mg) was purified by prep. TLC (SiO_2, $CHCl_3$/^{i}PrOH 92:8, eluate) to give **1** and **2** (13.5 mg, R_f 0.31, in the same eluent, and R_f 0.12, hexane/AcOEt 1:1; HPLC analysis t_R 10.1 min); and **5** as yellow homogeneous oil (10.2 mg, R_f ($CHCl_3$/^{i}PrOH 92:8) 0.45; and R_f (TLC on reversed phase, EtOH/H_2O 6:4) 0.50).

Lasiolactols A and B (= **{Oxybis[(2*SR*,3*S*,4*R*,5*R*)-3,5-dimethyltetrahydrofuran-2,4-diyl]}dimethanol**; **1** and **2**). Oil. $[\alpha]_D^{25}$ = +9 (c = 0.2, $CHCl_3$). UV: λ < 100 nm. IR: 3300, 1651, 1637, 1541, 1085. ^1H- and ^{13}C-NMR ($CDCl_3$): *Table 1*. HR-ESI-MS: 313.1456 ($[M + K]^+$, $C_{14}H_{26}KO_5^+$; calc. 313.1417), 297.1649 ($[M + Na]^+$, $C_{14}H_{26}NaO_5^+$; calc. 297.1668), 169.0818 ($C_7H_{14}NaO_3^+$; calc. 169.0823), 129.0897 ($[M - C_7H_{13}O_3]^+$, $C_7H_{13}O_2^+$; calc. 129.0878).

Botryosphaeriodiplodin (= **(7*R*)-7,12-Dihydroxy-14-methoxy-3-methyl-3,4,5,6,7,8,9,10-octahydro-1*H*-2-benzoxacyclododecin-1-one**; **3**). Colorless oil. $[\alpha]_D^{25}$ = −12 (c = 0.2, $CHCl_3$). UV: 205 (3.70), 247 (2.61), 283 (2.43). IR (neat):

3350, 1697, 1602, 1469, 1273. ([29]: $[\alpha]_D^{26} = -9.8$ ($c = 0.7$, $CHCl_3$). UV (MeOH): 205 (3.20), 245 (2.44), 280 (2.19). IR (neat): 3440, 1684). ^{1}H- and ^{13}C-NMR are very similar to those reported [29]. ESI-MS: 639 ($[2M + Na]^+$), 347 ($[M + K]^+$), 331 ($[M + Na]^+$), 309 ($[M + H]^+$).

(5*R*)-5-Hydroxylasiodiplodin (= (3*R*,5*R*)-5,12-Dihydroxy-14-methoxy-3-methyl-3,4,5,6,7,8,9,10-octahydro-1*H*-2-benzoxacyclododecin-1-one; **4**). Colorless oil. $[\alpha]_D^{25} = +12$ ($c = 0.2$, $CHCl_3$); UV: 204 (3.70), 249 (2.59), 284 (2.43). IR (neat): 3350, 2950, 1688, 1601, 1460, 1264. ([19]: $[\alpha]_D^{23} = +8.8$ ($c = 0.70$, $CHCl_3$). IR (film): 3290, 2900, 1670, 1570, 1250, 1080). ^{1}H- and ^{13}C-NMR are very similar to those reported [19]. ESI-MS: 639 ($[2M + Na]^+$), 347 ($[M + K]^+$), 331 ($[M + Na]^+$), 309 ($[M + H]^+$).

Methylation of **3**

The Me ether **7** [13-*O*-methylbotryosphaeriodiplodin (= (7*R*)-7-hydroxy-12,14-dimethoxy-3-methyl-3,4,5,6,7,8,9,10-octahydro-1*H*-2-benzoxacyclododecin-1-one)] was prepared by the reaction of **3** (3.7 mg) dissolved in MeOH (1.0 ml) with an Et_2O soln. of CH_2N_2. The reaction mixture was left at room temperature for 6 h. The solvent was evaporated under a N_2 stream giving an oily residue (3.8 mg) which was purified by prep. TLC (SiO_2; $CHCl_3$/^{i}PrOH 95:5) to give **7** (3.5 mg, R_f ($CHCl_3$/^{i}PrOH 95:5) 0.69). Uncolored oil. UV: 213 (3.13), 247 (2.52), 282 (2.29). IR (neat): 3370, 1715, 1700, 1697, 1451, 1260. ^{1}H-NMR: *Table 2*. ESI-MS: 361 ($[M + K]^+$), 345 ($[M + Na]^+$), 323 ($[M + H]^+$).

7-*O*-(*S*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) Ester of 13-*O*-Methylbotryosphaeriodiplodin (= (7*R*)-12,14-Dimethoxy-3-methyl-1-oxo-3,4,5,6,7,8,9,10-octahydro-1*H*-2-benzoxacyclododecin-7-yl (2*S*)-3,3,3-Trifluoro-2-methoxy-2-phenylpropanoate; **8**). (–)-(*R*)-MPTA-Cl (20 µl) was added to **7** (1.0 mg) dissolved in dry pyridine (20 µl). The reaction was stirred at room temperature overnight, and then stopped by adding MeOH. Pyridine was removed by a N_2 stream. The residue (7.3 mg) was purified by prep. TLC (SiO_2, $CHCl_3$/^{i}PrOH 98:2, 0.9 mg, R_f 0.66). Homogeneous oil. UV: 206 (3.58), 249 (2.42), 282 (2.16). IR: 1742, 1646, 1453, 1257, 1154. ^{1}H-NMR: *Table 2*. ESI-MS: 577 ($[M + K]^+$), 561 ($[M + Na]^+$), 539 ($[M + H]^+$).

7-*O*-(*R*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MPTA) Ester of 13-*O*-Methylbotryosphaeriodiplodin (= (7*R*)-12,14-Dimethoxy-3-methyl-1-oxo-3,4,5,6,7,8,9,10-octahydro-1*H*-2-benzoxacyclododecin-7-yl (2*R*)-3,3,3-Trifluoro-2-methoxy-2-phenylpropanoate; **9**). (+)-(*S*)-MPTA-Cl (20 µl) was added to **7** (1.0 mg) dissolved in dry pyridine (20 µl). The reaction was carried out under the same conditions used for preparing **8** from **7**. The residue (8.1 mg) was purified by prep. TLC: SiO_2; 1.2 mg, R_f ($CHCl_3$/^{i}PrOH 98:2) 0.66. Homogeneous oil. UV: 206 (3.56), 250 (2.40), 281 (2.16). IR (neat): 1751, 1587, 1461, 1265, 1168. ^{1}H-NMR: *Table 2*. ESI-MS: 577 ($[M + K]^+$), 561 ($[M + Na]^+$), 539 ($[M + H]^+$).

Phytotoxicity Bioassays

A preliminary test was performed on tomato (non-host plant) and grapevine cv. Inzolia (host plant). Culture filtrate of strain B6 at different dilutions (1, 5, 10, 25, 50, and 100%), in distilled sterile water as solvent, was assayed for phytotoxic activity. Tomato stems of 2-week-old rootless plants and petiole of grapevine leaves were dipped for 24 h in a vial containing cultural filtrate (2 ml) and then for 48 h in a new vial with sterile distilled water (2 ml). *Czapek* medium and sterile distilled water were used as controls. Three stems of non-host plant and three leaves of host plant were employed as replicates and each treatment was repeated twice. Referring to a 0 – 3 scale (0 no symptoms; 1 slight withering; 2 medium withering; and 3 full withering) symptoms were assessed and by standardizing the mean value to a 0 – 100% range phytotoxic activity was evaluated [39].

B6 strain cultural filtrate was also tested by puncture assay on detached grapevine leaves. A droplet (20 µl) of each dilution was singly distributed on the adaxial side of leaves previously needle punctured. *Czapek* medium and sterile distilled water were used as controls. Three leaves were employed as replicates and each treatment was repeated twice. The inoculated leaves were placed in moist chambers (90% relative humidity) to prevent the droplets from drying and kept in darkness at 25 °C for 15 days. The lesion size, consisting of necrotic spots surrounding the puncture, measured using Image Tool UTHSCSA software (Texas University) was expressed in mm^2.

The org. extracts, the aq. phases, and the compounds were tested by puncture assay on detached grapevine leaves. Org. extracts and aqueous phases were assayed at different concentrations (0.5, 1, 2, and 4 mg/ml), while compounds **1** – **3**, **5**, and **6** at the following concentrations 0.125, 0.25, 0.5, and 1 mg/ml. Compound **4** present in small quantity was not tested. Samples were first dissolved in MeOH and successively diluted in sterile distilled water, up to the assay concentrations (the final content of MeOH was 4%), while the aq. phases was directly dissolved in sterile distilled water. MeOH (4% *v*/*v*) and sterile distilled water were used as controls. Inoculation, detection of symptom, and estimation of lesion size were performed as described above.

Data of necrotic areas (NA) were compared by analysis of variance, with level of statistical significance at $P = 0.05$, compound (CP) and concentration (Conc) as main factors and concentration × compound as the sole interaction, using SYSTAT procedures (*Systat software Inc.*, Richmond, CA, USA).

Linear regression analysis (*SigmaPlot*; *SPSS INC.*, Chicago, IL, USA) was used to establish association between concentration and area lesion for each compound; slopes of regression lines were compared using coefficients and standard error from regression analysis. When appropriate, *Tukey*'s test at $P < 0.05$ was used to separate means.

Antifungal Bioassays

Compounds **1** – **3**, **5,** and **6** were tested against four different plant pathogens: *Diplodia seriata*, *Neofusicoccum parvum*, and *N. vitifusiforme* belonging to *Botryosphaeriaceae,* and the oomycete *Phytophthora citrophthora*. In the blank antimicrobial susceptibility test, disks (6-mm diameter; *Oxoid*, Milan, Italy) impregnated with 30 µl of each compound (50 µg/ml) were placed in the center of a *Petri* dish containing PDA. Then, fresh mycelial suspension (10^5 – 10^6 propagules/ml) of each pathogen, obtained by shaking the mycelium scraped from 2-week-old colonies in sterile distilled water, was distributed on the surface of *Petri* dishes. *Ridomil Gold SL* (Metalaxil-M 43.88%; *Syngenta*, Milan, Italy) was used as positive control for oomycete and PCNB (Pentachloronitrobenzene 99%; *Sigma-Aldrich Co.*, USA) for botryosphaeriaceous fungi. MeOH (4% *v*/*v*), $CHCl_3$, and the blank disk were used as negative controls. Five replicates for each treatment were employed, and each assay was performed twice. *Petri* dishes were incubated at 25 °C for 3 days in darkness. For all plant pathogens, two perpendicular diameters were measured and the obtained data were expressed as growth inhibition.

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