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RESEARCH PAPERS

Naphthalenone polyketides produced by *Neofusicoccum parvum*, a fungus associated with grapevine Botryosphaeria dieback

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Summary. A strain of *Neofusicoccum parvum* isolated from declining vines was pathogenic to grapevine cultivar Inzolia in Sicily. This strain produced some metabolites in liquid medium. Crude extract, through a bio-guided purification process, yielded four naphthalenone polyketides. They were identified by comparison with spectroscopic data and optical proprieties reported in literature as: (3*S*, 4*S*)-7-ethyl-3,4,8-trihydroxy-6-methoxy-3,4-dihydro-1-(2*H*)-naphthalenone, (3*S**, 4*S**)-3,4-dihydro-3,4,8-trihydroxy-7-(1-hydroxyethyl)-6-methoxy-1-(2*H*)-naphthalenone, (4*S*)-3,4-dihydro-4,8-dihydroxy-1-(2*H*)-naphthalenone, named botryosphaerones D and A, isosclerone, respectively, and (3*S**,4*S**)-3,4,5-trihydroxy-1-tetralone (**1-4**). Phytotoxic activity of the isolated compounds (**1-4**) was tested on grapevine leaves at using the leaf puncture assay. All tested compounds were phytotoxic, with botryosphaerone D showing the greatest activity. The phytotoxic effects decreased when treated leaves were exposed to light. All of the metabolites did not show *in vitro* antifungal activity against *Diplodia seriata*, *Lasiodiplodia mediterranea*, *Neofusicoccum vitifusiforme*, or *Phytophthora citrophthora*. This is the first report of *in vitro* production of botryosphaerones D and A, and 3,4,5-trihydroxy-1-tetralone by *N. parvum*.

Key words: Botryosphaeriaceae, grapevine trunk diseases, phytotoxins.

Introduction

Among the grapevine trunk diseases which induce a premature plant decline, Botryosphaeria dieback has become an impending threat to the productivity and longevity in most wine-growing areas. The annual vine losses of Botryosphaeria dieback can vary from 3 to over 50% depending on wine-growing area (Larignon *et al.*, 2001; Yan *et al.*, 2013; Abou-Mansour *et al.*, 2015). The syndrome on vines includes perennial canker, bud necrosis, slow or arrested shoot growth, bunch rot and brown stripes just beneath the bark. The discolouration of necrotic wedges in stem cross sections, may extend longitudinally from the trunk to the rootstock and annual stems. In early season, wood symptoms are sometimes associated with leaf chlorosis (Larignon *et al.*, 2001; Auger *et al.*, 2004; Burruano *et al.*, 2008). Effective control of Botryosphaeria dieback is extremely difficult both for the wide range of Botryosphaeriaceae species infecting grapevines and possible co-infection with other taxonomically unrelated fungi (Urbez-Torres, 2011). To date, 30 botryosphaeriaceous species are known to be involved in the syndrome, and among them *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips has been reported as one of the most virulent and recurrent pathogens in many regions (Úrbez-Torres and Gubler 2009; Úrbez-Torres, 2011; Bertsch *et al.*, 2013; Mohammadi *et al.*, 2013; Pitt *et al.*, 2013; Ammad *et al.*, 2014; Spagnolo *et al.*, 2014). Recently, it has been reported that *N. parvum* and *N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips cause latent infections in

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the bark of dormant cuttings which are used in plant propagation, thus providing an additional infection pathway for a disease that is known to show obvious symptoms only in older vineyards (Billones-Baaijens *et al.*, 2015).

Unlike the report of Burruano *et al.* (2008), Botryosphaeria dieback in Sicily has been recently associated with *N. parvum* and other botryosphaeriaceous fungi, such as *Diplodia seriata* (De Not.), *Lasiodiplodia mediterranea* (Linaldeddu, Deidda & Berraf-Tebbal), and *Neofusicoccum vitifusiforme* (van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips. Pathogenicity of the fungi was first demonstrated on excised shoots (Mondello *et al.*, 2013). Botryosphaeriaceous fungi were never isolated from leaves of infected plants (Larignon and Dubos, 1997; Mugnai *et al.*, 1999). It was hypothesized that leaf symptoms could be caused by phytotoxic metabolites produced by fungi in the xylem, moving to leaves, or inducing a chain effect determining the expression of foliar symptoms (Martos *et al.*, 2008; Evidente *et al.*, 2010; Ramírez-Suero *et al.*, 2014; Bénard-Gellon *et al.*, 2015; Abou-Mansour *et al.*, 2015). Several pathogens involved in grapevine trunk diseases produce secondary metabolites *in vitro* and *in vivo* whose modes of action are sometimes reported (Andolfi *et al.*, 2011; 2012; Abou-Mansour *et al.*, 2015). We have recently isolated new and known compounds from culture filtrates of two fungal strains of *L. mediterranea* associated with grapevine decline in Sardinia and Sicily, (Andolfi *et al.*, 2014, 2016).

Figure 1. Brown necrosis caused by *Neofusicoccum parvum* in a grapevine stem cross section.

Since *N. parvum* B19, among the others strains of the same species, was the only morphologically and molecularly identified causal agent isolated during sampling in August 2010 of declining vine plants (Figure 1) in Marsala vineyards, this strain was used for metabolomic studies. In addition, *N. parvum* B2, B3 and B7 strains were associated with *L. mediterranea* during sampling in September 2009, and with *D. seriata* in September and October 2010.

The aims of the present study were 1) to confirm the pathogenicity of *N. parvum* B19 strain *in planta*; 2) to purify and characterize secondary metabolites produced by this strain *in vitro*; 3) to evaluate phytotoxic and antifungal activities of purified compounds.

Materials and methods

Fungal strain and pathogenicity test

In this study, *N. parvum* B19 strain was used. Identification was confirmed on the basis of morphological characteristics, analysis of internal transcribed spacer (ITS) of rDNA, and part of the translation elongation factor 1-alpha (EF1-a) gene (Mondello *et al.*, 2013). Sequences of both ITS and EF1-a regions from the strain were deposited in GenBank with accession numbers JN251119 for ITS and KC884949 for EF1-a.

Pure cultures of the strain were maintained on potato dextrose agar (PDA), and stored at 4°C in the mycological collection of the "Dipartimento di Scienze Agrarie e Forestali, Università degli Studi di Palermo", Italy.

The pathogenicity of the *N. parvum* strain was tested on 2-year-old grapevine plants of cv. Inzolia, in July 2013. The bark surface of each trunk was sterilized with 70% ethanol and wounded between the first and second internode using a cork borer with a tip of 5 mm diam. One-week-old mycelial plugs (5 mm diam.) were individually inoculated in each wound and covered with Parafilm® M. Three vines were inoculated with colonized agar plugs and three with non-colonized agar plugs as negative controls, and were then kept in natural growth conditions. A randomized-block experimental design was used,

and the experiment was repeated twice. In order to ascertain symptom occurrence, plants were monitored monthly. In January 2014, the vines were longitudinally sectioned to detect vascular symptoms. Total length of xylem discolorations was measured upward and downward, starting from the inoculation point. To fulfill Koch's postulates, the surface of each vine trunk was disinfected with 70% ethanol for 3 min, and small pieces of necrotic tissue from the edge of each lesion were cut and inoculated on PDA.

The length of lesions, in comparison to the controls, was measured and analyzed by Student's *t* test at $P<0.05$, using SAS version 9.0 (SAS Institute), and expressed as mean ± standard error (S.E.).

Culture filtrate production

The fungus was grown in stationary conditions in 2 L Erlenmeyer flasks each containing 400 mL of Czapek medium amended with corn meal (pH 5.7). For seeding liquid cultures, 5 mL of mycelial suspension from a 1-week-old colony were inoculated into each flask, and flasks were incubated at 25°C for 4 weeks in darkness.

Chemical analysis and characterization

Chemicals and solvents used for purification processes were ACS grade or equivalent (Sigma-Aldrich). Melting point was measured on a Mettler Toledo FP90 central processor associated with a Zeiss Axioskop microscope. Optical rotations (OR) were measured in $CHCl_3$ or MeOH on a Jasco P-1010 digital polarimeter. Electronic circular dichroism (ECD) spectra were recorded on a JASCO J-815 spectrometer in EtOH. UV spectra were measured in MeCN on a Jasco V-530 spectrophotometer. IR spectra were recorded as deposit glass film on a Thermo Electron Corporation Nicolet 5700 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, in $CDCl_3$ or CD_3OD on Bruker spectrometer. For referencing, the respective solvent signals (δ_H 7.26, δ_C 77.16, and δ_H 3.30) were used. EIMS spectra were recorded at 70 eV on Shimadzu QP 5050, and ESIMS spectra were recorded with positive and negative polarization on Agilent Technologies 6120 Quadrupole LC/MS instrument. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel 60, F_{254}, layer thickness 250 and 500 mm, respectively) plates. Spots were visualized by exposure to UV radiation, and by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110°C for 10 min. Chromatographic column was performed on silica gel (Merck, Kieselgel 60, 0.063-0.200 mm). Solvent systems: (A) $CHCl_3$-*i*-PrOH (93:7); (B) $CHCl_3$-*i*-PrOH (95:5); (C) $CHCl_3$-*i*-PrOH (85:15).

Extraction and purification

The freeze-dried culture filtrate (3 L) was dissolved in ultrapure water (300 mL) at a final pH of 6.5 and extracted three times with ethyl acetate (300 mL each). The organic phases were combined, dried with Na_2SO_4, and evaporated under reduced pressure to give the crude extract as brown-red oil (495.5 mg). This extract was chromatographed through a silica gel column (70 cm high, 3 cm i. diam.) using solvent system (A), yielding seven homogeneous fractions.

The residue of the first fraction (12.5 mg) was further purified by thin layer chromatography (TLC) on silica gel using solvent system B. A yellow oil was obtained (**3**, 2.4 mg, *Rf* 0.46, solvent system B). The white amorphous solid residue of the fourth fraction (25.3 mg), was obtained as the main metabolite (**1**, *Rf* 0.28, solvent system A). The residue of the seventh fraction (10.7 mg) was purified on silica gel TLC using solvent system C, giving two metabolites as a white solid (**2**, 3.7 mg, *Rf* 0.32, solvent system C) and as a crystalline yellow solid (**4**, 1.7 mg, *Rf* 0.42, solvent system C).

Botryosphaerone A (2)

^{1}H NMR (CD_3OD) δ: 6.87 (s, 1H, H-5), 5.33 (q, 1H, *J*=6.6 Hz, H-1'), 4.54 (d, 1H, *J*=7.4 Hz, H-4), 3.99 (m, 1H, H-3), 3.95 (s, 3H, OCH_3), 3.00 (dd, 1H, *J*=17.2, 4.1 Hz, H-2A), 2.66 (dd, 1H, *J*=17.2, 9.1 Hz, H-2B), 1.51 (t, 3H, *J*=6.6 Hz, H_3-2').

Crystal structure determination of 3,4,5-trihydroxytetralone (4)

The identity of **4** was established by single crystal X-ray analysis which was compared with a previous reported single crystal structure determination (Borgschulte *et al.*, 1991).

Yellow, block-shaped single crystals of **4** were obtained by slow evaporation of $CHCl_3$ at room tem-

perature. X-ray data collection was performed at 298 K on a Bruker Nonius Kappa CCD diffractometer equipped with graphite-monochromated MoK_α radiation (λ = 0.71073 Å, CCD rotation images, thick slices, ϕ and ω scans to fill asymmetric unit). The structure was solved and refined by routinely used software programs. A mutually *trans* configuration of 3- and 4- hydroxyl groups was observed. The S/S relative configuration at the two stereogenic centers was assigned.

Crystal data: $C_{10}H_{10}O_4$; M = 194.18 g mol^{-1}; monoclinic system, space group $P2_1$; Z = 2, D_x = 1.413 g cm^{-3}; cell parameters: a = 6.5535(9), b = 10.44967(7), c = 7.12582)Å, b = 110.685(17)°; $R1$ = 0.0576, $wR2$ = 0.1325.

Biological activities

A preliminary test was carried on tomato stems of 2-week-old rootless plants (non-host plant) and on petiole leaves of grapevine cv. Inzolia (host plant) to detect phytotoxic activity of B19 strain. Cultural filtrate dissolved in sterile distilled water at different dilutions (1, 5, 10, 25, 50, 100%) was applied to the plants.

Tomato stems and grapevine leaves were each dipped for 24 h in a vial containing cultural filtrate (2 mL) and then in a new vial with sterile distilled water (2 mL). Czapek medium and sterile distilled water were used as controls. Three stems of tomato and three leaves of grapevine were employed as replicates, and each treatment was repeated twice. Symptoms were assessed referring to a 0-3 scale (0 = no symptoms; 1 = slight withering; 2 = medium withering; 3 = full withering), and the range of phytotoxic activity was evaluated by standardizing the mean value to 0-100% (Martos *et al.*, 2008).

Leaf puncture assay

Phytotoxicity of cultural filtrate of *N. parvum* B19 strain was also tested by a puncture assay on grapevine leaves. A droplet (20 µL) of each dilution of filtrate was placed on the adaxial surfaces of three leaves which were previously punctured by a needle. Czapek medium and sterile distilled water were used as controls. Three leaves were employed as replicates and each treatment was repeated twice. For detection of toxic symptoms, inoculated leaves were kept in darkness in a moist chamber to prevent the droplets from drying, and were observed each day until day 15. The size (mm^2) of necrotic spots surrounding the punctures was measured by Image Tool UTHSCSA software (Texas University).

The crude extracts, the aqueous phases and the pure compounds were tested by leaf puncture assay on grapevine leaves. Crude extracts and aqueous phases were assayed at concentrations of 0.5, 1, 2, or 4 mg mL^{-1}, while pure compounds **1-4** were tested at the concentrations of 0.125, 0.25, 0.5 or 1 mg mL^{-1}. Samples of crude extracts and compounds previously dissolved in MeOH, were successively diluted in sterile distilled water, up to 4% of MeOH. The aqueous phases were directly dissolved in sterile distilled water. MeOH (4% v/v) and sterile distilled water were used as controls. Inoculation, detection of symptoms, and estimation of lesion sizes were performed as described above. Simultaneous exposure of leaves treated with compounds **1-4** was also examined to assess effects of light on phytotoxic activity. A 12 h light/12 h darkness photoperiod was used, maintaining the same features of the puncture assay described above. Data of necrotic areas were compared by analysis of variance, with compound and concentration 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) was used to examine associations between each compound concentration and necrotic area. Slopes of regression lines were compared by analysis of variance using coefficients and standard errors from regression analyses. When appropriate, Tukey's test at P<0.05 was used to separate means.

Antifungal bioassays

Antifungal effects of compounds **1-4** were tested against four different phytopathogens: *D. seriata, L. mediterranea*, *N. vitifusiforme,* and *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian. Blank antimicrobial susceptibility test discs (6 mm diam.; Oxoid, Milan, Italy), were impregnated with 30 µL of solutions (50 µg mL^{-1}) of each compound, and placed into Petri dishes containing PDA. Fresh mycelial suspensions (10^5-10^6 propagules mL^{-1}) of each of the pathogens, were obtained by shaking mycelium scraped from 2-week-old colonies in sterile distilled water. These were distributed on the PDA surfaces of the test plates.

Ridomil Gold SL (metalaxyl-M 43.88%; Syngenta) and PCNB (pentachloronitrobenzene 99%;

Sigma-Aldrich) were used as positive antifungal controls for, respectively, *P. citrophthora* and botryosphaeriaceous fungi. MeOH (4% v/v), chloroform and blank discs were used as negative controls. Petri dishes were incubated at 25°C for 3 d in the dark. Each treatment was performed twice, with five replicates. In all assays, two perpendicular diameters of inhibition zones around test discs were measured, and the obtained data were assumed to express growth inhibition.

Results

Pathogenicity test

Six months after inoculation in all plants, xylem symptoms were detected even if in different sizes. Discoloration under the bark developed longitudinally, starting from the inoculation points (Figure 2). Mean discolouration length produced by *N. parvum* was 9.3 cm (S.E. ± 0.8), and was significantly different from the mean discolouration length resulting from the control (0.9 ± 0.1 cm). The inoculated fungus was always re-isolated from each inoculated cane.

Purification and chemical identification of phytotoxins (1-4)

The crude extract of *N. parvum* (495.5 mg) from 3 L culture filtrates, was purified by combined column and thin layer chromatography to yield four pure compounds (**1-4**, Figure 3). The preliminary ^{1}H NMR spectra showed characteristic signals of the naphthalenone polyketide metabolites. The structure of each compound was confirmed by comparison of the obtained data (OR, IR, UV, ^{1}H and ^{13}C NMR, ESI and EIMS) with those reported in the literature. Moreover, ^{1}H NMR data for botryosphaerone A recorded in CD_3OD are outlined (above) Materials and methods. The main metabolite was identified as (3*S*, 4*S*)-7-ethyl-3,4,8-trihydroxy-6-methoxy-3,4-dihydro-1-(2*H*)-naphthalenone, namely botryosphaerone D (**1**). Absolute configuration of this compound was confirmed by comparison of its ECD spectrum, recorded under the same conditions, with that reported in a previous study (Andolfi *et al.*, 2012). Compound **2** was identified as (3*S**, 4*S**)-3,4-dihydro-3,4,8-trihydroxy-7-(1-hydroxyethyl)-6-methoxy-1-(2*H*)-naphthalenone, named botryosphaerone A (Xu *et al.*, 2011). The relative configuration at C(3) and C(4) was assigned by the comparison of the vicinal coupling constant, in the ^{1}H NMR spectrum recorded in CD_3OD, with those previously reported (Xu *et al.*, 2011). In particular, given that this ring assumes a pseudo-chair conformation, the H-4 at δ 4.54 and H-3 at δ 3.99 ($J_{3,4}$=7.4 Hz) should be *anti*-pseudoaxially positioned. Compound **3** was identified as (+)-isosclerone and its absolute configuration was determined unambigu-

Figure 2. Vascular discolorations in longitudinal section on grapevine cv. Inzolia canes 6 months after inoculation with *Neofusicoccum parvum.* (a) and a control plant (b).

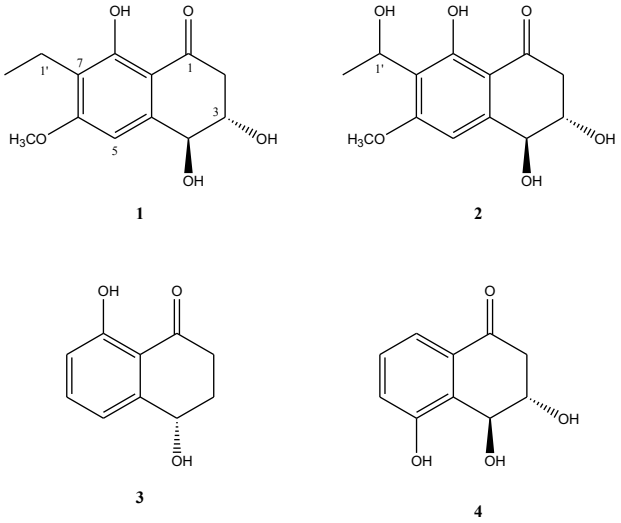

Figure 3. Structures of botryosphaerones D and A, isosclerone, and (3*S**,4*S**)-3,4,5-trihydroxytetralone (**1-4**). The relative configuration was reported for **2** and **4**.

ously using circular dichroism and by measurement of the optical rotation (Evidente *et al.*, 2000; and 2011). Compound **4** was identified as (3S*,4S*)- 3,4,5-trihydroxy-1-tetralone (Fujimoto *et al.*, 1986). The structure and the relative configuration of this compound,

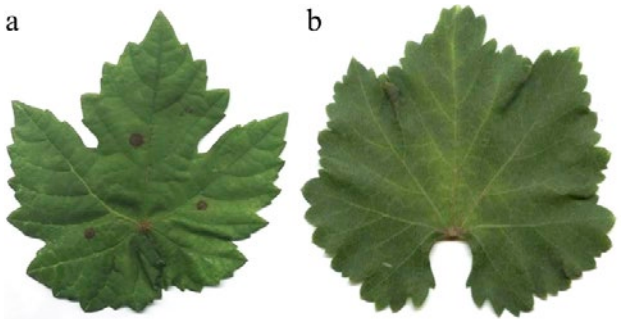

Figure 4. Necrotic areas produced by botryosphaerone D on a grapevine leaf cv. Inzolia (a); control leaf (b).

obtained as a crystalline solid, were also confirmed by comparing the single crystal X-ray analysis with published data (Borgschulte *et al.*, 1991).

Phytotoxicity and antifungal activities of pure compounds 1-4

Culture filtrates, corresponding extracts and chromatographic fractions showed phytotoxic activity both on tomato (non-host) and grapevine (host) plants in the different assays. All compounds assayed on grapevine leaves showed necrotic areas around

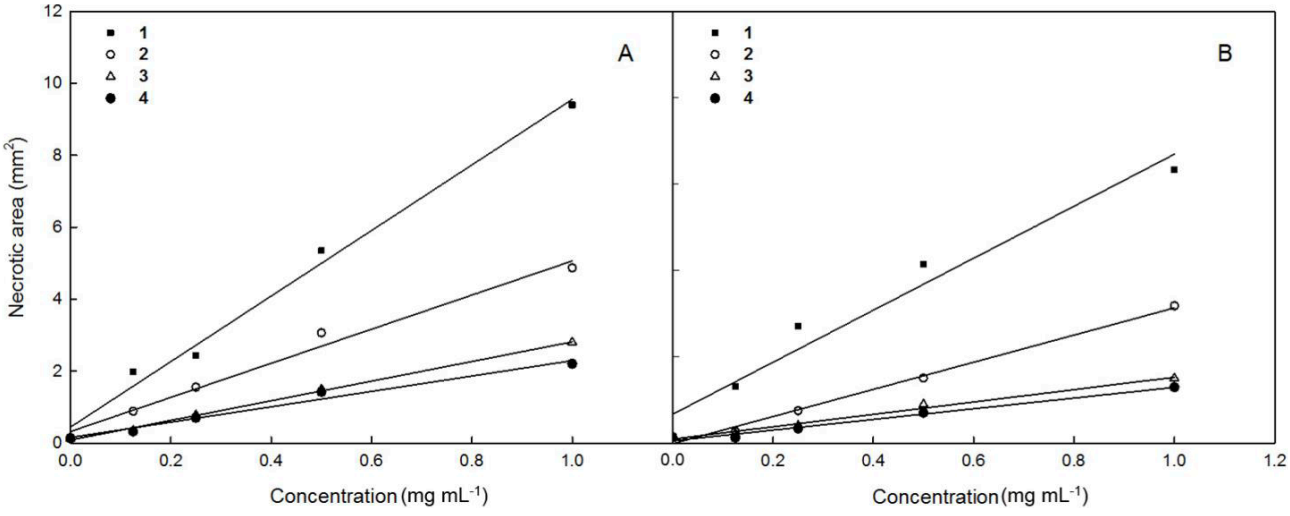

Figure 5. Linear association comparison between necrotic areas (NA) and concentrations (Conc) of assayed compounds under dark (A) and light (B) conditions. In the dark and for compound **1**, *NA* = 0.44 + 9.11conc; R^2 = 0.99, *P*<0.001: for **2**, NA = 0.32 + 4.74conc; R^2 = 0.99, *P*<0.001: for **3**, NA = 0.08 + 2.73conc; R^2 = 0.99, *P*<0.001: for **4**, NA = 0.15 + 2.13conc; R^2 = 0.98, *P*<0.001. In the light and for compound **1**, NA = 0.67 + 6.03conc; R^2 = 0.96, *P*<0.001: for **2**, NA = -0.01 + 3.15conc; R^2 = 0.99, *P*<0.001: for **3**, NA = 0.09 + 1.43conc; R^2 = 0.99, *P*<0.001: for **4**, NA = 0.05 + 1.24conc; R^2 = 0.99, *P*<0.001.

Table 1. Comparison of slopes from linear associations between necrotic areas on tissues of test plants and concentrations of assayed compounds under dark and light conditions (slopes ± standard error).

Compounds	Dark	Light
1	9.11 ± 0.51	6.03 ± 0.71
2	4.74 ± 0.34	3.15 ± 0.14
3	2.73 ± 0.08	1.43 ± 0.09
4	2.13 ± 0.18	1.24 ± 0.09
HSD*	1.58	

* Honestly Significant Difference according to Tukey's Test at $P \leq 0.05$.

inoculation points after 6 d (Figure 4). Analysis of variance showed a linear relationship between concentrations and necrotic areas as well as significant concentration × compound interactions ($P<0.05$), for each compound. Various types of association were highlighted for the different compounds. Data related to linear relationships between concentrations and necrotic areas of the sampled compounds showed four separate linear relationships, with significantly different slopes ($P<0.001$) (Figure 5). Specifically, the statistical comparison of linear regression slopes showed greater phytotoxicity for compunds **1** than **2**, and both were more phytotoxic than **3** and **4**. The relationships for compounds **3** and **4** were not different ($P>0.05$; Table 1).

The equations fitted the data with high R^2 values (Figure 5) and low standard errors for the estimated parameters (Table 1). Response in dark and light conditions among the compounds were similar, as indicated by statistical analyses. With the exception of compound 1, phytotoxicity did not change when plant tissues treated with the compounds were either exposed to light or kept in darkness (Table 1).

Compounds **1-4** did not show *in vitro* antifungal and anti-oomycete activity against the four plant pathogens tested.

Discussion

Results obtained through inoculation of strain B19 confirm the pathogenicity of *N. parvum* on grapevine cv. Inzolia, as observed in preliminary studies (Mondello *et al.*, 2013). This fungus was previously shown to be pathogenic to other grapevine cultivars such as Periquita in South Africa, Macabeo and Tempranillo in Spain, and Crimson in California (Úrbez-Torres, 2011). Inoculation tests in New Zealand also confirmed the pathogenicity of *N. parvum* on cv. Pinot noir and Sauvignon blanc (Amponsah *et al.*, 2011; Baskarathevan *et al.*, 2012; Billones-Baaijens *et al.*, 2013). Pathogenicity was again observed on cv. Cabernet Sauvignon in Iran (Mohammadi *et al.*, 2013), and cv. Chardonnay in Australia (Pitt *et al.*, 2013). In California, cv. Thompson was the most susceptible cultivar to this botryosphaeriaceous species (Travadon *et al.*, 2013).

Secondary metabolite production by *N. parvum* strains associated with declining grapevines as the likely cause of foliar symptoms has been previously highlighted. Production of (3*R*,4*R*)- and (3*R*,4*S*)-4-hydroxymelleins, tyrosol and isosclerone by *N. parvum* (strain CBS121486) was ascertained, with isosclerone first reported from a botryosphaeriaceous species (Evidente *et al.*, 2010). More recently (-)-terremutin and its analogues, asperlins, melleins and salicylic acid derivatives were isolated from a strain of *N. parvum*, and their phytotoxicity was determined. Terremutin and (-)-mellein have also been detected in grapevine wood showing Botryosphaeria dieback symptoms (Abou-Mansour *et al.*, 2015).

As for compounds from strain B19, botryosphaerones D and A (compounds **1-2**) were previously isolated from strain ZJ12-1A of *Neofusicoccum australe* (teleomorph *Botryosphaeria australis*) Slippers, Crous, & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, from root epidermis of *Sonneratia apetala* (Xu *et al.*, 2011), and are here reported for the first time from *N. parvum*. Compound **1** was also reported from *N. australe* from a branch of *Phoenicean juniper* showing dieback symptoms, and was phytotoxic on leaves of grapevine cv. Cannonau when tested by the puncture assay, but only at high concentrations (Andolfi *et al.*, 2012). The different response observed for botryosphaerone D from *N. parvum*, showing greater phytotoxicity at lower concentrations, could be due to different susceptibility of the grapevine cultivars.

Isosclerone was first isolated from *Sclerotina sclerotinium* as a bioactive metabolite with plant growth regulating properties (Morita and Aoki, 1974), and later from a large variety of plants and fungi (Fujimoto *et al.*, 1986; Joshi *et al.*, 2002; Gallo *et al.*, 2010). Recently anti-proliferative effects of isosclerone,

isolated from a marine-derived fungus, *Aspergillus fumigatus*, was studied *in vitro* (Li *et al.*, 2014). Starting with the commercially available 5-hydroxy-1,4-naphthalenedione (juglone), biotransformation by the isosclerone-producing endophytic fungus *Paraconiothyrium* variabile has also been described (Prado *et al.*, 2013).

3,4,5-Trihydroxy-1-tetralone (compound **4**) was isolated for the first time from *Penicillium diversum* var. *aureum* Raper & Fennell (Fujimoto *et al.*, 1986), and later from an endophytic "*Botryosphaeria*" sp. in *Maytenus hookeri* (Lin *et al.*, 2012). No data were reported of phytotoxic activities of this compound.

Isosclerone together with some other naphthalenone pentaketides, usually produced by esca fungi, are intermediate metabolites resulting from the biosynthesis of dihydroxynaphthalene melanins (DHN). The mode of action of these toxins may be related to their oxidant properties playing determinant roles in active defense mechanisms of host plants (Andolfi *et al.*, 2011). For fungal pathogens, melanin contributes to virulence in humans and plants through contrasting host defense mechanisms involving oxidizing agents. Inversely, these compounds protect sclerotia, conidia or other melanized structures from lysis (Butler and Day, 1998). Thus, the decrease of phytotoxicity observed after exposure to light, particularly in the case of botryosphaerone D, could be related to such properties. Naphthalenones such as juglone contribute to the generation of reactive oxygen species (ROS) (Chen *et al.*, 2015), and in leaf tissues ROS such as singlet oxygen ($^1O_2$), could be detoxified by main mechanisms (Triantaphylides and Havaux, 2009). The present information about the *N. parvum* B19 pathogenicity, phytotoxicity and also emphasized by its exclusive occurrence in declining vine plants, supports a possible contribution of this fungus in Botryosphaeria dieback.

Concluding remarks

Pathogenicity tests allowed the confirmation of the pathogenic role of *N. parvum* on grapevine cultivar Inzolia. From the fungal culture filtrates, four known naphthalenone poliketides were isolated, three of which were isolated for the first time from *N. parvum*. The crude extract, fractions and pure compounds obtained from *N. parvum* were screened for their phytotoxic activity against host and non-host plants. Botryosphaerone D was the most phytotoxic. The metabolites produced by the fungus could be considered as markers in chemotaxonomic classification of *Neofusicoccum* species.

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