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Fatty Acids Produced by *Neofusicoccum vitifusiforme* and *N. parvum*, Fungi Associated with Grapevine Botryosphaeria Dieback

Maria Michela Salvatore ¹, Selene Giambra ², Daniele Naviglio ¹, Marina DellaGrecia ¹,
Francesco Salvatore ¹, Santella Burruano ² and Anna Andolfi ^{1,*}

¹ Department of Chemical Sciences, University of Naples 'Federico II', 80126 Naples, Italy; mariamichela.salvatore@unina.it (M.M.S.); naviglio@unina.it (D.N.); dellagre@unina.it (M.D.G.); frsalvat@unina.it (F.S.)

² Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo, 90128 Palermo, Italy; selene.giambra@unipa.it (S.G.); santella.burruano@unipa.it (S.B.)

* Correspondence: andolfi@unina.it; Tel.: +39-081-2539179

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Abstract: There is evidence that secondary metabolites are involved in the fungal pathogenicity and virulence of *Neofusicoccum* spp. Fatty acids may also influence the plant–pathogen interaction but, so far, no information is available on their production by species of *Neofusicoccum* associated with *Botryosphaeria dieback*, which is a well-known syndrome of several plants with a complex etiology. In the present paper, the production of fatty acids in liquid medium, by strains of *N. vitifusiforme* and *N. parvum* associated with declining Sicilian vine plants, was evaluated. Data, acquired via gas chromatography–mass spectrometry (GC/MS), show the presence of linoleic acid as the most abundant fatty acid produced by both examined strains. In addition, the pathogenicity of *N. vitifusiforme* was tested on 2-year-old grapevine plants of cv. Inzolia.

Keywords: Botryosphaeriaceae; grapevine trunk diseases; GC/MS; azelaic acid; linoleic acid

1. Introduction

Grape is a crop of major economic importance in the world: in 2016, around 7.5 Mha were under cultivation and produced 75.8 Mt of fruits, 56% being processed for wine, juice and spirits and 44% used for fresh or dried raisin consumption [1]. As a consequence of its relevance, grapevine disease symptoms caused by fungal pathogens are responsible for significant economic losses [2,3].

Several fungal pathogens, involved in grapevine trunk diseases (GTDs), live in and colonize the wood of the perennial organs causing wood necrosis, wood discoloration, vascular infections, and white decays [4–6]. Among the species causing GTDs, species of the family Botryosphaeriaceae have become an impending threat to productivity and longevity in most wine-growing areas by causing so-called Botryosphaeria dieback [6]. Annual losses for Botryosphaeria dieback can vary depending on wine-growing area. For instance, in the Bordeaux area (France), annual losses are estimated to be 4–20% [7] and in some regions in China are over 30–50% [8].

Several Botryosphaeriaceae belonging to *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia* and *Neofusicoccum* genera are associated with GTDs [9].

In Sicily, Botryosphaeria dieback has been recently associated with botryosphaeriaceous fungi, such as *D. seriata*, *L. mediterranea*, *N. vitifusiforme* and *N. parvum* [10,11]. Furthermore, the pathogenicity in planta of some of them (i.e., *L. mediterranea* and *N. parvum*) was newly assayed [12,13].

Botryosphaeriaceous fungi have never been isolated from leaves of infected plants [4,14]. It was hypothesized that leaf symptoms could depend on phytotoxic metabolites produced by fungi in the

xylem, which are moved to the leaves, or induce a chain effect determining the expression of foliar symptoms [15–20]. In fact, several pathogens involved in GTDs produce secondary metabolites in vitro and in vivo whose mode of action is sometimes reported [12–16,21,22]. Many secondary metabolites (e.g., cyclohexenones, melleins, naphthalenones, and phenols) were isolated from in vitro culture of diverse species of *Neofusicoccum* associated with grapevine dieback [9,14,16,18,21], and some of them are reported as vivotoxins [15].

Despite several studies on species of *Neofusicoccum* associated with grapevine dieback, the production in liquid medium of fatty acids had not been reported. In fact, fatty acids and modified fatty acids are important compounds during the colonization of plants by pathogenic fungi and may be involved in their virulence [23].

In the present study, the composition and phytotoxicity of fatty acids produced in liquid medium by strains of *N. vitifusiforme* [11] and *N. parvum* [13] were evaluated. Furthermore, the pathogenicity in planta of a grapevine strain of *N. vitifusiforme* (B8) was assayed and discussed according to data of pathogenicity previously reported for the grapevine strain of *N. parvum* (B19) [13].

2. Materials and Methods

2.1. General Experimental Procedures

Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz in CDCl₃ on a Bruker spectrometer (AscendTM400) (Bremen, Germany) and the same solvent was used as internal standard. All reagents and solvents were analytical grade and purchased from Carlo Erba (Milan, Italy), Sigma-Aldrich (Saint Louis, MO, USA).

Gas chromatography–mass spectrometry (GC/MS) measurements were performed with an Agilent 6850 GC (Milan, Italy) equipped with an HP-5MS capillary column (5% phenyl methyl polysiloxane stationary phase) and the Agilent 5973 Inert MS detector (used in the scan mode). Helium was employed as the carrier gas, at a flow rate of 1 mL/min. The injector temperature was 250 °C and, during the run, a temperature ramp raised the column temperature from 50 °C to 240 °C: 50 °C for 2 min; 10 °C min^{−1} until reaching 180 °C, 180 °C for 5 min, and 5 °C min^{−1} until reaching 240 °C; 240 °C for 25 min. The electron impact (EI) ion source was operated at 70 eV and at 200 °C. The quadrupole mass filter was kept at 250 °C and, in the scan mode, was programmed to scan the range 45–550 m/z at a frequency of 3.9 Hz.

Solutions of standard compounds were prepared in *n*-hexane at a concentration range of 50–200 mg L^{−1} and analyzed via GC/MS. Methyl ester of nonanoic acid (Sigma-Aldrich) was used as internal standard, a stock solution of which in *n*-hexane at concentration of 1 g L^{−1} was prepared and stored at −20 °C.

Analytical and preparative TLCs (thin layer chromatography) were performed on silica gel plates (Kieselgel 60, F₂₅₄, 0.25 mm) (Merck, Darmstadt, Germany). The spots were visualized by exposure to ultraviolet (UV) radiation (253 nm), or by spraying with 10% H₂SO₄ in MeOH followed by heating at 110 °C for 10 min. Column chromatography (CC) was performed using silica gel (Merck, Kieselgel 60, 0.063–0.200 mm).

2.2. Fungal Strain and Pathogenicity Test

Strains B8 (*N. vitifusiforme*) and B19 (*N. parvum*) were isolated from stem cankers of symptomatic grapevines showing subcortical discolourations and xylematic sectorial necrosis, but in both cases without foliar symptoms, in a vineyard in western Sicily and identified in a previous study [12]. The pathogenicity of B19 strain has been reported before [13] while both pathogenicity re-isolation tests of strain B8 were performed on 2-year-old grapevine plants of cv. Inzolia in this study according to Burruano et al. [13].

Data of lesion length, including the non-inoculated 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 \pm standard error (S.E.).

2.3. Culture Filtrate Production

The fungi were grown in stationary conditions in 2 L Erlenmeyer flasks containing 400 mL of Czapek medium amended with corn meal (pH 5.7). For seeding liquid cultures, 5 mL of mycelial suspension by a 1-week-old colony were inoculated in each flask and incubated at 25 °C for 4 weeks in darkness [13]. The culture filtrates were obtained by sterile filtering the culture in a vacuum on a 500 mL Stericup (0.45 μ m HV Durapore membrane; Millipore Corp., Billerica, MA, USA) and stored at -20 °C.

2.4. Extraction and Purification Processes

2.4.1. *Neofusicoccum vitifusiforme*

The freeze-dried culture filtrate (10 L) was dissolved in ultrapure water (1000 mL) and extracted at native pH (i.e., pH \approx 6.0) three times with ethyl acetate (1000 mL for each). The organic phases were combined, dried with Na₂SO₄, and evaporated under reduced pressure to give the crude extract as brown-red oil (550.2 mg).

2.4.2. *Neofusicoccum parvum*

The freeze-dried culture filtrate (3 L) was dissolved in ultrapure water (300 mL) and extracted at native pH (i.e., pH \approx 6.5) as reported before for *N. vitifusiforme* to give the crude extract as brown-red oil (495.5 mg). As previously reported [14], this extract was chromatographed through a silica gel column using as solvent system CHCl₃-i-PrOH (93:7), yielding seven homogeneous fractions. The residue of second fraction (23.9 mg) was further purified by TLC on silica gel eluted with CHCl₃-i-PrOH (95:5) yielding a yellowish oil (fatty acids mixture, 6.6 mg, R_f 0.50 in the same chromatographic condition).

2.5. Fatty Acids Methylation

An ethereal solution of CH₂N₂ was slowly added to 2 mg of sample (i.e., crude extract or chromatographic fraction) dissolved in MeOH (1.5 mL) until a yellow color was persistent. The reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated under a N₂ stream and the residue used for GC/MS analysis.

2.6. Qualitative and Quantitative Analysis of Fatty Acids

Prompted by the collected NMR data, samples were fully analyzed by GC/MS in order to unravel the nature and amount of compounds potentially present. Before GC/MS analysis, samples were treated with diazomethane in ether in order to obtain methyl esters of fatty acids.

Methyl esters of fatty acids were identified by retention indices (RI) and their mass spectra compared with those present in databases by employing the National Institute of Standards and Technology (NIST) Mass Spectral Search Program v.2.0g which, among others, can explore the NIST 14 Mass Spectral library (2014) and the Golm Metabolome Database. Matrix components could create some problems for GC/MS identification via mass spectra and retention times essentially due to compounds' co-elution which gives rise to impure chromatographic peaks and mass spectra. In order to overcome these problems, GC/MS data were analyzed by using the NIST deconvolution program Automated Mass Spectral Deconvolution and Identification System (AMDIS) [24,25].

Quantification of individual methylated (ME) fatty acids was obtained by linear regression of the chromatographic peak areas and known concentrations of certified standards (ranging from 50 to 200 mg L⁻¹) [26]. The response of target compounds was normalized to the response of an internal

standard (nonanoic acid ME), which was added to the sample prior to the analysis at the constant concentration of 100 mg L⁻¹.

2.7. Phytotoxicity Bioassays

Crude extracts and chromatographic fractions obtained both from B8 and B19 strains were tested by puncture assay on detached grapevine leaves from greenhouse two-year-old potted plants. 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%). A droplet (20 µL) of each fatty acid concentration (0.125, 0.25, 0.5, and 1 mg/mL) was singly distributed on the adaxial side of leaves previously needle punctured. MeOH (4% v/v) 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 onto a box containing paper with 5 mL of water and then kept in moist chambers (90% relative humidity), to prevent the droplets from drying, in darkness at 25 °C for 15 days [21]. The leaves were observed daily in order to detect any symptoms.

3. Results

N. vitifusiforme B8 strain showed pathogenic activity on the inoculated grapevines. Six months after inoculation, vascular discolourations were found upward and downward starting from the point of inoculation and were observed in all inoculated plants, except for control (Figure 1). Mean discolouration length produced by B8 strain was 12.0 ± 3.0 cm (mean ± S.E.) 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. The inoculated fungi were always re-isolated from the inoculated canes, but never from the control. Similar results were previously reported for *N. parvum* B19 [13].

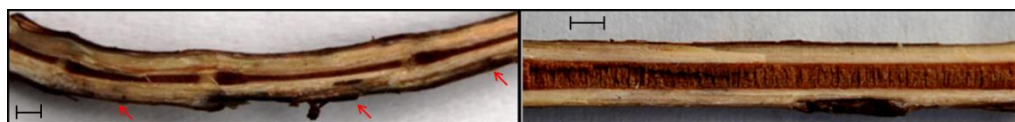


Figure 1. Brown necrosis (indicated by the red arrow) caused by *N. vitifusiforme* extending along a large part of the trunk of 2-year-old grapevine plants of cv. Inzolia in longitudinal section (left) and absence of xylematic symptom in the control (right). Scale bars = 1 cm.

The preliminary spectroscopic investigation conducted for the crude extract of *N. vitifusiforme* and for the chromatographic fraction obtained from the crude extract of *N. parvum* showed typical signals of saturated and unsaturated fatty acids [27].

Samples were analyzed via GC/MS after esterification with diazomethane in ether. Table 1 is an overview of compounds (i.e., fatty acids and dicarboxylic acids) produced in liquid medium by *N. vitifusiforme* and *N. parvum*. Particularly important is the composition of the crude extract of *N. vitifusiforme* which presents azelaic acid.

Table 1. An overview of qualitative results of compounds (i.e., fatty acids and dicarboxylic acids) produced in liquid medium by *N. vitifusiforme* and *N. parvum*.

	<i>N. vitifusiforme</i>	<i>N. parvum</i>
Azelaic acid	✓	-
Palmitoleic acid	✓	-
Palmitic acid	✓	-
Linoleic acid	✓	✓
Elaidic acid	✓	✓
Stearic acid	✓	✓

Figure 2 shows total ion chromatograms (TICs) of each sample analyzed after treatment with diazomethane in ether. This treatment allows to identify carboxylic acids (e.g., fatty acids) as methyl esters [28]. The direct comparison of TIC profiles clearly confirms qualitative differences in fatty acid production between two species of *Neofusicoccum*.

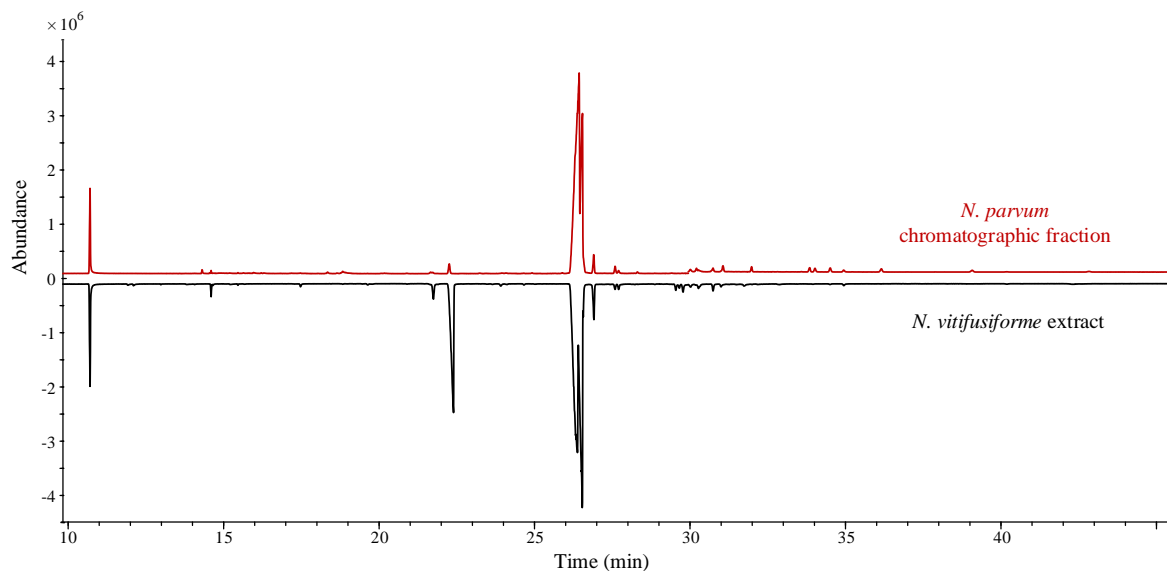


Figure 2. Head to tail comparison of total ion chromatogram (TIC) profiles of methylated compounds from *N. parvum* (chromatographic fraction) and *N. vitifusiforme* (crude extract). For clarity, chromatograms have been translated in the vertical direction by an arbitrary amount.

Finally, fatty acids, and, eventually, related compounds, in the extract of the strain B8 of *N. vitifusiforme* and of the strain B19 of *N. parvum*, have been quantified and results are summarized in Table 2 as percentages.

Table 2. Quantitative results of compounds in the extract of the strain B8 of *N. vitifusiforme* and of the strain B19 of *N. parvum*.

Code	Name	KI ¹	Abundance (%) in the Extract of <i>N. vitifusiforme</i> (B8)	Abundance (%) in the Extract of <i>N. parvum</i> (B19) ²
-	Azelaic acid	1548	0.5	-
16:1 <i>n</i> -7	Palmitoleic acid	1903	1.5	-
16:0	Palmitic acid	1926	10.4	-
18:2 <i>n</i> -6	Linoleic acid	2092	48.2	0.96
18:1	Elaidic acid	2109	38.0	0.33
18:0	Stearic acid	2125	1.4	0.01

¹ KI are referred to methyl esters of compounds reported. ² Values reported have been calculated from the composition of the chromatographic fraction which represents 1.3% of the crude extract.

From Table 2, it can be seen that linoleic acid (48.2%) and elaidic acid (38%), are the most abundant components in the crude extract of the strain B8 of *N. vitifusiforme*, while palmitic acid (10.4%), palmitoleic acid (10.5%) and stearic acid (1.4%) are present in much lower amounts. In addition, a small amount of azelaic acid (0.5%) was detected in the examined crude extract.

As reported by Burruano et al. [13], from the culture filtrate extract of the strain B19 of *N. parvum*, many naphthalenone polyketides were isolated by column chromatography and then identified via NMR spectroscopy. However, the NMR spectrum of a chromatographic fraction (representing only 1.3% of the crude extract) showed the presence of fatty acids. Accordingly, the data obtained from the fatty acids quantification in this fraction showed that linoleic acid was 73.5%, elaidic acid was 25.5% and stearic acid was 0.9% (which represent, respectively, 0.96%, 0.33% and 0.01% of the crude extract).

Moreover, crude extract of strain B8 of *N. vitifusiforme* and chromatographic fraction from crude extract of strain B19 of *N. parvum* did not show phytotoxic activity in puncture-assay tests on detached grapevine leaves.

4. Discussion

Among the fungi which are known to cause GTDs, increasing importance has been given to species from the genus *Neofusicoccum* [20,29]. Results obtained through inoculation of strain B8 of *N. vitifusiforme* and strain B19 of *N. parvum* [13] on grapevine cv. Inzolia, confirm their pathogenicity. *N. vitifusiforme* reported in *V. vinifera* for the first time in South Africa [30], is now frequently associated with Botryosphaeria dieback in several grape-growing areas worldwide: Spain [31], Mexico [32] and Italy [11].

Despite all the available information concerning *Neofusicoccum* metabolites, there are no studies on the production of compounds by *N. vitifusiforme*. In this respect, the production of fatty acids by *N. vitifusiforme* was investigated. Furthermore, the production of fatty acids was evaluated also for *N. parvum* isolated by grapevine, previously studied for the production of toxic metabolites [14], in order to compare the fatty acid composition of these two species of *Neofusicoccum* involved in grapevine diseases.

From the first investigation steps, it was evident that fungi under examination have different metabolic patterns. In fact, crude extracts had significant differences in weight and preliminary tests (i.e., TLC and NMR) confirmed the different composition of the extracts. These findings are not unusual for fungi belonging to different species.

In this work, five different fatty acids and one dicarboxylic acid were detected in the crude extract of the culture filtrate of *N. vitifusiforme*. The most abundant component identified is linoleic acid (48.2%). Octadecenoid acids have influence in the fungal virulence because many of them are precursors of jasmonic acid [33], a plant hormone capable of inducing phytotoxic effects [12,22,34–37]. Furthermore, Farmer and Ryan [38] tested the capacity of octadecenoid precursors of jasmonic acid, such as linolenic acid and linoleic acid (which can be converted to linolenic acid by the plant) to be inducers of proteinase inhibitors in tomato, tobacco, and alfalfa leaves. The results suggest that the octadecenoid intermediates may participate in the signaling pathway in response to the pathogen attack and in plant colonization.

Even if in low concentration, *N. vitifusiforme* also produces azelaic acid, a dicarboxylic acid well-known for its bacteriostatic and bactericidal properties against diverse microorganisms [39].

As previously reported [13], four naphthalenone polyketides [i.e., botryosphaerones D and A, isoclerone and (3S,4S)-3,4,5-trihydroxy-1-tetralone] were isolated from the examined strains of *N. parvum*. Each of them showed a different phytotoxicity on grapevine leaves. Moreover, some fatty acids occur in its crude extract and linoleic acid represents the main fatty acid component.

The mixtures of fatty acids produced by the two tested species of *Neofusicoccum* do not show phytotoxicity on grapevine leaves and probably do not influence the foliar symptoms' expression of GTDs. In fact, the relationship between the botryosphaeriaceous fungi found in the diseased wood and the foliar symptoms observed in some declining vines is still not well understood [6] and the presence of these symptoms has been very sporadic in monitored Sicilian vineyards. So much so that the foliar chlorosis was only observed for plants infected by *L. mediterranea* strain (B6) and not for plants infected by *N. parvum* (B19), although both produce an assortment of different metabolites [12,13].

The investigations carried out during the last 10 years induce us to hypothesize that, as well as the kind of cultivar and environmental conditions, the type of secondary metabolite, produced by different botryosphaeriaceous fungi, can also be correlated with foliar chlorosis expression in declining grapevines.

Author Contributions: M.M.S., S.G., S.B., A.A. conceived and organized the manuscript, and wrote the text, S.G. and S.B. cultivated the fungal strain and performed the pathogenicity assays; M.D.G. and A.A. performed the extraction of culture filtrate and the NMR analysis; M.M.S., D.N. and F.S. performed the GC/MS analysis; F.S., S.B. and A.A. edited and reviewed the manuscript.

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