SUPPLEMENTARY MATERIAL

Chemical and physico-chemical characterization of phytotoxic metabolites produced by *Verticillium dahliae* Kleb.

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Abstract

Most of the symptoms associated with Verticillium wilt disease in olive cultivation are due to complexes excreted by *Verticillium dahliae*. In this study chemical and physico-chemical techniques were combined to investigate how the molecular structure of phytotoxins isolated from two pathotypes of *Verticillium dahliae*, defoliating, D, and non-defoliating, ND, grown on two different media, Verticillium-dahliae-Medium (VdM) and Simulated Xylem-fluid-Medium (SXM), can affect their aggressiveness. Data obtained highlight important structural differences, both in term of elemental composition and in functional groups distribution. Such peculiarities strongly affect their solubility, which results higher for the phytotoxins from D pathotype. This property likely induces serious modifications of the conformational state of the proteinaceous component, making tyrosine residues accessible to the phosphate ion. A phosphorylation mechanism would thus be promoted, that is going to interfere with the function of the involved proteins in intracellular signalling networks, likely by altering its role in modulating the plant's response.

Key words: *Verticillium dahliae* Kleb., Phytotoxicity, Elemental composition, Fourier transform infrared and Fluorescence Spectroscopies

Experimental section

Secondary metabolite production, purification and bioassay

Two pathotypes of *V. dahliae* were used for this work: the defoliating VD312D (D) isolated from Cotton and the non-defoliating VD315ND (ND) obtained from Olive. Both strains have grown on Potato-Dextrose-Agar (PDA; Difco Laboratories Inc., Detroit, MI, USA) at 25±1°C in the dark, and stored on PDA slants at 4±1°C

at the fungal collection of Plant pathology division, Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti -Di.S.S.P.A, Università Bari. A growth test was performed for the selected pathotypes in stationary liquid cultures in 1-litre Roux flasks containing 150 ml of two different media: *Verticillium-dahliae*-Medium (VdM) and Simulated Xylem fluid Medium (SXM). Each Roux flask was inoculated with 25-30 fragments of mycelium taken from *V. dahaliae* colonies 10-day-old (grown on PDA at 25±1°C, in the dark). Simoultaneously, a control without inoculum was set up for each growth medium. Flasks were incubated at 25±1° C for 28 days in the dark. At harvest, the mycelial mat was removed by filtration on Miracloth (Calbiochem Merck Corporation, Billerica, MA, USA), centrifuged (Centrikon mod H403 centrifuge; Kontron Instruments Volketswil, CH) at 8000×*g*, 4±1°C, 20 min, and further filtered on 0.45μm Millipore filter (Merck Corporation). Finally, it was drained on filter paper, weighed, and dried at 70±1°C for 48 h to measure mycelial dry weight. For each culture filtrate (CF) were determined the total amount (ml), pH values (pH-meter GLP 22, Crison Instruments, Hach, Bizkaia, E) and total protein contents (Bradford 1976). VdM and SXM CFs, and their corresponding dilutions with distilled water (1:1, 1:10 and 1:100) were analyzed for their toxicity on twigs of *O. europaea* cv. Frantoio (resistent to *V. dahliae*) and cv. Leccino (susceptible). Water and non-inoculate VdM and SXM media and their 1:10 dilutions with distilled water were used as control. All solutions before the bioassy were filtered on 0.45 μm Millipore filter. The twigs were kept in a growth chamber (T: 23±2°C, RH: 60±5%, photon flux density: 150 $\mu Em^{-2} s^{-1}$) with their stem immersed in 3 ml of toxic solution or water or non-inoculated media until complete absorption. Each test was replicated three times.

Toxicity symptoms were recorded up to 30 days after a complete adsorption of solution. An empirical 6-degrees scale of severity was adopted: no symptoms (0), curling on leaves (1), wilting (2), yellowing (3), browning (4), and defoliation (5).

In order to purify the toxic metabolites, CFs, previously freeze-dried and stored at -20±1°C, were added with three volumes (1,500 ml) of absolute cold (-20°C) ethanol, and maintained overnight at -20°C. The resulting precipitate was filtered through Whatman GFC filters, dried, weighed and washed three times with 10 ml of cold (-20°C) methanol. The insoluble fraction were collected by centrifugation (Centrikon mod H403 centrifuge, 8,000×g, 4±1° C, 20 min). Each organic residue was freeze-dried and weighed.

Details on media composition

- Verticillium-dahliae-Medium (VdM): 3.3 g l^{-1} $NaNO_3$, 1.0 g l^{-1} K_2HPO_4, 0.5 g l^{-1} $MgSO_4 \times 7H_2O$, 0.01 g l^{-1} $FeSO_4 \times 7H_2O$, 0.5 g l^{-1} KCl, 0.5 g l^{-1} Difco Yeast Extract, 0.5 g l^{-1} Difco Malt Extract, 30 g l^{-1}, D–Glucose, pH value = 5.7
- Simulated Xylem fluid Medium (SXM) (according to Dixon and Pegg 1972, Wood 1961, and Bennett and Lasure 1991): 2.5 g l^{-1} sodium polypectate, 5 g l^{-1} Casamino acids vitamin-free, 0.65 g l^{-1} KCl, 0.65 g l^{-1} $MgSO_4 \times 7H_2O$, 1.9 g l^{-1} KH_2PO_4, 2.2 mg l^{-1} $ZnSO_4 \times 7H_2O$, 1.1 mgl^{-1} H_3BO_3, 0.5 mg l^{-1}, $MnCl_2 \times 4H_2O$, 0.5 mg l^{-1} $FeSO_4 \times 7H_2O$, 0.16 mg l^{-1} $CoCl_2 \times 5H_2O$, 0.16 mg l^{-1} $CuSO_4 \times 5H_2O$, 0.11 mg l^{-1} $(NH_4)_6Mo_7O_{24} \times 4H_2O$, 5.0 mg l^{-1} Na_4EDTA, 0.1 μM Biotin. pH value = 5.5

Statistical eleboration

Biomass, final pH values and total protein concentration data were statistically analysed with SAS/STAT 9.0 (SAS Institute Inc., Cary, NC, USA) software. For the comparison of means, samples were first tested for normal distribution by Shapiro-Wilk test. Post-hoc analyses were performed with Duncan test. In all cases, statistical significance was established at $P = 0.05$.

Chemical and physico-chemical characterization of the examined phytotoxins

Several chemical and physico-chemical techniques were used to evaluate the nature and characteristics of the four purified metabolites: D_VdM (from pathotype VD312D on VdM medium), ND_VdM (from pathotype VD315ND on VdM medium), D_SXM (from pathotype VD312D on SXM medium) and ND_SXM (from pathotype VD315ND on SXM medium).

Elemental analysis, Total Organic Carbon (TOC), Total Nitrogen (TN) and phosphorus determination

The elemental carbon (C), hydrogen (H), nitrogen (N) and oxygen (O) contents were determined in triplicate using a Fisons EA 1108 Elemental Analyser (ThermoQuest, Thermo Scientific, Waltham, MA, USA) calibrated against a 2,5-bis- (5-tert.-butyl-benzoxazol-2-yl)-thiophene (BBOT; Sigma–Aldrich, a part of Merk Corporation) standard. Values were corrected for moisture content, and used to calculate the atomic ratios. To confirm C and N contents in the substances analysed and to test the water solubility of the carbon- and/or nitrogen-containing moieties of these compounds, C and N were further determined on the same samples by an autoanalyzer TOC-VCSH (Shimadzu Scientific Instruments, Tokyo, JP) equipped with the ThEuS 1.3.1 software and calibrated with $KHC_8H_4O_4$, Na_2CO_3, $NaHCO_3$, and KNO_3 (Sigma–Aldrich). The analysis was carried out in triplicate on aqueous solution (0.1 mg $ml^{-1)}$ of each sample. The phosphorus

content was determined after digestion with a Multiwave 3000 (Anton Paar GmbH, Graz, A) according to Murphy and Riley (1962).

Statistical elaboration

Data obtained from the elemental analysis were analyzed by the one-way analysis of variance (ANOVA, F-distribution) using a 95% confidence interval. Fisher's least significant difference (LSD) protected t-test was applied on means comparison.

Fluorescence spectroscopy

Fluorescence spectra were recorded on aqueous solutions (10 mg l^{-1}) of each toxic sample using a Perkin-Elmer LS 55 luminescence spectrophotometer (Perkin- Elmer, Inc., 2001, Norwalk, CT, USA) equipped with the WinLab 4.00.02 software. For both monochromators, emission and excitation slits were set at a 5-nm band width and a scan speed of 240 nm min^{-1} was selected. Sensitivity and stability of the instrument were previously measured using the Raman band signal intensity. Fluorescence spectra were recorded both as single-scan mode (emission, excitation and synchronous-scan modes) and as Total Luminescence (TL). Emission spectra were recorded over the range 300–500 nm at a constant excitation (254 nm) wavelength. Excitation spectra were recorded over the range 270–500 nm at a constant emission (520 nm) wavelength. Synchronous-scan excitation spectra were measured by scanning simultaneously both the excitation, varied from 250 to 500 nm, and the emission wavelengths, while maintaining a constant, optimised wavelength difference $\Delta\lambda$ ($\lambda_{em} - \lambda_{exc}$) =18 nm. Total luminescence (TL) spectra, in the form of excitation/emission matrix (EEM; contour maps), were recorded over the emission wavelength range from 200 to 500 nm, increasing sequentially by 5 mm step the excitation wavelength from 225 to 500 nm. A scan speed of 1200 nm min^{-1} was selected for both monochromators. The EEM plots were generated as contour maps from spectral data by using Surfer 8.0 software (Golden Software LLC, Golden, CO, USA). Fluorescence intensity (FI) values (arbitrary units) were normalized according to TOC content.

Fourier-Transform Infrared spectroscopy

The FT-IR spectra were recorded on pellets obtained by pressing under reduced pressure a mixture of 1 mg of each sample and 400 mg of spectrometry grade dried KBr (Sigma–Aldrich), using a Nicolet Nexus FT-IR spectrophotometer (Thermo Scientific) equipped with a Nicolet Omnic 6.0 software. Spectra were recorded in the range 4000–400 cm^{-1}, with 2 cm^{-1} resolution, and 64 scans. To enhance the resolution of FT-IR spectra

and allow the identifications of the individual components of the absorption bands, Fourier Self-Deconvolution (FSD) was applied (Buslov et al. 2002).

References

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Table S1. Fresh (fwt) and dry (dwt) weight biomass (mg ml^{-1}), final pH values, and total protein concentration (μg ml^{-1}) in 28 day-old liquid cultures of *V. dahliae* defoliating (VD312D) and non-defoliating (VD315ND) pathotypes grown in Verticillium-dahliae-Medium (VdM) and Simulated Xylem fluid Medium (SXM) at 25±1°C in the dark**

Strains	Medium	Biomass (mg ml^{-1})		pH value***	Protein****
		f wt***	d wt***		(μg ml^{-1})
VD312D	VdM	$31.54±4.2^{B}$	$10.51±2.5^{B}$	$7.3±0.02^{D}$	$142.39±21.3^{D}$
	SXM	$16.26±2.3^{C}$	$5.72±1.5^{C}$	$8.7±0.02^{B}$	$248.21±25.2^{A}$
VD315ND	VdM	$44.76±5.2^{A}$	$14.92±2.4^{A}$	$7.8±0.02^{D}$	$148.29±21.7^{C}$
	SXM	$14.35±2.1^{C}$	$3.43±1.2^{C}$	$8.8±0.02^{A}$	$216.33±31.5^{B}$

** Values followed by different letters are significantly different at *P = 0.05*

***Values are means of 6 replicates ± SD

****Values are means of ten replicates ± SD

Table S2. Elemental composition* and P* content (%) (moisture free) and atomic ratios of the phytotoxins D_VdM (pathotype VD312D on VdM medium), ND_VdM (pathotype VD315ND on VdM medium), D_SXM (pathotype VD312D on SXM medium) and ND_SXM (pathotype VD315ND on SXM medium) (error standard in parenthesis)

Samples	C	H	N	O	P	Atomic ratios	
			%			(O+N)/C	H/O
D_VdM	17,82 (0.2)***	2,87 (0.1)**	6,11 (0.2)	25,47***	3,48 (0.1)	1,83	0,11
ND_VdM	24,55 (0.6)***	4,37 (0.1)**	6,15 (0.2)	8,03***	2,51 (0.1)	0,54	0,54
D_SXM	8,50 (0.2)***	3,73 (0.1)**	4,16 (0.1)***	13,06***	13,23 (0.2)***	2,03	0,29
ND_SXM	13,55 (0.6)***	4,93 (0.3)**	6,54 (0.2)	3,26***	6,59 (0.1)	0,72	1,51

*Value are means of 3 replicates

**Values are significantly different at *P = 0.05*

*** Values are significantly different at *P = 0.001*

Table S3. Total carbon (TC)*, inorganic carbon (IC), total organic carbon (TOC)* and total nitrogen (TN)* contents (mg l^{-1}) of the phytotoxins D_VdM (pathotype VD312D on VdM medium), ND_VdM (pathotype VD315ND on VdM medium), D_SXM (pathotype VD312D on SXM medium) and ND_SXM (pathotype VD315ND on SXM medium) (error standard in parenthesis)

Samples	TC	IC	TOC	TN
	mg l^{-1}			
D_VdM	17,70 (0.1)***	0,73 (0.0)***	16,97***	5,27 (0.1)***
ND_VdM	18,20 (0.1)***	0,83 (0.0)***	17,37***	3,99 (0.0)**
D_SXM	7,04 (0.0)***	0,71 (0.0)***	6,33***	3,86 (0.0)**
ND_SXM	10,60 (0.0)***	0,74 (0.0)***	9,87***	5,84 (0.0)***

*Value are means of 3 replicates

**Values are significantly different at $P = 0.05$

*** Values are significantly different at $P = 0.001$

Table S4. Major FT-IR and FSD absorption bands with relative assignment of *V. dahliae* phytotoxins D_VdM (pathotype VD312D on VdM medium), ND_VdM (pathotype VD315ND on VdM medium), D_SXM (pathotype VD312D on SXM medium) and ND_SXM (pathotype VD315ND on SXM medium)

Wavenumber (cm^{-1})	**Assignment**	Samples			
		D VdM	ND VdM	D SXM	ND SXM
3700-3100	O-H stretch of bonded alcohol and bonded acid groups (inter- and intramolecular association)	++	+	++	++
3448-3419	N-H stretch of secondary amide: Amide A band (protein), N-H stretch of indole nucleus (tryptophane)	-	+	-	-
3000-2840	C–H sym and asym stretch of CH_2 and CH_3 groups	+	+	-	-
1750-1735	C=O stretch of saturated lipid or phospholipid esters and/or six-membered ring lactone	++	-	-	-
1669-1666	C=O stretch of quinones and/or conjugated ketones	-	++	+	++
1643-1640	C=O stretch of secondary amides I bands of proteins	-	++	+	++
1626-1610	C=C stretch of alkenyl structures	+++	++	++	+
1578	C=C stretch of aromatic ring	+	++	+	++
1554-1530	N–H bend of amide II band (protein)	+	++	+	+
1455-1449	C-H aymmetric bend of CH_2 and CH_3 groups ($\delta asCH_2$ + $\delta asCH_3$)	-	++	+	++
1405, 1402	C-H bend of proteins	++	+	++	+
1388-1384	C=O of COO^- (sym stretch), C-H bend of CH_3 groups (δsCH_3)	++++	++	-	-
1289	C-N stretch of secondary amides, and/or Car-O stretch	-	-	+	-
1250-880	P=O, P-O stretch of phospholipids and C-O-C, C-OH stretch of carbohydrates	+	-	++	+
1252-1249	P=O asym stretch of phosphoryl groups (phosphorylated proteins, nucleic acid, polyphosphate)	++	+	+++	+
1116-1111	Car-O stretch	-	+++	++	+++
1074-1057	P-O- stretch and/or νas C-OH: glycosidic ring, P=O asym stretch of phosphorylated proteins, nucleic acid, polyphosphate	++	+	++	+
1150-1050	C-O stretch (tertiary, secondary and primary alcohols)	+++	+	+++	++
1050-1030	C-OH stretch of primary alcohol of carboydrates, P-O-H stretch	++	+	-	++
996, 883	C-O-C stretch: β→(1-4)-glycosidic linkage)	+++	+	++	-
860	P-O-H out of plane bending	++	+	+	+
828	Car-H out of plane bend of para-disubstituted aromatic ring (tyrosine)	++	+	+++	++
830-720	P-O-C bend of phospholipids and/or amide IV band	++	+	+++	++
619, 541-524	O-P-O bend (doublet) and/or N-H out of plane bend (amide IV band)	+++	++	+++	++

Symbols + and – indicate, respectively, the presence /absence of absorption bands

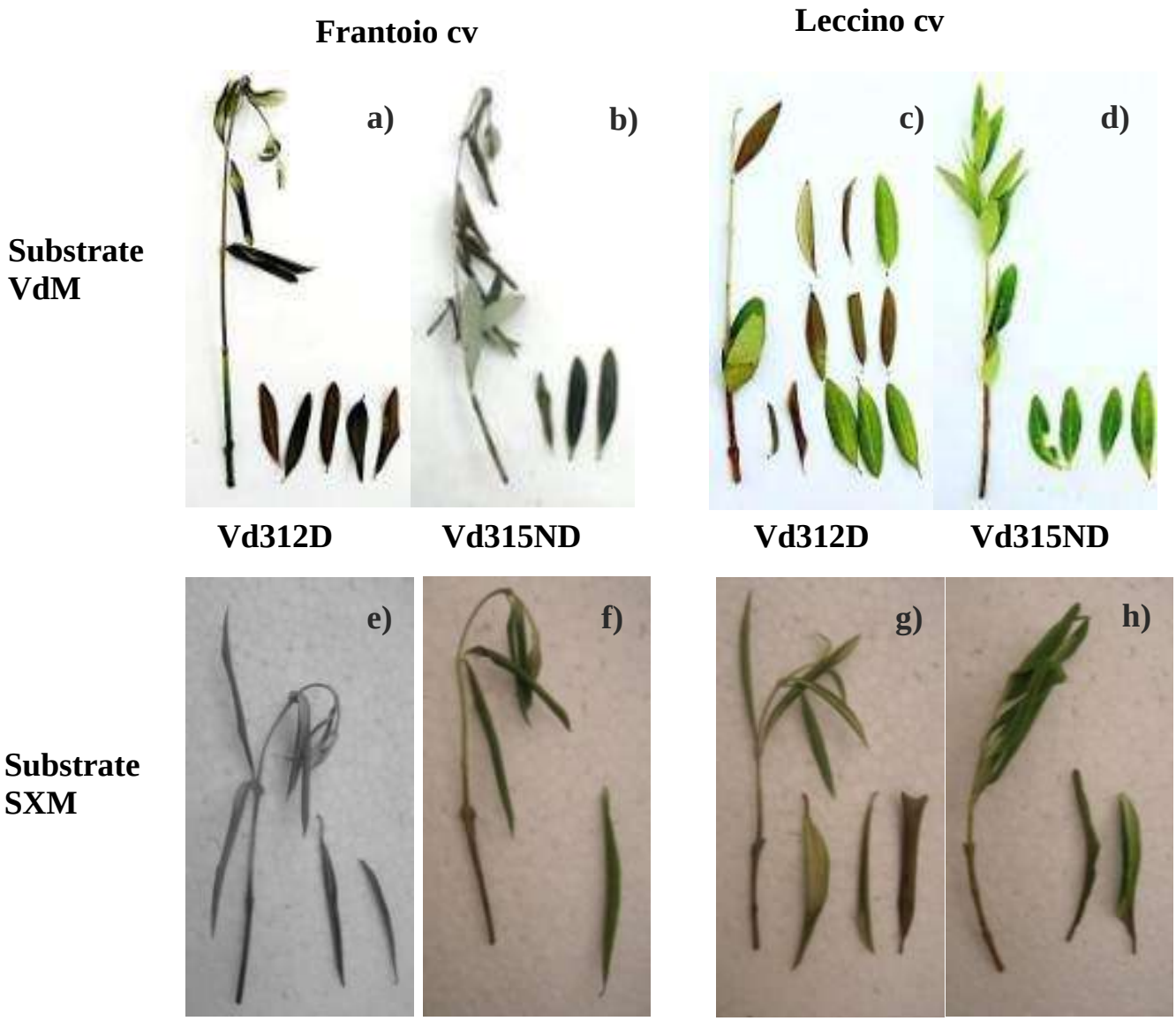


Figure S1. Effects on twigs of Frantoio (left) and Leccino (right) after the absorption of 3 ml of culture filtrates obtained from V. dahliae Vd312 defoliating (a and c), and Vd315 non-defoliating (b and d)) after 28 days of culture in Verticillium dahliae-Medium (top) and Vd312 defoliating (e and g), and Vd315 non-defoliating (f and h) in Simulated Xylem fluid Medium (bottom). Photo shows twigs 25 days after absorption of solutions.

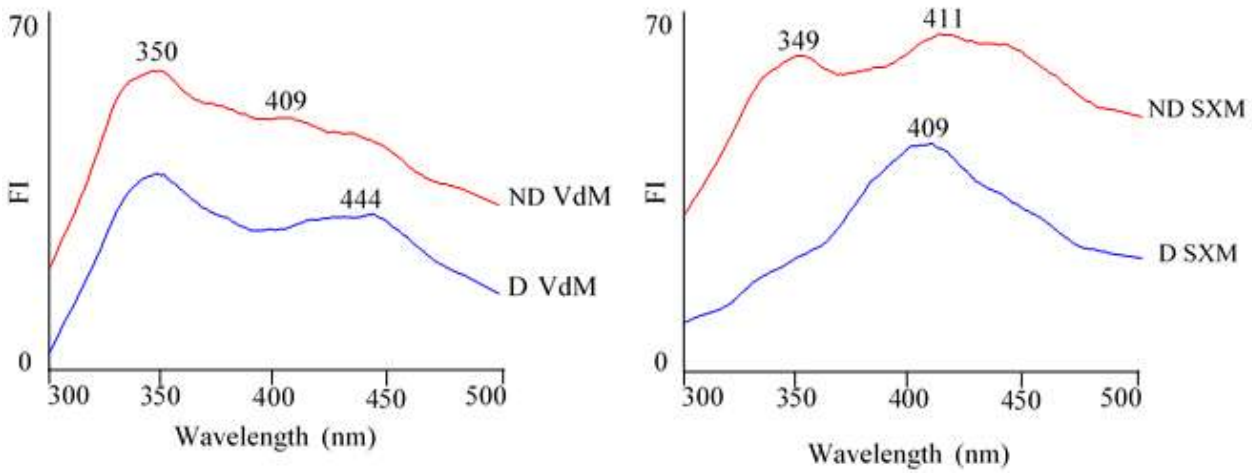


Figure S2. Emission fluorescence spectra of the purified toxins D_VdM (pathotype VD312D on VdM medium), ND_VdM (pathotype VD315ND on VdM medium), D_SXM (pathotype VD312D on SXM medium) and ND_SXM (pathotype VD315ND on SXM medium). FI = Fluorescence intensity normalized according to TOC content, arbitrary units.

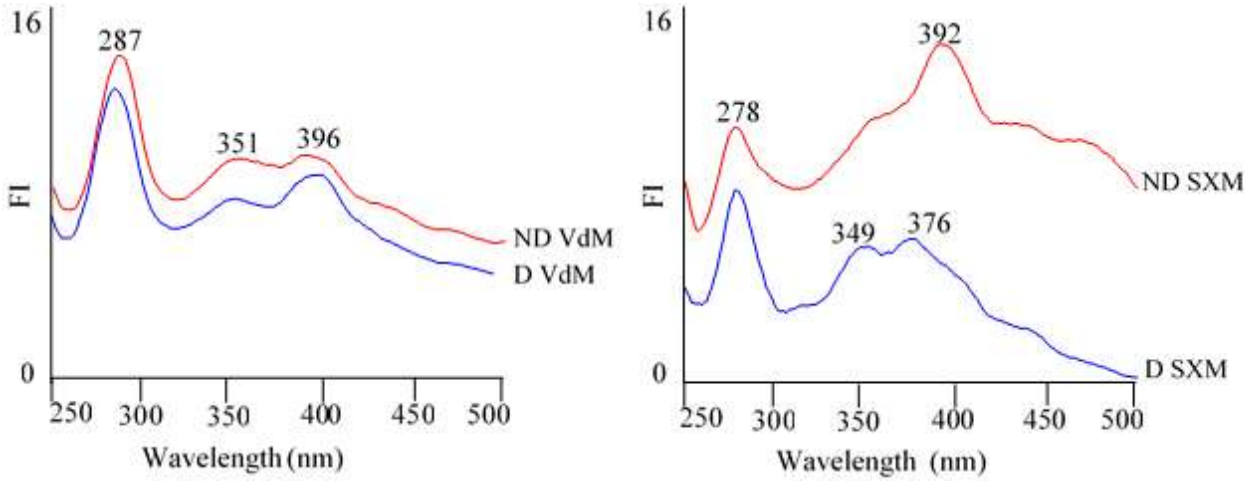


Figure S3. Synchronous-scan fluorescence spectra of the purified toxins D_VdM (pathotype VD312D on VdM medium), ND_VdM (pathotype VD315ND on VdM medium), D_SXM (pathotype VD312D on SXM medium) and ND_SXM (pathotype VD315ND on SXM medium). FI = Fluorescence intensity normalized according to TOC content, arbitrary units.

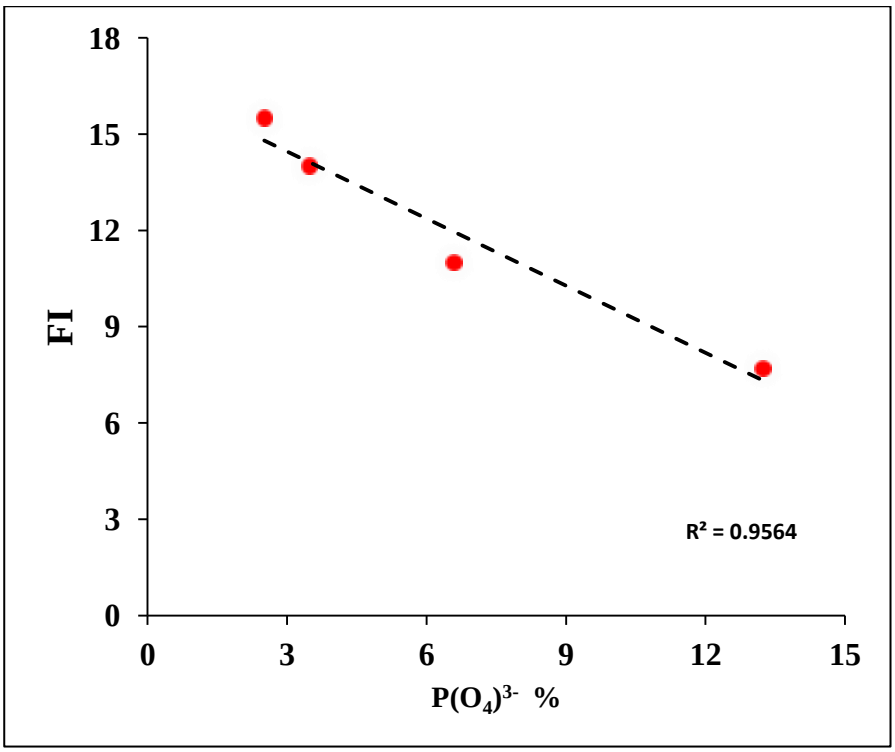


Figure S4. Relationships between $P(O_4)^{3-}$ ions (%) and Fluorescence intensity values (FI) (Arbitray Units).

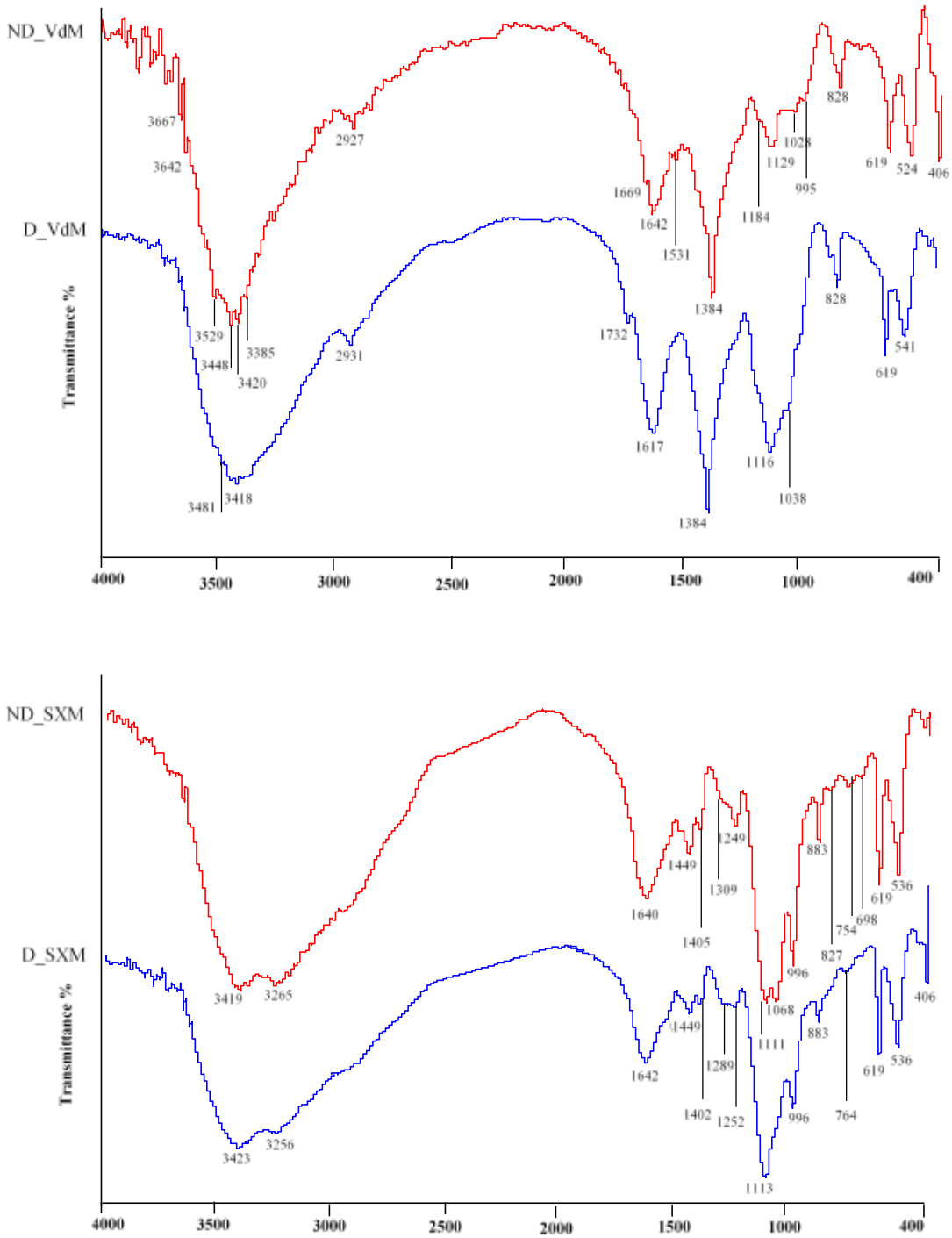
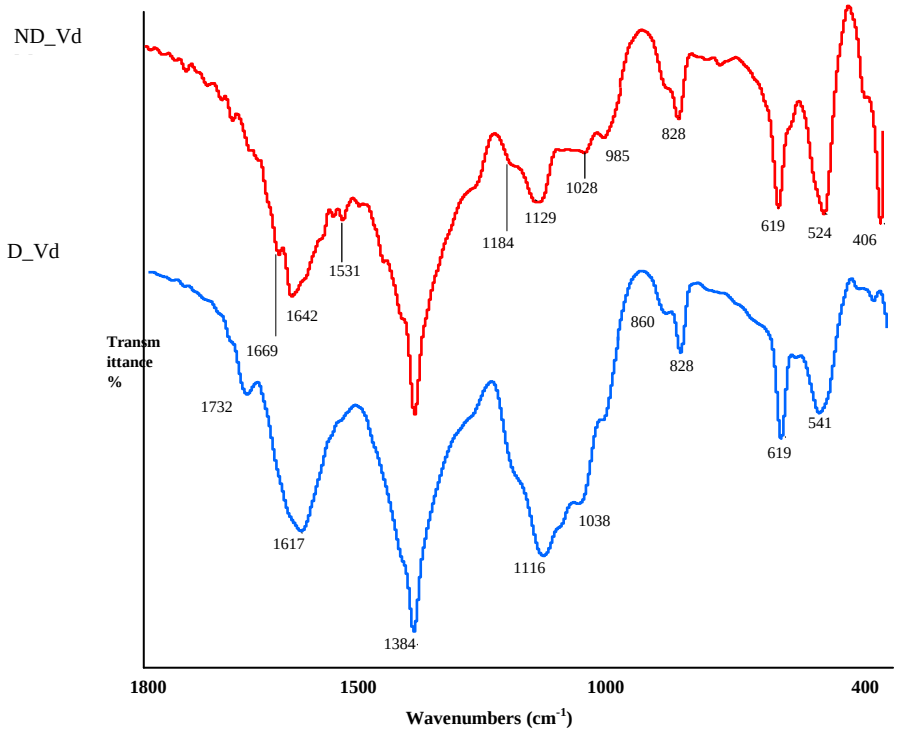


Figure S5. FT IR spectra of the purified toxins D_VdM (pathotype VD312D on VdM medium), ND_VdM (pathotype VD315ND on VdM medium), D_SXM (pathotype VD312D on SXM medium) and ND_SXM (pathotype VD315ND on SXM medium).


ND_Vd
D_Vd
Transm
ittance
%
1732
1669
1642
1531
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1384
1184
1129
1116
1038
1028
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860
828
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619
619
541
524
406
1800
1500
1000
400
Wavenumbers (cm-1)

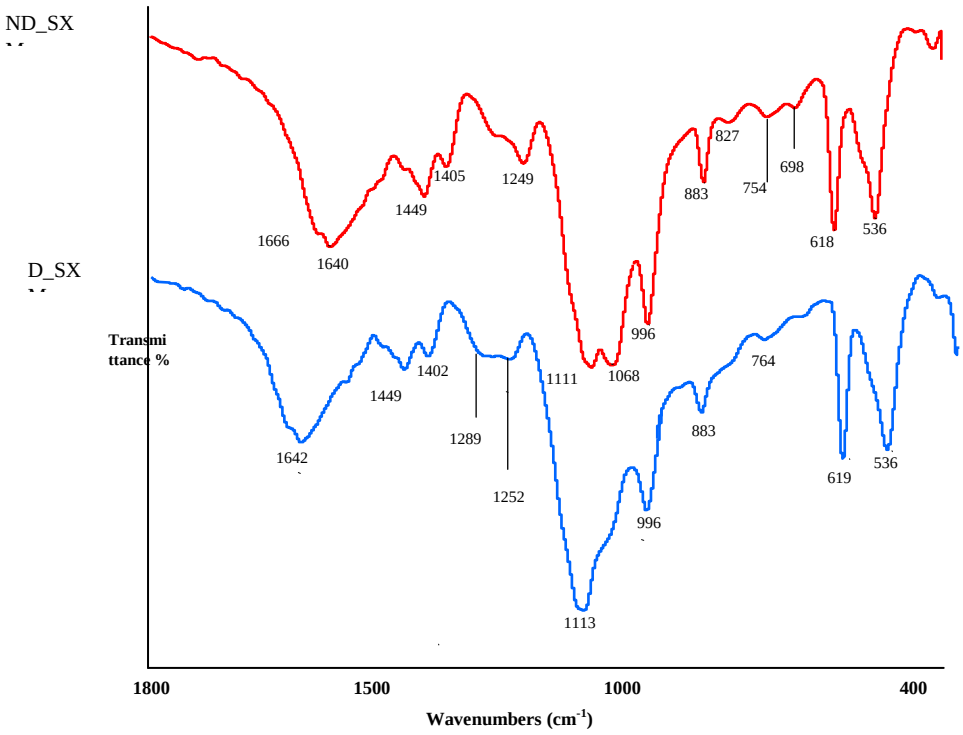
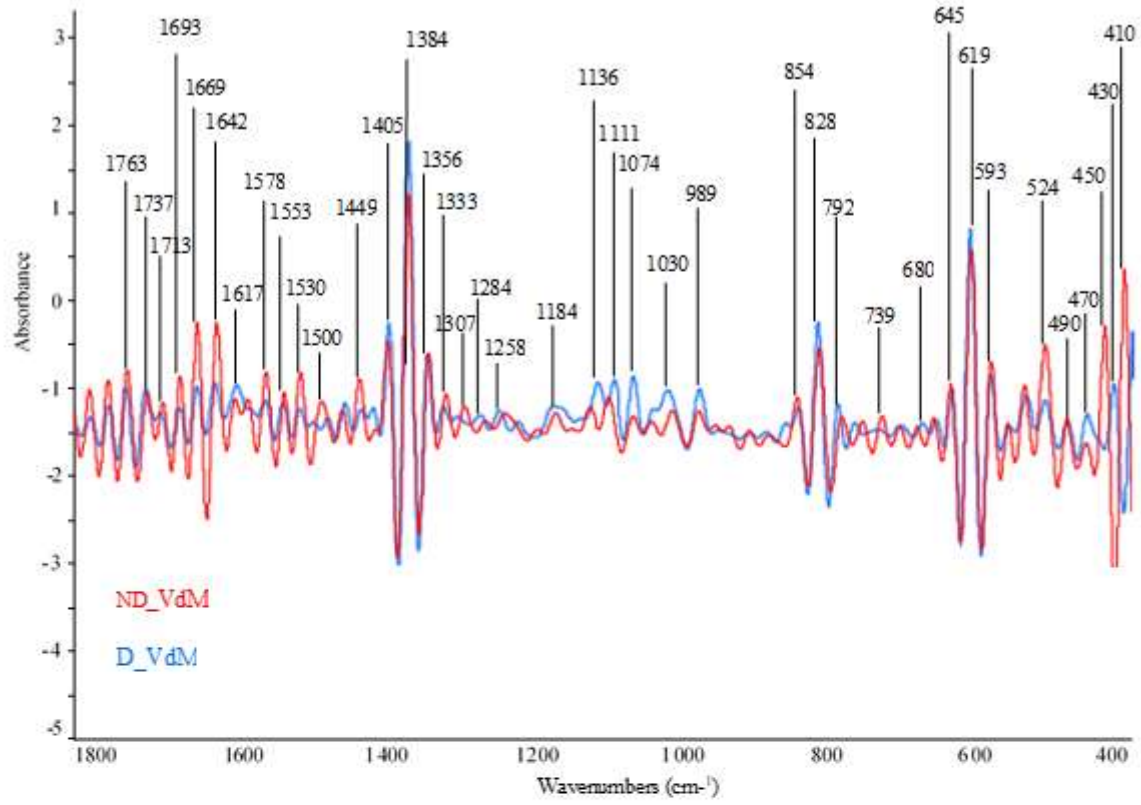

ND_SX
D_SX
Transmi
ttance %
1666
1640
1449
1405
1249
1111
1068
996
883
827
754
698
618
536
1642
1449
1402
1289
1252
1113
996
883
764
619
536
1800
1500
1000
400
Wavenumbers (cm-1)

Figure S6. FT IR fingerprint region spectra of the purified toxins D_VdM (pathotype VD312D on VdM medium), ND_VdM (pathotype VD315ND on VdM medium), D_SXM (pathotype VD312D on SXM medium) nad ND_SXM (pathotype VD315ND on SXM medium).



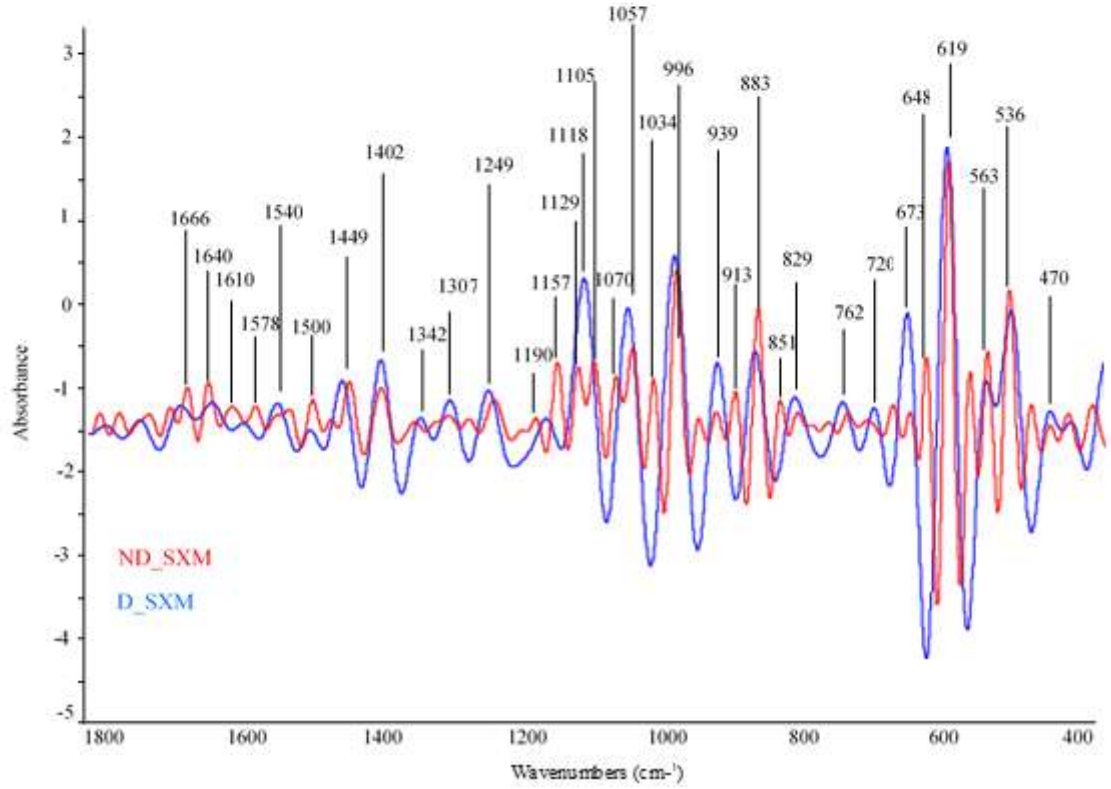


Figure S7. FSD FT IR fingerprint region spectra of the purified toxins D_VdM (pathotype VD312D on VdM medium), ND_VdM (pathotype VD315ND on VdM medium), D_SXM (pathotype VD312D on SXM medium) nad ND_SXM (pathotype VD315ND on SXM medium).