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Phytotoxic metabolites produced by *Verticillium dahliae* Kleb. in olive wilting: a chemical and spectroscopic approach for their molecular characterisation

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ABSTRACT

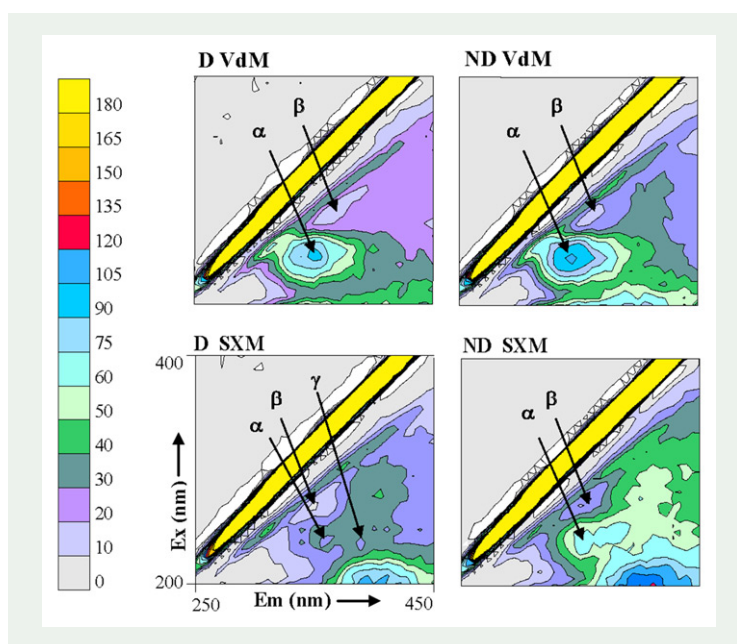
Most of the symptoms associated with *Verticillium* wilt disease in olive cultivation are due to complexes excreted by *Verticillium dahliae*. In this study chemical and physico-chemical techniques were combined to investigate how the molecular structure of phytotoxins isolated from two pathotypes of *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, resulted 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.

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1. Introduction

Verticillium dahliae Kleb. (Fungi, Dikarya, Ascomycota) is a soil-borne fungal plant pathogen, widely present all over the world and well known for being the cause of severe wilt in many herbaceous and woody plants (Pegg and Brady 2002; Kaliterna et al. 2016)). *V. dahliae* infecting olive can be classified as defoliating (D) pathotype, typically highly aggressive, that causes early leaf shedding, and non-defoliating (ND) one, mildly aggressive, that does not cause quick and extended defoliation (Sanei et al. 2008). Olive cultivars respond with different reactions to *Verticillium* wilt, showing diverse levels of resistance traits and susceptibility (Lopez-Escudero and Mercado-Blanco 2011; Bubici and Cirulli 2012; Calderón et al. 2013; Arias-Calderón et al. 2015; Sanei and Razavi 2017; Lozano-Tovar et al. 2017). In the last decades, being the olive a crop with a huge economical, social and ecological importance, numerous studies have been carried out on this topic, related to pathogen biology, cultivar susceptibility, disease epidemiology, integrated control strategies, genetic characterisation, and so forth (Dervis et al. 2007; Sanei and Razavi 2011; El-Bebany et al. 2013; Papaioannou et al. 2013; Carrero-Carrón et al. 2016; Chen et al. 2016; Michuda et al. 2018; Zeng et al. 2018). In general, plant pathogen may produce, in their hosts as well as in soil or artificial media, different toxic compounds, as a consequence of their secondary metabolism. These substances are better isolated from artificial media rather than natural substrates, and VdM and SXM media are routinely used for the production of *V. dahliae* metabolites (Graniti et al. 1989). In several studies, crude extracts that contain *Verticillium* toxin complexes have been used to elicit plant defence responses or to study physiological responses of the plants (Fradin and Thomma 2006; Wang et al. 2012). However, the exact nature of the components of the complexes is often

unclear. Generally, these crude extracts are found to contain high-molecular weight protein-lipopolysaccharide (PLP) complexes, found to dissociate under non-reducing conditions while the phytotoxic activity remained in a low-molecular-weight polypeptide fraction (Buchner et al. 1982; Nachmias et al. 1985). Further studies were carried out on the molecular characteristics of the phytotoxins produced by these pathotypes involved on cotton and tomato Verticillium disease (Meyer and Dubery 1993; Mansoori et al. 1995; Davis et al. 1998; Palmer et al. 2005; Jia et al. 2007; Gao et al. 2010; Liu et al. 2013). A new phytotoxin, produced by a strain of *V. dahliae* pathogenic on olive tree, was purified from culture fluids, and results obtained confirmed that this new phytotoxin can be used to differentiate between Verticillium-tolerant and susceptible varieties of olive tree (Laouane et al. 2011). In general, all these studies highlight how, in the toxic activity, an important role is played by a low molecular weight polypeptide fraction. The aim of this work was to isolate and partially characterise, by a chemical and spectroscopic approach, the phytotoxic secondary metabolites produced in vitro by two pathotypes of *V. dahliae*, VD312D (D) and VD315ND (ND), grown on two different media, Verticillium-dahliae-Medium (VdM) and Simulated Xylem-fluid-Medium (SXM), both involved in the disease symptom expression of two selected olive cultivars, Frantoio cv. (resistant to *V. dahliae*) and Leccino cv. (susceptible).

2. Results and discussion

2.1. Phytotoxicity of *V. dahliae* culture filtrates

In our experiments, the two isolates of *V. dahliae*, Vd312D and Vd315ND, seem well adapted to the growth conditions adopted, as showed by data reported in Supporting Information Table S1. After 28 days of incubation, both pathotypes produce a large amount of biomass (as fresh and dry weight) in VdM medium, with values significantly higher for the ND sample. On the opposite, lower contents and nonsignificant differences are evident for both pathotypes grown on SXM medium (Supporting Information Table S1). A marked increase of the initial pH value occurs for both pathotypes (Supporting Information Table S1), statistically significant in SXM medium. Finally, both pathotypes grown in SXM medium show a significantly higher total protein concentration with respect to that measured in the corresponding samples from VdM medium, whereas there are not trends correlated to the specificity of the strain considered. In Supporting Information Figure S1 are reported the effects on twigs of Frantoio (resistant) and Leccino (susceptible) of 3 mL of culture filtrates (CFs) obtained from D and ND pathotypes after 28 days of culture in VdM and SXM media. For all CFs toxicity symptoms started 7–9 days after testing. As final symptom defoliation occurs on both cultivars. No symptoms appear during the whole testing period (30 days) for the corresponding control (water or non-inoculated media). CFs from the two pathotypes grown on VdM induce leaves curling and browning on Frantoio, whereas on Leccino yellowing starts from leaf vein, and in 3–5 days appears on the whole leaf surfaces. Defoliation induced from D pathotype requires 17- and 21-days from the beginning of the test, while that from ND one starts 20- and 25-days, on Leccino and Frantoio, respectively. Similar effects, though characterised by a faster defoliation process, are induced in both cultivars by the CFs of two pathotypes grown on SXM

medium. In detail, on Frantoio cv D and ND CFs induce leaves curling, browning, and 20 days-past-adsorption defoliation, whereas the leaves of Leccino become yellow, browning and fell on 16-days. All CFs maintain their toxic properties at 1:1, 1:10 and 1:100 dilutions with distilled water. Further, aqueous solutions ($100\text{ }\mu\text{g mL}^{-1}$) of the four fractions (D_VdM, ND_VdM, D_SXM, and ND_SXM) purified from both *V. dahliae* pathotypes, by precipitation with ethanol and washing with methanol, cause leaves curling, browning and defoliation on twigs of Frantoio, yellowing, browning and defoliation on twigs of Leccino (data not shown). Data presented in this study clearly show that defoliating and non-defoliating pathotypes of *V. dahliae* produce substances that, when absorbed by olive twigs, are involved in wilting symptoms. In detail, these toxic substances, produced both in VdM and SXM media, obtained from the culture filtrates and collected in ethanol precipitates, induce chlorosis, necrosis, and defoliation on olive twigs collected from resistant (Frantoio) and susceptible (Leccino) olive cultivars.

2.2. Chemical and physico-chemical characterisation of the four phytotoxin

2.2.1. Elemental composition

Elemental composition data (average values), atomic ratios, and P content of the four isolated toxic metabolites, indicated as D_VdM, ND_VdM, D_SXM and ND_SXM, are reported in Supporting Information Table S2. In general, C and H contents of ND fractions are significantly higher than those of D ones, while showing an opposite trend as a function of the growth medium. In fact, the C contents are higher in the fractions from VdM medium, whereas H contents are higher in those from SXM medium. No differences appear for the N content, with the exception of the sample D_SXM characterised by a significantly lower value. Data relating to O content, absolutely greater in the D and ND fractions from VdM medium, are significantly higher in D fractions with respect to those of the corresponding ND ones (Supporting Information Table S2). Similarly, P content shows the same trend reported above if it is taken into consideration the pathotype, whereas the opposite is true with respect to the growth medium (Supporting Information Table S2). The total organic carbon (TOC) and total nitrogen (TN) analysis (Supporting Information Table S3), although an overall decrease of C and N contents, are in agreement with those reported above (Supporting Information Table S2), confirming the trend of these elements. Elemental analysis, TOC and TN data have been further used to calculate the ratio between C and N content in the solid samples and the corresponding value in the liquid extracts. The goal was to test the different solubility of the various structural C- and N-components occurring in the four samples. In all cases, both the ratios $C(\text{solid})/C(\text{liquid})$ and $N(\text{solid})/N(\text{liquid})$ of the phytotoxins from D pathotype, grown on both media, is close to 1, whereas those calculated for ND samples are slightly higher. These results suggest that the C- and N-moieties of the fractions isolated from D pathotype have a greater solubility with respect to those from the pathotype ND, thus indicating for the latter the presence of insoluble C- and N-containing units. This hypothesis is strongly supported by $(O+N)/C$ and H/O atomic ratios, shown in Supporting Information Table S2. In particular, $(O+N)/C$ ratio can be considered an index to evaluate the polar/non-polar balance

of the samples, whereas H/O ratio can be used to evaluate the oxidation degree, that is, a greater ratio corresponds to a relatively low polarity and high hydrophobicity. Thus, higher (O + N)/C and lower H/O atomic ratio values found in the D fractions, with respect to those of ND samples, suggest a more polar character and a relatively high amount of oxygen-containing functional groups, that is, a low hydrophobicity (Supporting Information Table S2). On the whole, elemental data clearly indicate that phytotoxins of D pathotype are characterised by a greater solubility, likely related to the presence of more polar groups and lower molecular weights, with respect to those produced by the ND pathotype. These information on the molecular and structural properties of the examined fractions suggests that the nature of pathotype plays a key role in the molecular composition and properties of the corresponding phytotoxins, unlike the growth substrate whose influence is less significant.

2.2.2. Fluorescence spectroscopy

Fluorescence emission spectra (Supporting Information Figure S2) of each phytotoxins are characterised by a main peak, with the presence of a secondary shoulder. In particular, ND_VdM sample is characterised by a main peak centered in the region of lower wavelengths (350 nm), with a normalised Fluorescence intensity (FI) value of about 60 U, and a weak shoulder centered at higher wavelengths (409–414 nm), FI value about 48 U. A similar spectrum is shown by the sample ND-SXM, characterised by the same peak and shoulder, whose intensities are inverted (62 and 70 U, respectively). The sample D_VdM is characterised by the same main peak at about 350 nm, with the presence of a discrete peak at intermediate wavelengths (444 nm), whereas the sample D_SXM shows a unique main peak at 409 nm. A strong decrease of FI (ca. 20) of the main peak of emission spectra is recorded for both D fractions with respect to ND ones, regardless the growth medium. As generally found in complex molecules, the emission spectra appear to be broad and featureless, precisely because they derive from the unresolved overlapping of different spectral bands, some of which are evident as simple shoulders. Emission fluorescence spectra pattern of all samples examined may be ascribed to the presence of simpler structural components of small molecular size, bearing electron-donating substituents such as hydroxyls, methoxyls, and amino groups, and to small levels of aromatic polycondensation and conjugated chromophores. Furthermore, the occurrence of fluorescence bands and peaks at slightly longer wavelengths with smaller FI values can be related to the presence of systems with a certain degree of unsaturation and, consequently, of conjugation, as well as to the presence of fractions bearing electron-withdrawing substituents such as carbonyl and carboxyl groups (D'Orazio et al. 2014). These features are evident in the emission spectra of the phototoxins from D strain. Fluorescence emission data are confirmed by synchronous-scan fluorescence spectra, that appear more structured and better resolved (Supporting Information Figure S3). Synchronous-scan spectra of ND_VdM and D_VdM fraction show a main peak in the lowest wavelengths region (287 nm), and two peaks in the intermediate region, respectively, at 351 and 396 nm. ND_SXM show a different pattern, with the main peak at 392 nm and a secondary peak at about 278 nm. The synchronous scan spectrum of D_SXM shows the same pattern of the corresponding ND one, with a blue shift of the main peak. As observed

for the emission spectra, FI values of phytotoxins from D pathotype result much lower than those recorded for the corresponding ND ones. Total Luminescence (TL) spectra of the four fractions examined (Graphical abstract) are characterised by the presence of numerous fluorophores, among which two or three of greater importance, centered in the lower wavelength region, with some differences in their intensities and excitation/emission wavelengths pairs (EEWPs). In detail, all fractions are characterised by the presence of two main peaks, α and β , centred at EEWPs 270-275ex/341-357em and 305-310ex/351-359em. A third peak, γ , at EEWPs 260ex/437-440em is evident in D_SXM. The main peaks, α and β , may be associated, respectively, to simple components of proteinaceous origin such as tryptophan and tyrosine aromatic aminoacids, and simple phenolic compounds with the conjugated electronic system limited to one aromatic ring (Lakowicz 2006). The third peak, γ , may be associated to simple phenolic-like structures, hydroxylsubstituted benzoic and cinnamic acid derivatives (Wolfbeis 1985; Airado-Rodríguez et al. 2011; D'Orazio et al. 2014). As reported for the previous single-scan mode spectra, ND fractions are featured by greater FI values, that appear significantly lower in the corresponding fractions from D pathotype. On the whole, fluorescence spectra pattern appears to be consistent with those commonly recorded for proteins or peptides (Lakowicz 2006). Tryptophan (Trp), tyrosine (Tyr) are the amino acid residues mainly responsible for the intrinsic fluorescence of proteins (Baker 2002). In proteins that contain these aromatic amino acids, the emitted fluorescence is usually dominated by the contribution of the Trp, though it is generally present at about 1 mole% in proteins (Lakowicz 2006). On the other hand, Tyr presents a strong absorption band at 280 nm and might contribute significantly to protein fluorescence because it usually occurs in larger amounts (Abani, 2007). Further, in neutral solution the Tyr hydroxyl group can dissociate (Lakowicz 2006), and Tyr units can form a ground-state complex with weak bases. In these conditions, quenching of tyrosine by weak bases can thus proceed through different mechanisms. If the solvent contains proton acceptors, these can access easily to their residues, with a consequent strong decrease of fluorescence intensity. Several studies showed the quenching effect played by phosphate ions, especially on Tyr (Vdovenko et al. 1993). Phosphate is reported to form a ground-state complex with tyrosine that prepares tyr to ionise to tyrosinate as a result of excitation by light (Lakowicz 2006). The quenching of tyr emission is due to the deprotonation of the phenolic group promoted in the excited state by phosphate ions and leading to the weakly fluorescent tyrosinate ion (Behmoaras et al. 2008). Our data confirm this hypothesis, as highlighted by the good correlation found between P contents and FI values ($r^2 = 0.956$) (Supporting Information Figure S4). Protein phosphorylation is one of the critical regulatory mechanisms in signal transduction pathways modulating plant growth, development and responses to the environment (Lu et al. 2015). The past few years have seen significant advances in techniques and technologies in global phosphoproteomic studies, as well as functional analyses of specific phosphorylation sites, and the importance of pTyr signaling system has now been widely recognised also in plant physiology (Garcia-Brugger et al. 2006). Furthermore, according to Lim and Pawson (2010), tyrosine phosphorylation in plants is not simply restricted to a limited number of protein species and to stress-related responses, but, as well as in animals, it concerns many other proteins from all

subcellular fractions and also with developmental control, strongly suggesting that a complex protein tyrosine phosphorylation system might exist in plants too ().

2.2.3. Fourier-transform infrared spectroscopy

FT-IR spectra of the four phytotoxins isolated from VD312D and VD315ND pathotypes feature a number of common absorption bands with some differences in their relative intensity and some distinctive bands (Supporting Information Figure S5 and S6). On the whole, samples obtained from ND pathotype are characterised by a larger amount of quinones and/or conjugated ketones ($\sim 1666\text{ cm}^{-1}$) (Boukir et al., 2001), secondary amides ($\sim 1640\text{ cm}^{-1}$) (Hajji et al., 2015), aromatic rings variously substituted ($\sim 1580\text{ cm}^{-1}$ and $\sim 828\text{ cm}^{-1}$) (Colthup et al., 1990; Boukir et al., 2001), and both methyl (2927 and 1384 cm^{-1}) and methylene groups (~ 2927 and 1450 cm^{-1}). Otherwise, D fractions from both pathotypes show a greater amount of saturated lipid or phospholipid esters and/or six-membered ring lactones ($\sim 1732\text{ cm}^{-1}$ and $\sim 830\text{--}720\text{ cm}^{-1}$), alkenyl structures ($\sim 1620\text{ cm}^{-1}$), methylene groups ($\sim 1400\text{ cm}^{-1}$), carboxylate groups ($\sim 1384\text{ cm}^{-1}$), glycosidic rings ($\sim 1150\text{ cm}^{-1}$) (Hajji et al., 2015; Boukir et al., 2019) and phosphoryl groups of phosphorylated proteins, nucleic acids, and polyphosphates (~ 1070 , ~ 1250 , $\sim 619\text{ cm}^{-1}$) (Colthup et al., 1990; Driver et al., 2015), tertiary, secondary and primary alcohols ($1150\text{--}1050\text{ cm}^{-1}$) (Durmaz et al., 2016), polysaccharides with $\beta\rightarrow(1\text{--}4)$ glycosidic linkage ($\sim 898\text{ cm}^{-1}$) (Hajji et al., 2015; Boukir et al., 2019), para-disubstituted aromatic rings ($\sim 828\text{ cm}^{-1}$) (Colthup et al., 1990; Boukir et al., 2001), and phosphate groups ($\sim 619\text{ cm}^{-1}$). On the whole, FTIR data largely support elemental and fluorescence results previously discussed, emphasising the different groups distribution between the two pathotypes examined, D and ND. In fact, the FT IR spectra of phytotoxins from D strain, if compared to those of the corresponding ND ones, appear richer in functional groups potentially more soluble, such as carbonyl and carboxyl groups, alkenyl chain with hydroxy-substituted benzoic derivatives, aryl and alkyl phosphate esters, alcohols and polysaccharidic units. Certainly, the presence of a polar portion in a molecule justifies its greater solubility only if accompanied by a not long and not very branched carbon chain. In this respect, fluorescence data clearly indicate that all phytotoxins examined are structurally simple and of low molecular weight. To better evaluate the differences occurring in the FT IR spectra, Fourier self-deconvolution (FSD) procedure are performed in the $2,000\text{--}400\text{ cm}^{-1}$ region (Supporting Information Figure S7). Following this procedure, some interesting structural features of the samples examined are discernible, and it is possible to identify the principal bands that contribute to the more complex band resulting from overlapping features (Nikonenko et al., 2000; Buslov et al., 2002). In Supporting Information Table S4 are reported the main FT IR and FSD absorption bands, with relative assignment (Colthup et al., 1990; Coates 2000; Nikonenko et al., 2000; Boukir et al., 2001; Buslov et al., 2002; Driver et al., 2015; Hajji et al., 2015; Durmaz et al., 2016; Boukir et al., 2019;), whose presence/absence is indicated with the + and – symbols. On the whole, data obtained by FTIR and FSD spectroscopy support and confirm the previously obtained data, both from the elementary analysis and the fluorescence. In fact, the greater resolution obtained by means of the deconvolution process show more clearly the differences already observed in the classical

spectra, and allows to identify spectral features previously overlapping or hidden, related both to the original pathotype and to the growth medium. In detail, data obtained by FT-IR and FSD spectroscopy indicate the occurrence of hydrophilic molecular structures in both fractions isolated from pathotype Vd312D, whereas hydrophobic units are abundant in the phytotoxins from Vd315ND pathotype.

3. Conclusions

In general, data obtained in this study suggest important structural differences between the two phytotoxins examined, mainly related to their pathotype, both in term of elemental composition and in functional groups distribution. Likely, such peculiarities strongly affect their solubility, which results higher for the phytotoxins from D pathotype. Further, an important role in the toxicity of these fractions is played by the phosphate ions, particularly more abundant in the phytotoxins from the D pathotype. The role of phosphate ions would be explained essentially through Tyr-residue phosphorylation, a mechanism that regulates the function of many proteins in intracellular signalling networks by altering binding affinities and enzymatic activities. The lower solubility of the phytotoxins from Vd315ND pathotype, likely related to their slightly higher molecular weight and to the presence of hydrophobic molecular moieties, makes certain tyrosyl residues less accessible to the phosphate anions. In this case, the protein unfolding happens with intermediates states, that is, there isn't a net transformation from a native state to a denatured state, and as a consequence the symptoms of the disease appear mildly. On the whole, the analytical data obtained indicate a close correlation between the structure of the two phytotoxins isolated from the two strains and the severity of the disease on the cultivars tested. There is still a lot of work to be done in this regard, especially two aspects should be explored: i) the increased aggressiveness of the D strain compared to the ND strain is linked to the phosphorylation of tyrosine residues? ii) the phosphorylated protein no longer acts as an elicitor, so it is not recognised by the receptors, which, in turn, no longer stimulate the synthesis of phytoalexins?

Surely, further analyses on the molecular characterisation of the phytotoxins produced by the two pathotypes of *V. dahliae*, Vd312D and Vd315ND, and their role as potential elicitors will provide more insights into the defence and signalling mechanisms of host–pathogen interactions involving *V. dahliae*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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