TESIS DOCTORAL

UNIVERSIDAD Ð CÓRDOBA

Identificación y caracterización de metabolitos de hongos necrotrofos

Identification and characterization of metabolites from necrotrophic fungi

Doctorando

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Departamento de Mejora Genética Vegetal – IAS/CSIC

Córdoba, 04 de diciembre de 2023

TITULO: *Identification and characterization of metabolites from necrotrophic fungi*

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Identification and characterization of necrotrophic fungi

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Los trabajos de esta tesis doctoral se han desarrollado en el Instituto de Agricultura Sostenible del CSIC y han dado lugar a las siguientes publicaciones:

- Agudo-Jurado, F.J., Reveglia, P., Rubiales, D., Evidente, A., Barilli, E., 2023. Status of Phytotoxins Isolated from Necrotrophic Fungi Causing Diseases on Grain Legumes. Int. J. Mol. Sci. 24: 5116. https://doi.org/10.3390/ijms24065116, IF2022=5.6, Ranking 66/285 (Bioch & Mol Biol), Q1

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Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, a 30 de noviembre de 2023

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TÍTULO DE LA TESIS:

Identification and characterization of metabolites from necrotrophic fungi

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- Agudo-Jurado, F.J., Reveglia, P., Rubiales, D., Evidente, A., Barilli, E., 2023. Status of Phytotoxins Isolated from Necrotrophic Fungi Causing Diseases on Grain Legumes. Int. J. Mol. Sci. 24: 5116. https://doi.org/10.3390/ijms24065116, IF2022=5.6, Ranking 66/285 (Bioch & Mol Biol), Q1

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Por todo ello, se autoriza la presentación de la tesis doctoral.

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Aún recuerdo el día que entre al Departamento. Tras haber tenido una pequeña charla por correo con Diego un domingo en la búsqueda de un TFM, nos conocimos todos un frío lunes de diciembre y jamás se me olvidará ese comienzo, que tuvo lugar un 22 de diciembre con una gran jornada de campo siendo la primera de las muchas siembras que vendrían después. Jamás podré describir mi temor (y la vergüenza) de un recién químico novato que no tenía ni idea de lo que era una espora ni como trabajar con este tipo de material. Doy de verdad las gracias de todo corazón a Diego, que me dio la oportunidad para poder desarrollarme y ver ese potencial oculto que tenía y en especial también a Eleonora que fue la que me abrió a todo este amplio mundo, y que imagino que debió tener una enorme paciencia para explicar todos y cada uno de los entresijos de esto. Ha sido como tener una segunda madre dentro del laboratorio, siempre preocupándose de mi bienestar, ya sea en el laboratorio con sus explicaciones y directrices o bien en el campo con todas sus recomendaciones (y bien saben todos que lo máximo que yo sabía de campo era lo más relacionado al fútbol); e intentar educar esa cabeza tan caótica que tengo a la hora de gestionar y analizar los todos y cada uno de los experimentos (aunque aún tengo que mejorar muchísimo en esto).

Otros dos de los pilares fundamentales de esta tesis han sido mi madre y mi hermana, aguantando todos y cada uno de los berrinches cuando me atascaba en algo y no sabía cómo continuar y los pequeños viajes que hacemos en familia para poder desconectar y tener una mejor convivencia tras todo lo que tenemos en la espalda.

Otra ayuda inestimable en este proceso ha sido Pierluigi, el cual ha sido de gran ayuda, ya no solo en el planteamiento protocolario, sino en consolidar y fortalecer todo lo aprendido en la carrera (mucho de lo cual había olvidado) y ayudarme a percibir mejor la ciencia como algo integral.

Por último, dar las gracias a todos y cada uno mis colegas que, aunque ahora nos vemos poco por estar cada uno con sus quehaceres (cosas que tienen la vida adulta), siempre quedarán esas pipas en el banquito del parque o las charlas nocturnas con nuestras paranoias en la cuesta del Reventón o en las Asomadillas; y por supuesto esas partidas de rol tan locas en la cual no sabes que puede pasar.

"*La luz busca un único camino y descarta los demás por falsos. La sombra busca todos los caminos posibles y los ve como verdaderos. Ningún punto de vista es verdadero. Tampoco erróneo*"

El peregrino a Alleria Brisaveloz

TABLE OF CONTENT

INDEX OF ABBREVIATIONS

(Alphabetical order)

ANOVA: Analysis of Variance

ATP: Adenosine Triphosphate

AUDPC: Area Under the Disease Progress Curve

BA: Biocontrol Agent

bbCID: Broadband Collision-Induced Dissociation

CD: Czapek-Dox

$CDCl_3$: Chloroform D

CE: Collision energy

CH_2Cl_2: Dichloromethane

$CHCl_3$: Chloroform

DAI: Days After Inoculation

DBI: Days Before Inoculation

DESI-MS: Desorption Electrospray Ionization Mass Spectrometry

DNA: Deoxyribonucleic Acid

DS: Disease Severity

EC_{50}: Half maximal effective concentration

ELISA: Enzyme-Linked ImmunoSorbent Assay

ESI/MS: Electrospray Ionization Mass Spectrometry

ETI: Effector-Triggered Immunity

EtOAc: Ethyl acetate

ETP: Epipolythio-Dioxopiperazine

FA: Formic Acid

FRAC: Fungicide Resistance Action Committee

h.a.i: hours after inoculation

HR: Hypersensitive Reaction

HRMS: High-resolution mass spectrometry

HSD: Honestly Significant Difference

HTS: Host Specific Toxins

IAS: Institute for Sustainable Agriculture

i-PrOH: 2-propanol

IT: Infection Type

LC-MS: Liquid Chromatography coupled with mass high-resolution Mass Spectrometry

LSD: Least Significant Difference

MAPKs: Mitogen-Activated Protein Kinase

MeOH: Methanol

Na_2SO_4: Sodium sulfate

nHTS: Non-Host Specific Toxins

NMR: Nuclear Magnetic Resonance

OSMAC: One Strain Many Compounds Strategy

PAMP: Pathogen-Associated Molecular Pattern

PCA: Principal Component Analysis

PDA: Potato Dextrose Agar

PDB: Potato Dextrose Broth

PLS-DA: Partial Least Squares-Discriminant Analysis

PRR: Pattern Recognition Receptors

PTI: (PAMP)-Triggered Immunity

QTOF: Quadrupole Time-of-Flight

SI: Severity Index

SM-FL: Sample Manager Fixed- Loop

UV: Ultraviolet

WHO: World Health Organization

OBJECTIVES

OBJETIVOS

The main objectives of this work have been the search for metabolites (phytotoxins) that are involved in the virulence of certain necrotrophic fungi such as *Ascochyta* spp. and *Colletotrichum* spp. and as a secondary objective, the use of these metabolites as possible biofungicides against biotrophic fungi in crops of agronomic importance. For this, the following specific objectives are defined:

- Integration of the OSMAC strategy to obtain different metabolites from the fungus *Ascochyta fabae*. (CHAPTER II)
- Integration of the one strain many compounds (OSMAC) strategy for the creation of a different metabolomic profile through a targeted and non-targeted analysis for several *Colletotrichum* spp. isolates. (CHAPTER III)
- Search for metabolites potentially involved in the infection process caused by the pathogen *Ascochyta fabae*, leading to the formation of necrotic spots in the plant tissue. (CHAPTER II)
- Analysis of *Colletotrichum* metabolites with obtained results from targeted analysis that could be implicated in the infection process, specifically in the production of necrotic spots in plant tissues. (CHAPTER III)
- Investigate metabolites extracted from the fungus *Ascochyta lentis* for their potential use as a biofungicide against biotrophic fungi, such as powdery mildew (*Erysiphe pisi/ Blumeria graminis* f.sp. *avenae*) and rust (*Uromyces pisi* /*Puccinia coronata* f.sp. *avenae*), in legumes (*Pisum sativum* cv. Messire) and cereals (*Avena sativa* cv. Selma). (CHAPTER IV)
- Test different metabolites of natural origin to verify their inhibitory capacity against the development of pea powdery mildew (*Erysiphe pisi*) for their use as potential biofungicides. (CHAPTER V)

Los objetivos principales de esta tesis han sido la búsqueda de metabolitos (fitotoxinas) que se encuentran involucrados en la virulencia de ciertos hongos necrotrofos como son *Ascochyta* spp. y *Colletotrichum* spp. Como objetivo secundario se ha propuesto el uso de estos metabolitos como posibles biofungicidas contra hongos biotrófos en cultivos de importancia agronómica. Para ello se definen los siguientes objetivos específicos:

- Integración de la estrategia OSMAC para la obtención de diferentes metabolitos del hongo *Ascochyta fabae*. (CHAPTER II)
- Integración de la estrategia OSMAC para la creación de un perfil metabolómico específico mediante un análisis dirigido y no dirigido para diferentes especies de *Colletotrichum*. (CHAPTER III)
- Análisis de aquellos metabolitos de *Ascochyta fabae* que podrían estar involucrados en el proceso de infección de la planta huésped, dando lugar a la formación de manchas necróticas en el tejido vegetal. (CHAPTER II)
- Análisis de aquellos metabolitos de *Colletotrichum* de los que se han obtenido resultados de análisis dirigidos que podrían estar implicados en el proceso de infección, específicamente en la producción de manchas necróticas en el tejido vegetal. (CHAPTER III)
- Búsqueda de metabolitos extraídos del hongo *Ascochyta lentis* para su uso como potencial biofungicida contra hongos biotróficos como el oídio (*Erysiphe pisi*/ *Blumeria graminis* f.sp. *avenae*) y la roya (*Uromyces pisi* /*Puccinia coronata* f.sp. *avenae*) en leguminosas (*P. sativum* cv. *Messire*) y cereales (*Avena sativa* cv. *Selma*). (CHAPTER IV)
- Testar diferentes metabolitos de origen natural para comprobar su capacidad inhibitoria del desarrollo del oídio del guisante (*Erysiphe pisi*) para su uso como potenciales biofungicidas. (CHAPTER V)

SUMMARY

RESUMEN

Phytopathogenic fungi present a great problem, since they reduce the quality of crops and even decrease their yield, causing large economic losses to farmers. For this reason, the present work is divided into two main sections: the first one is dedicated to identifying compounds involved in the disease development of necrotrophic fungi, such as *Ascochyta* and *Colletotrichum*, while the second section aims to explore the potential use of these identified compounds as natural biofungicides. To this end, the work has been divided into the following chapters, which have given rise to their respective scientific publications:

- Chapter I presents a general introduction on the importance of necrotrophic fungi on legumes. We review and discuss the isolation, chemical, and biological characterization of fungal phytotoxins produced by the most important necrotrophic fungi involved in legume diseases. We also report their possible role in plant-pathogen interaction and their structure-toxicity relationship. Moreover, we describe several multidisciplinary studies on other prominent biological activity conducted on reviewed phytotoxins. Finally, we explore the challenges in the identification of new fungal metabolites and their possible applications in future experiments.
- Chapter II explore the possibility to integrate the OSMAC strategy for the identification of phytotoxic metabolites produced by the fungus *Ascochyta fabae*. For this, the fungus was grown in different culture media (solid and liquid) to obtain the respective fungal extracts that were fractionated by chromatographic methods and identified by NMR and LC-HRMS. Finally, a bioassay on detached leaves of several legume hosts was carried out to assess theis phytotoxicity and their role in disease development.
- Chapter III reported the integration of the OSMAC strategy with targeted and untargeted metabolomic approaches to shed light on the secondary phytotoxic metabolite panels produced by pathogenic isolates of *Colletotrichum truncatum* and *Colletotrichum trifolii*. The phytotoxicity of the fungal crude extracts was also assessed on their primary hosts and related legumes, and the results correlated with the metabolite profile that arose from the different cultural conditions.
- Chapter IV addresses the issue of the use of metabolites of natural origin for their use as possible biofungicides, as an alternative to chemicals. For this, the potential antifungal activity of some anthraquinones isolated from *Ascochyta lentis*, was

assayed in this study for their effectiveness to reduce rust and powdery mildew diseases on pea and oat. Their effect on fungal development was macro- and microscopically assessed on inoculated leavesand compared to the control achieved by the chemical fungicide (Tetraconazol 12.5% and Azoxystrobin 25%). In addition, the most promising compound represented by pachybasin was also tested at different concentrations in inoculated whole plants in order to evaluate its preventive and curative potential against fungal infection.

- Chapter V explore de fungitoxic effect of 12 bioactive plant and fungal metabolites belonging to different class of natural compounds on the pea powdery mildew incited by the pathogen *Erysiphe pisi*. Metabolites were tested, together with a commercial fungicide, at different concentrations on detached pea leaves for their potential to inhibit spore germination and subsequent stages of fungal growth. In addition, the most effective metabolites were tested at different concentrations in planta under controlled conditions to evaluate the level of control achieved by treatments before, concurrently and after pathogen inoculation. Pathogen development was macroscopically scored on whole plants as percentage of disease severity and area under the disease progress curve.

Los hongos fitopatógenos presentan un gran problema, ya que reducen la calidad de los cultivos e incluso disminuyen su rendimiento, provocando grandes pérdidas económicas a los agricultores. Por este motivo, el presente trabajo se divide en dos secciones principales: la primera está dedicada a identificar compuestos implicados en el desarrollo de enfermedades de hongos necrotróficos, como *Ascochyta* y *Colletotrichum*, mientras que la segunda sección tiene como objetivo explorar el uso potencial de estos compuestos identificados como biofungicidas naturales. Para ello, el trabajo se ha dividido en los siguientes capítulos, que han dado lugar a sus respectivas publicaciones científicas:

- El Capítulo I presenta una introducción general donde se aborda la importancia de los hongos necrotrofos en los cultivos de las principales leguminosas de interés agronómico, y la relación planta-patógeno que se establece, así como las características de desarrollo de las diferentes enfermedades tratadas. Revisamos y discutimos el aislamiento y la caracterización química y biológica de las fitotoxinas fúngicas producidas por los hongos necrotróficos más importantes involucrados en las enfermedades de las leguminosas. También informamos su posible papel en la interacción planta-patógeno y su relación estructura-toxicidad. Además, describimos varios estudios multidisciplinarios sobre otras actividades biológicas destacadas realizadas con fitotoxinas previamente aisladas. Finalmente, exploramos los desafíos en la identificación de nuevos metabolitos fúngicos y sus posibles aplicaciones en experimentos futuros.
- El Capítulo II explora la posibilidad de integrar la estrategia OSMAC para la identificación de metabolitos fitotóxicos producidos por el hongo *Ascochyta fabae*. Para esto, el hongo se cultivó en diferentes medios de cultivo (tanto sólidos como líquidos) para obtener los respectivos extractos fúngicos que fueron posteriormente fraccionados por métodos cromatográficos e identificados por RMN y LC-HRMS. Finalmente, se llevó a cabo un bioensayo en hojas cortadas de habas (su huespéd primario) y de otras leguminosas relacionadas para evaluar su fitotoxicidad y su papel en el desarrollo de la enfermedad.
- El Capítulo III estudió aplicabilidad de la integración de la estrategia OSMAC con enfoques metabolómicos tanto dirigidos como no dirigidos para identificar los conjuntos de metabolitos fitotóxicos secundarios producidos por aislados de hongos patógenos de *Colletotrichum truncatum* y *Colletotrichum trifolii*. También se evaluó la fitotoxicidad de los extractos crudos de dichos hongos en sus

huéspedes primarios y leguminosas relacionadas, y los resultados se correlacionaron con el perfil de metabolitos que surgió de las diferentes condiciones culturales testadas.

- En el capítulo IV se aborda la problemática de los fungicidas aplicando diferentes metabolitos aislados de *A. lentis* como posibles biofungicidas frente a hongos biotrofos como la roya o el oidio en leguminosas y cereales. Para ello, primero se realizó un trabajo previo en hoja cortada de estas especies de planta y tras seleccionar el que mejor se adapta al nivel de un fungicida comercial, se aplicó en planta completa para ver cómo actúa a diferentes tiempos de aplicación conforme llega y actúa la enfermedad.
- El Capítulo IV aborda el tema del uso de metabolitos de origen natural para su uso como posibles biofungicidas, como alternativa a los químicos frecuentemente usados. Para ello, en este estudio se analizó la potencial actividad antifúngica de algunas antraquinonas aisladas del hongo *Ascochyta lentis* (responsable de la ascoquitosis en lentejas) para reducir el desarrollo de la roya y el oidio en guisantes y avena. Su efecto sobre el desarrollo fúngico se evaluó macro y microscópicamente en hojas inoculadas y se comparó con el control logrado con los fungicidas químicos específicos de cada enfermedad. Además, el compuesto más prometedor representado por la pachibasina también se probó a diferentes concentraciones en plantas enteras inoculadas para evaluar su potencial preventivo y curativo contra el desarrollo de la enfermedad.
- Finalmente, el Capítulo V comprueba el efecto fungitóxico de 12 metabolitos pertenecientes a diferentes clases de compuestos naturales contra el oidio del guisante causado por el patógeno *Erysiphe pisi*. Se testaron los metabolitos, junto con un fungicida comercial, en diferentes concentraciones en ensayos de hojas cortadas de guisantes para determinar su potencial para inhibir tanto los estadios tempranos de desarrollo del hongo, como la germinación de esporas, como las etapas posteriores de crecimiento. Además, los metabolitos más prometedores y efectivos se aplicaron en plantas enteras a diferentes concentraciones en condiciones controladas de crecimiento para evaluar el nivel de control logrado por los tratamientos antes, en paralelo y después de la inoculación con el patógeno. De esta manera se pudo evaluar el potencial fungicida tanto sistémico como curativo de cada compuesto seleccionado.

CHAPTER I

INTRODUCTION

Status of Phytotoxins Isolated from Necrotrophic Fungi Causing Diseases on Grain Legumes

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This chapter is published in

International Journal of Molecular Sciences, 2023, 24(6), 5116

DOI: https://doi.org/10.3390/ijms24065116

Abstract: Fungal phytotoxins can be defined as secondary metabolites toxic to host plants and are believed to be involved in the symptoms developed of a number of plant diseases by targeting host cellular machineries or interfering with host immune responses. As any crop, legumes can be affected by a number of fungal diseases, causing severe yield losses worldwide. In this review, we report and discuss the isolation, chemical, and biological characterization of fungal phytotoxins produced by the most important necrotrophic fungi involved in legume diseases. Their possible role in plant–pathogen interaction and structure–toxicity relationship studies have also been reported and discussed. Moreover, multidisciplinary studies on other prominent biological activity conducted on reviewed phytotoxins are described. Finally, we explore the challenges in the identification of new fungal metabolites and their possible applications in future experiments.

Keywords: necrotrophic fungi; fungal phytotoxins; secondary metabolites; legumes

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1.Introduction

Legumes are members of the third largest plant family, Fabaceae, with over 20,000 species, many of which were domesticated at the very onset of agriculture. Among them,

grain legumes are important food and feed crops, having played a key role as the basis of the food of all major civilizations combined with cereals [1], being staple crops in many regions [2], and also have a major role in animal feeding [3]. Legumes are also gaining in popularity due to their various health [4,5] and environmental [6] benefits, as their ability to fix atmospheric nitrogen through a symbiotic relationship with *Rhizobium*, which can be used by the crop itself or left in the soil for subsequent crops, makes legumes essential for sustainable agriculture.

The demands for protein crops are markedly increasing [7]; this should be paired with technical solutions to support cultivation. As with any crop, legumes can be affected by biotic or abiotic stresses, reducing their quality and yield, which translates into economic losses for the farmer. Biotic stresses can be caused by a range of biological organisms such as bacteria, viruses, fungi, insects, nematodes, or even parasitic plants [8]. Among these, fungal diseases are likely to be the most economically relevant threats. Indeed, they have been a devastating menace throughout history, with vast epidemics and disastrous yield losses still occurring [9].

Regardless of their lifestyle, all fungi can be recognized by plant immune systems and elicit several host defense responses. The plant's innate immune system can display two layers of defenses: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), and effector-triggered immunity (ETI). PTI is the first line of defense reactions. It is initiated in plants when PAMPs are recognized by membrane-localized pattern recognition receptors (PRRs) [10]. When PAMPs are recognized through PRRs, they trigger a fast, relatively weak but broad-spectrum immune response to pathogen infection. This response includes the accumulation of reactive oxygen intermediates, the accumulation of antimicrobial compounds, changes in hormone biosynthesis such as salicylic acid, jasmonates, or ethylene, and plant cell wall reinforcement [11–13]. PTI is effective against non-adapted fungi [14].

ETI is the second line of innate immunity. Effectors that trigger ETI are usually highly specific and perceived by plant resistance proteins (R proteins). In this case, the recognition leads to a rapid, robust, and localized cell death response, often referred to as a hypersensitive reaction (HR) [12].

Conversely, the objective of a fungus is to obtain nutrients from the host plant by penetrating and neutralizing plant defenses [15]. According to the colonization/infection strategy, fungal pathogens can be generally classified as biotrophic or necrotrophic [16]. Biotrophic fungi feed on living cells, so the penetration structures allow the invading

fungus to suppress the plant's immune response system and for the reprogramming of its metabolism [17]. Alternatively, necrotrophic fungi emit a number of compounds to degrade the host tissues and obtain nutrients [18]. In order to establish a compatible interaction leading to its proliferation, the fungus must avoid eliciting PTI or either cope with or suppress it. For this purpose, necrotrophic fungi can inactivate the plant defenses by secreting toxic compounds. These compounds are mainly enzymes, catalyzing the degradation of structural components and other essential substances, and phytotoxins, causing damage and alterations in the cells.

Phytotoxins can be either host specific toxins (HSTs) that affect only a particular plant species or more often genotypes of that species [19], or non-host specific toxins (nHSTs) that affect a broad range of plant species. Induction of host alterations such as DNA damage, abnormal mitochondrial oxidation, cytotoxicity, etc. with the goal of leading to host cell death are some of the common functions of HSTs [20]. These HST toxins are diverse, chemically ranging from low-molecular-weight compounds to cyclic peptides [21–24]. High molecular weight compounds such as some polysaccharides have also been reported as phytotoxins, although their true role is an open question [25–27]. Genes encoding polypeptides for the biosynthesis of these HSTs have been shown to reside on a conditionally dispensable chromosome that controls host-specific pathogenicity [28]. The mechanism of host-selective pathogenesis, through the HSTs, is well-understood and documented [29,30]. In some cases, host sensitivity was mediated by gene-for-gene interactions, and the toxin sensitivity was mandatory for disease development.

Phytotoxins belong to different chemical families and are commonly classified as polyketides, macrolides, non-protein amino acids, naphthalenones, anthraquinones, furanones, pyranones, non-ribosomal peptides, alkaloids, terpenes, or metabolites of mixed biosynthetic origin [21–24,30–32]. This structural diversity is related to the complexity of the host–pathogen relationship. Assigning the correct structure to a fungal phytotoxin is a stepping stone to shed light on its biosynthesis pathways and their regulation, the mode of action, and how this relates to fungal virulence [33,34].

Recent advances in organic and analytical chemistry techniques such as nuclear magnetic resonance (NMR) and liquid chromatography coupled with mass high-resolution mass spectrometry (LC-MS), together with the development of software and bioinformatic tools, have allowed for the isolation and structural elucidation of low abundant and novel phytotoxins [21–24,35–38].

With a particular focus on necrotrophic fungi, important grain legumes such as chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), field pea (*Pisum sativum*), lentil (*Lens culinaris*), lupin (*Lupinus* spp.), common bean (*Phaseolus vulgaris*), or soybean (*Glycine max*) are impacted by pathogens belonging to the *Ascochyta* spp., *Botrytis* spp., *Colletotrichum* spp., *Phoma* spp. or *Macrophomina* species. They cause significant economic losses [39] through direct seed loss and reduced marketability as well as the costs derived from disease man- agement including chemical and cultural methods. Disease resistance is currently a primary objective of most plant breeding programs. Durable and multi-disease resistance is considered as a prerequisite to broad environmental adaptation aiming at stabilizing agricultural systems.

Herein, we review the isolation, chemical, and biological characterization of fungal phytotoxins produced by the most important necrotrophic fungi involved in legume dis- eases. In addition, research on the mode of action of the reviewed phytotoxins will also be discussed. Finally, multidisciplinary studies on other prominent biological activity conducted on the reviewed phytotoxins are also reported.

2. Phytotoxic Metabolites Isolated from Necrotrophic Pathogenic Fungi on Grain Legumes Crops

The most important pathogenic necrotrophic fungi of grain legumes are *Ascochyta*, *Colletotrichum* or *Botrytis*, together with their anamorphs [40], followed by *Cercospora*, *Macrophomina*, *Pleiochaeta*, or *Sclerotinia*, as listed in Table 1.

2.1 Phytotoxic Compounds Produced by Ascochyta *spp.*

Ascochyta species cause diseases globally called Ascochyta blights, whose symptoms typically develop in the aerial parts of the plants under high humidity and average temperature conditions, producing necrotic lesions on the leaves and stems [66]. Leaves with many lesions wither before the lesions become large, especially on the lower portion of the plants. On the stems, these fungi cause deep necrotic lesions that can lead to the breaking of stems and the death of plant parts above the affected zone. Infected grains and pods can spread disease through seeds, causing their use in the following crops to be harmful since they can drown growing plants. Ascochyta blights are incited by

Table 1. Metabolites reported on necrotic fungi infecting grain legumes.

Fungi	Host	Disease	Metabolite	Literature
Ascochyta lentis	Lentil (*Lens culinaris*)	Ascochyta blight	Lentiquinone A (**1**)	[21]
			Lentiquinone B (**2**)	[21]
			Lentiquinone C (**3**)	[21]
			Lentisone (**4**)	[41]
			ω–hydroxypachybasin (**5**)	[21]
			1,7-dihydroxy-3-methylanthracene-9,10-dione (**6**)	[21]
			Phomarin (**7**)	[21]
			Pachybasin (**8**)	[41]
			Tyrosol (**9**)	[41]
			Pseurotin A (**10**)	[41]
Ascochyta pinodes	Pea (*Pisum sativum*)	Ascochyta blight	Pinolidoxin **(11)**	[42]
			7-*epi*-Pinolidoxin (**12**)	[43,44]
			5,6-Dihydropinolidoxin (**13**)	[43,44]
			5,6-Epoxypinolidoxin (**14**)	[43-44]
			Herbarumin II (**15**)	[44]
			2-*epi*-Herbarumin II (**16**)	[44]
			Pinolide (**17**)	[44]
Ascochyta pisi	Pea (*Pisum sativum*)	Ascochyta blight	Ascosalitoxin (**18**)	[45]
			Ascochitine (**19**)	[42]
Ascochyta lentis var. *lathyri*	Grass pea (*Lathyrus sativus*)	Ascochyta blight	Lathyroxin A (**20**)	[22]
			Lathyroxin B (**21**)	[22]
			p-Hydroxybenzaldehyde (**22**)	[22]
			p-Methoxyphenol (**23**)	[22]
			Tyrosol (**9**)	[22]
Ascochyta fabae	Faba bean (*Vicia faba*)	Ascochyta blight	Ascochitine (**19**)	[46]
Ascochyta rabiei	Chickpea (*Cicer arietinum*)	Ascochyta blight	Solanapyrone A (**24**)	[47]
			Solanapyrone B (**25**)	[48]
			Solanapyrone C (**26**)	[47]
			Cytochalasin D (**27**)	[49]
Botrytis fabae	Faba bean (*Vicia faba*)	Chocolate spot	Botrytone (**28**)	[50]
			Regiolone (**29**)	[50]
			cis-2,4,8-Trihydroxy-1-tetralone (**30**)	[50]
			trans-2,4,8-Trihydroxy-1-tetralone (**31**)	[50]
			(4*S*)-(+)-Isosclerone (**32**)	[50]
			Scytalone (**33**)	[50]
			3-Hydroxyjuglone (**34**)	[50]
Botrytis cinerea	Faba bean (*Vicia faba*)	Grey mould	Botrydial (**35**)	[51]
			Botryendial (**36**)	[52]
			Botrydienal (**37**)	[52]
			8,9-*epi*-botrydial (**38**)	[52]
			1-*epi*-botrydial (**39**)	[52]
			Dihydrobotrydial (**40**)	[52]
			Norbotrydialone acetate (**41**)	[53]
			10-Oxodihydrobotry-1(9),4(5)-diendial (**42**)	[53]

			10-Oxodehydrodihydro botrydial (**43**)	[53]
			4β-Acetoxytetrahydro botryslactone (**44**)	[53]
			Botryenalol (**45**)	[54]
			β-O-methyldihydrobotri dialone (**46**)	[54]
			Botrylactone (**47**)	[54]
			Botcinic acid (**48**)	[54]
			3-Acetylbotcinic acid (**49**)	[54]
			Botcinin A (**50**)	[54]
Macrophomina phaseolina	Soybean (*Glycine max*)	Charcoal rot	Phaseolinone (**51**)	[55]
			(-)-Botryodiplodin (**52**)	[56]
			Phaseocyclopentenones A (**53**)	[57]
			Phaseocyclopentenones B (**54**)	[57]
			Guignardone A **(55**)	[57]
Colletotrichum truncatum	Soybean (*Glycine max*)	Anthracnose	Colletruncoic acid methyl ester (**56**)	[58,59]
			Meso-2,3-butane-2,3-diol (**57**)	[58,59]
			(2*R*,3*R*)-Butane-2,3-diol (**58**)	[58,59]
			(2*S*,3*S*)-Butane-2,3-diol (**59**)	[58,59]
			Truncatenolide (**60**)	[60]
			Truncatenone (**61**)	[60]
			N-Acetyltyramine (**62**)	[60]
			Tyrosol (**9**)	[60]
Colletotrichum lupini	Lupine (*Lupinus albus*)	Anthracnose	Lupindolinone (**63**)	[61]
			Lupinlactone (**64**)	[61]
			(3*R*)-Mevalonolactone (**65**)	[61]
			Tyrosol (**9**)	[61]
Colletotrichum lindemuthianum	Common bean (*Phaseolus vulgaris*)	Anthracnose	Colletotrichin (**66**)	[62]
			Colletopyrone (**67**)	[62]
Cercospora kikuchii	Soybean (*Glycine max*)	Pod and stem blight	Cercosporin (**68**)	[63]
Pleiochaeta setosa	Lupine (*Lupinus albus*)	Brown spot	Setosol (**69**)	[64]
			Triacetylsetosol (**70**)	[64]
Sclerotinia sclerotiorum	Common bean (*Phaseolus vulgaris*)	White mould	Oxalic acid (**71**)	[65]

different pathogens in the various legumes such as *Ascochyta lentis* in lentil; *A. pisi* and *A. pinodes* in pea; *A. lentis* var. *lathyri* in grass pea; *A. fabae* in faba bean; and *A. rabiei* in chickpea [67].

Ascochyta blight remains an extremely difficult pathogen to control, primarily due to the limited levels of host resistance available, and secondarily, because fungicides are often neconomic [68], forcing the integration of the use of genetic resistance with cultural practices. Therefore, the main disease control strategy has been to avoid sowing close to

infested field stubbles and/or to delay the sowing of field crops for as long as possible.

This minimizes inoculum carry-over and its survival on crop residues and in soil, avoiding the initial infection of the crop from aerial inoculum arising from infested residues [69–71]. Nevertheless, late sowing is not an option in some countries due to the short crop season, and this practice incurs unsustainable yield penalties in many instances.

Different metabolites with pathogenesis-determining cytotoxic capacity have been reported in several Ascochyta species, as listed in Table 1. For instance, 10 metabolites have been isolated and identified from A. lentis, being lentiquinones A–C, lentisone, ω- hydroxypachybasin, 1,7-dihydroxy-3-methylanthracene-9,10-dione, phomarin, pachybasin, tyrosol, and pseurotin A (**1**–**10**, Figure 1). However, between them, only compounds 1–4, 8, and 9 showed phytotoxic activity on the lentil plants, being also capable of reducing the root growth and seed germination; in contrast, the reported activity of metabolites 5–7 was almost null in the bioassay condition [41,72], and the role they play in plant–pathogen interaction is not well-defined yet [73,74].

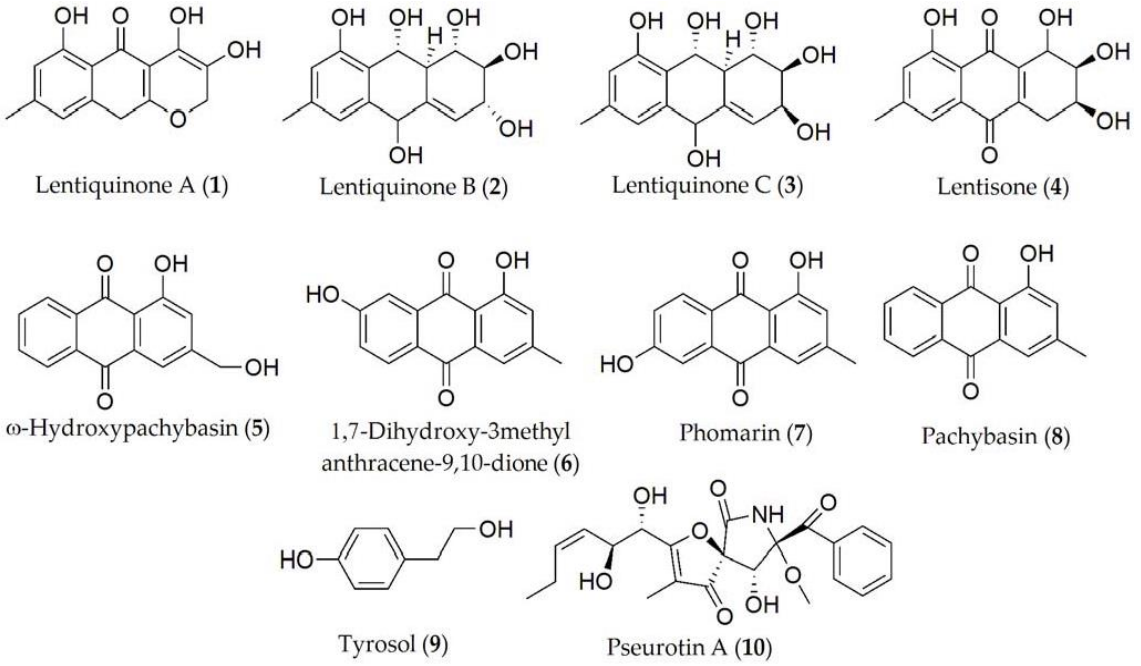


Figure 1. Phytotoxins isolated from *Ascochyta lentis*.

The so-called Ascochyta blight of peas is in fact a disease complex that can be caused by several fungi including *A. pisi*, *A. pinodes*, and *Phoma medicaginis*. Compounds described so far in *A. pinodes* with noteworthy phytotoxic activity have been pinoli- doxin, 7-*epi*-pinolidoxin, 5,6-dihydropinolidoxin, 5,6-epoxypinolidoxin, herbarumin II, 2-*epi*-herbarumin II, and pinolide (**11–17**, Figure 2) [42–44]. Between

them, pinolidoxin (**11**) showed the highest phytotoxic activity measured in pea plants such as lesion size (mm2) on both the pods and in leaves as well as in other grain legumes such as faba bean. In contrast, the other metabolites found only produced reduced symptoms [42,44]. When the compounds were tested on other legumes, it was observed that herbarumin II, 2-*epi*-herbarumin II, and pinolide (**15** and **17**) did not display significant phytotoxic activity. The importance of the stereochemistry of the hydroxy group at C-7 of these compounds on phytotoxicity was deduced by the authors [44].

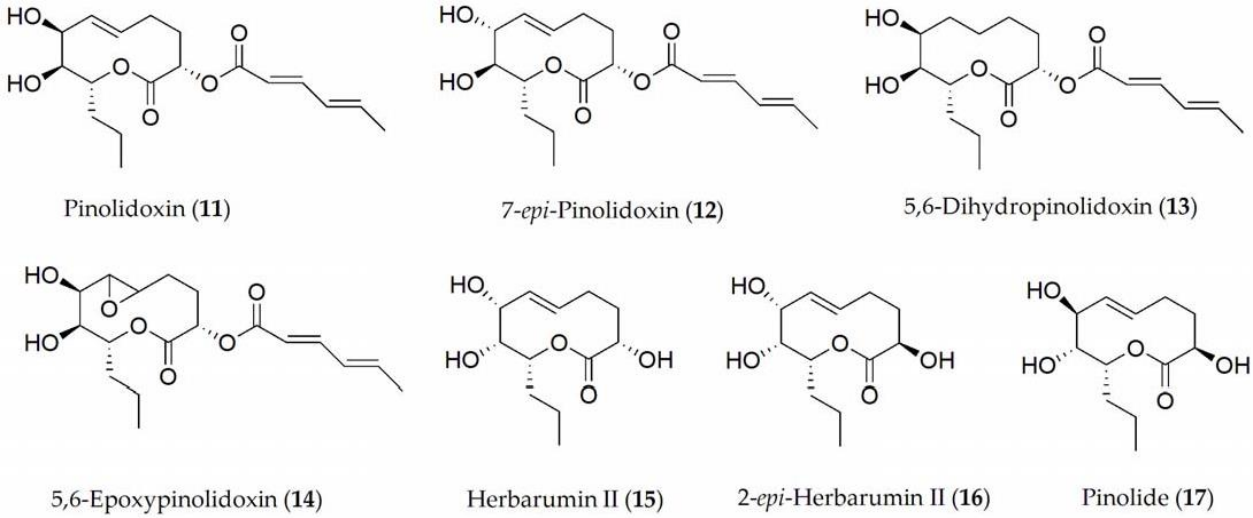


Figure 2. Phytotoxins isolated from *Ascochyta pinodes*.

Ascosalitoxin (**18**, Figure 3), which is a derivative of salycilic aldehyde, was isolated as the main phytotoxin from *A. pisi* [75]. Ascosalitoxin (**18**) displayed phytotoxic activity on pea and faba bean leaves and pods, and on tomato seedlings [76]. Ascochitine, an *o*-quinone methide, is an abundantly produced phytotoxin that was first discovered in culture extracts of *A. pisi* [77], and later in *A. fabae* [78] (**19**, Figure 3), where it displayed antibiotic activity.

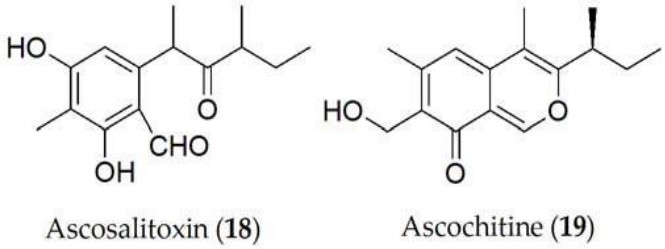
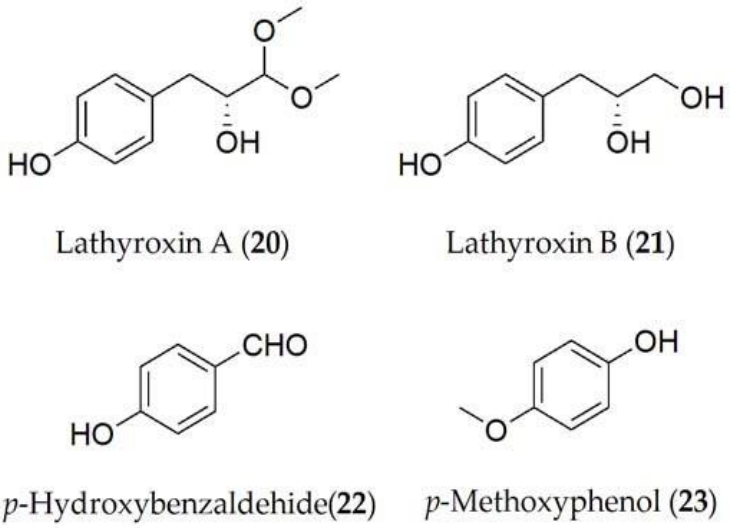


Figure 3. Phytotoxins isolated from *Ascochyta pisi*.

More recently, ascochitine (**19**, Figure 3) was found in the culture extracts of many wild vetch-infecting *Ascochyta* and *Ascochyta*-like species including *A. viciae-villosae* [78]. The widespread distribution of ascochitine (**19**) production indicates its ancient

origin in these related taxa. Ascochitine (**19**) production is not restricted to the legume-associated *Ascochyta* species, but also to some *Phoma* species. In phytotoxicity studies performed on faba bean plants, ascochitine (**19**) was shown to produce electrolyte leakage when tested on leaf discs as well as necrosis and wilting in whole plants assays [45].



Lathyroxin A (**20**) Lathyroxin B (**21**)

p-Hydroxybenzaldehide(**22**) *p*-Methoxyphenol (**23**)

Figure 4. Phytotoxins isolated from *Ascochyta pisi* var. *lathyri*.

Concerning *A. lentis* var. *lathyri*, the compounds described have been lathyroxins A and B, *p*-hydroxybenzaldehyde, *p*-methoxyphenol (**20–23**, Figure 4), and tyrosol (**9**, Figure 1) [22]. The latter has also been isolated in *A. pinodes* [41]. Lathyroxin B (**21**) showed activity in a panel of legumes tested including lupine, lentil, and beans. In contrast, lathyroxin A (**20**) only showed activity on lupin and *Sonchus oleraceus*, while *p*-hydroxybenzaldehyde (**22**) was toxic only on lupin and lentil [22].

Finally, compounds described for *Ascochyta rabiei* were solanapyrone A [47], solanapyrone B [48], solanapyrone C [47], and cytochalasin D [49,79] (**24–27**, Figure 5). The solanapy- rones A–C (**24–26**) are structural isomers that act as HSTs [44]. When tested on whole plant assays in chickpea plants, solanapyrones A, B, and C were shown to be active individually as well as in combination, being able to reduce root development as well as the seed germination [80] of the host plant.

Structure–Toxícate Relationship Studies of Phytotoxins Produced by *Ascochyta* spp.

Although a significant number of compounds produced by *Ascochyta* spp. have been identified, only a few toxicity relationship studies have been carried out with them.

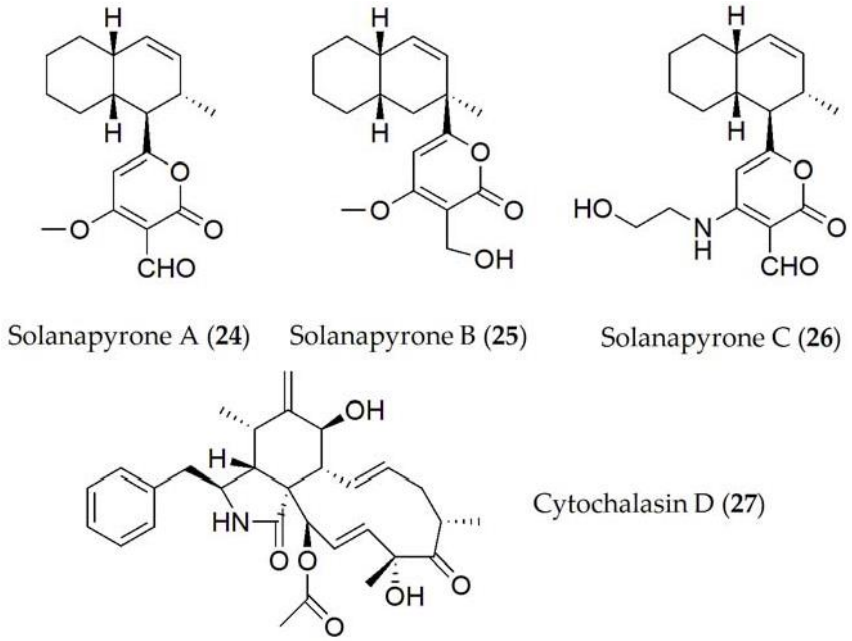


Figure 5. Phytotoxins isolated from *Ascochyta rabiei*.

For example, the nonenolide pinolidoxin (**11**) is the main phytotoxin produced by both *Ascochyta* spp., which is closely related to putaminoxin, having a similar nonenolide ring system and some substituent groups. *Phoma putaminum* is a fungus proposed for the biocontrol of the dangerous weed *Erigeron annus*. The two nonenolides, pinolodoxin and putaminoxin, along with some of their natural analogues and some hemisynthetic derivatives, were assayed for their phytotoxic, antifungal, and zootoxic activities. The results obtained by testing all of the compounds on the weeds and crops showed that the phytotoxic activity was related to the integrity of the nonenolide ring, to the presence of two hydroxyl groups, and to an unmodified propyl side chain. Likewise, pinolidoxin (**11**) was detected in *A. pinodes* in front of the hyphae as it developed, which suggests that this compound has a fundamental role in modulating the defense response in plants [81]. Furthermore, among a set of phytotoxins with different carbon skeletons and produced by different pathogenic fungi, only the nonenolides pinolidoxin and putaminoxin appeared to be inhibitors of the first steps in the phenylpropanoid pathway. This is the route in charge of generating compounds such as phytoalexins or lignin in the defense against parasitic attacks [82].

Structure–activity relationship studies have been also performed with compounds belonging to the Solanapyrone group (**24–26**). In fact, solanapyrone A (**24**) was shown to be active against *Bacillus subtilis* and *Micrococcus tetragenus*, in addition to certain saprobe fungi [83], while solanapyrone C (**26**) only acted against *B. megaterium*, and a

unicellular alga [83,84]. Solanapyrone A (**24**) binds specifically to DNA polymerases [85], acting in cell control during mitosis and meiosis, postulating that this compound could inhibit DNA repair processes, unbalancing the cell cycle, and finally causing apoptosis; or by affecting defense signaling induced by DNA damage and the subsequent repair process [86,87]. Solanapyrones J and K had activity against Gram-positive bacteria, but not against Gram-negative bacteria. However, when solanapyrones L and M, which differ in the functionalization of the pyrone ring, were tested, the latter did not show any activity [88].

2.2 Phytotoxic Compounds Produced by Botrytis *spp.*

Chocolate spot can be elicited by both pathogens, *Botrytis fabae* and *B. cinerea*, but *B. fabae* is more harmful to faba bean [89]. Chocolate spot is an important disease, having a worldwide distribution and causing a series of dark brown spots on the aerial parts of plants [90]. When the humidity reaches high levels and there is an average temperature of around 22 °C, the fungus begins an aggressive phase where it spreads very quickly, significantly increasing the number of necrotic spots and withering the plant completely in a period of two days in some cases [40]. When the plant is affected during the flowering period, the flowers fall, decreasing the final yield production and favoring the spread of the fungus to the lower parts of the host or to other neighboring plants. In addition, if the pathogen affects the pod during its formation, the seed quality decreases, and often, their commercialization is unviable [91].

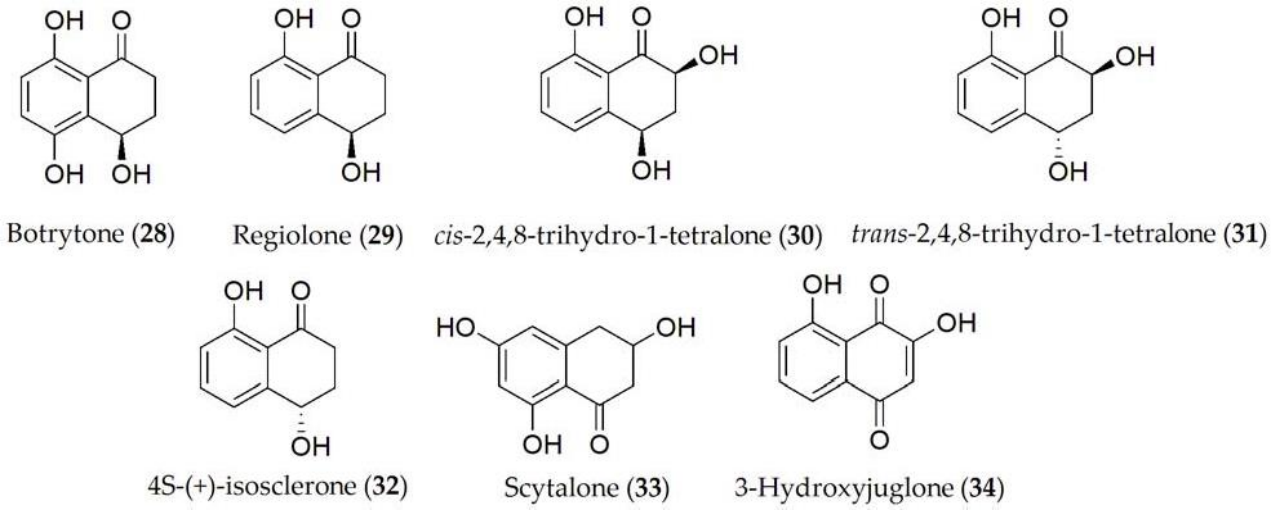


Figure 6. Phytotoxins isolated from *Botrytis fabae*.

The pathogen should be limited by applying both agronomic controls including

removing the crop infected remains and their destruction as well as by chemical control techniques through the utilization of fungicides with different chemical characteristics such as benzimidazoles (benomyl, carbendazim) or dithiocarbamates (mancozeb) among others [70,92]. However, these methods are usually very costly, which is why other strategies have been formulated such as the use of resistant varieties obtained through the development of plant breeding programs. Although in the last 20 years some resistant materials have been described [93], more effort is needed in order to incorporate resistance into commercial varieties as well as testing the stability of sources of resistance through time and space [88]. Reported compounds produced by *B. fabae* are botrytone,

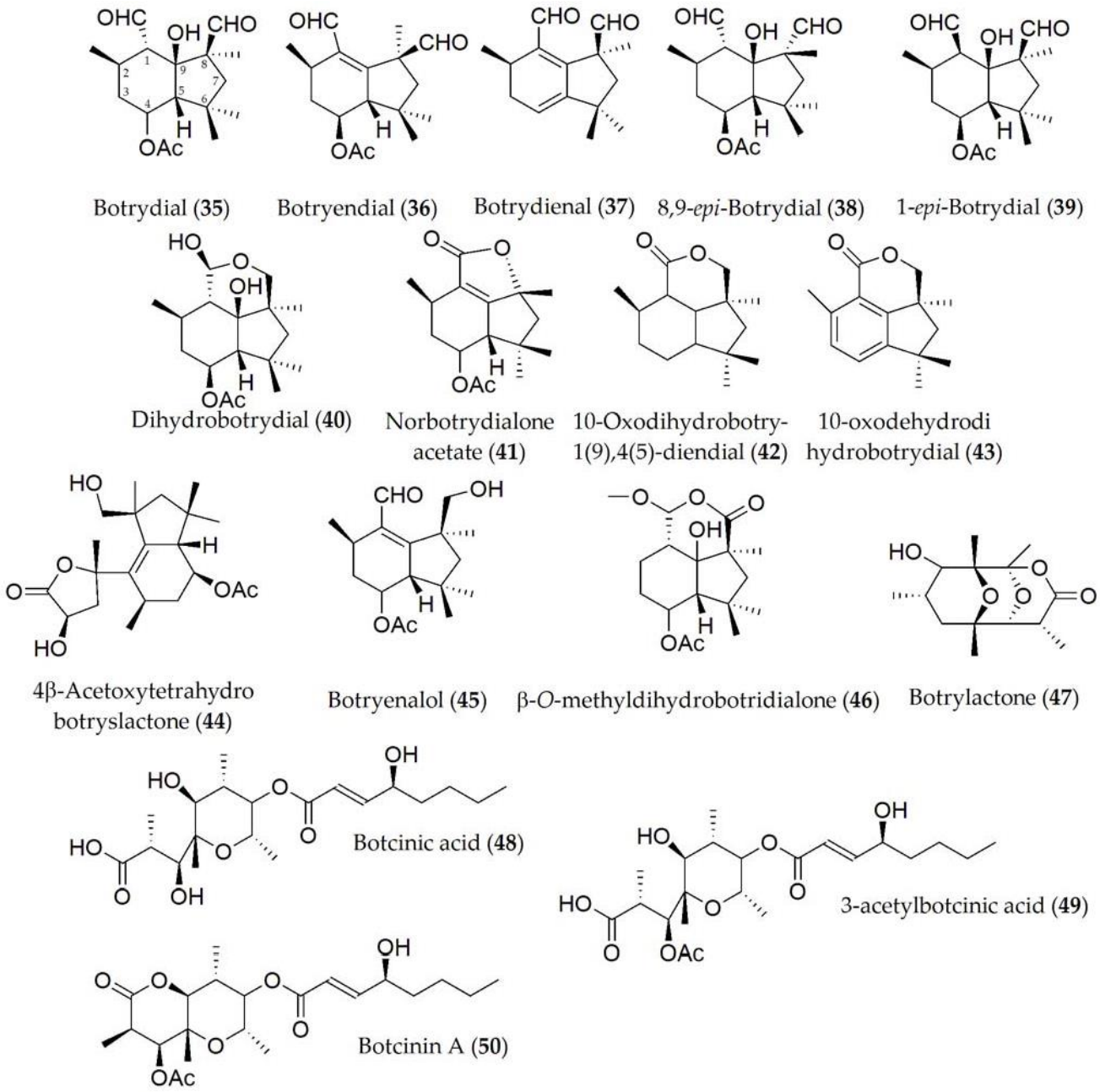


Figure 7. Phytotoxins isolated *from Botrytis cinerea*.

regiolone, *cis*-2,4,8- and *trans*-2,4,8-trihydro-1-tetralone, (4*S*)-(+)-isosclerone, scytalone, and 3-hydroxyjuglone (**28–34**, Figure 6). Out of these metabolites, botrytone (**28**) has shown some phytotoxicity on the host plant, with regiolone, *cis*-2,4,8, and *trans*-2,4,8 trihydro-1-tetralone (**29–31**) being the most toxic [50].

Because of the diverse host range that *B. cinerea* presents, most of the phytotoxins that have been identified from this fungus have been extracted from other hosts including legumes such as the common bean (*P. vulgaris*), or other different species such as sweet pep- per (*Capsicum annuum*), although later tests have been extrapolated to legumes.

Compounds identified in *B. cinerea* (Table 1) include botrydial, botryendial, botrydienal, 8,9-*epi*-botrydial, 1-*epi*-botrydial, dihydrobotrydial, norbotrydialone acetate; 10-oxo-dihydrobotry-1(9),4(5)-diendial, 10-oxodehydrodihydrobotrydial, 4β-acetoxytetrahydro- botryslactone, botryenalol, β-O-methyldihydrobotridialone, botrylactone, botcinic acid, 3-acetylbotcinic acid, and botcinin A (**35–50**, Figure 7). Out of these compounds, botrytone, regiolone, *cis*- and *trans*-2,4,8-trihydroxy-1-tetralone botrydial, botryendial, botrydienal, 8,9-*epi*-botrydial, and 4β-acetoxytetrahydrobotryslactone (**28–31**; **35–38**, and **44**) showed phytotoxic activity when tested on the host both in the cut leaf and in the whole plant. Bioassays using *B. cinerea* mutants deficient in the production of botrydial and botcinin A (**35** and **50**) showed no reduction in pathogenicity, being capable of damaging cells of the host plant tissue. Moreover, a marked lower virulence of the fungus was demonstrated [54]. Phytotoxic activity of some metabolites such as botrydial (**35**) has been shown to be influenced by external factors such as light intensity [52].

Structure–Toxicity Relationship Studies of Phytotoxins Produced by *Botrytis* spp.

Botrydial (**35**) was the only phytotoxin produced by *Botrytis cinerea* for which structure–activity relationship studies were conducted. This compound induces a hypersensitive response in the host, which is regulated via the salicylic acid and jasmonic acid pathways [94]. It has been shown that the activity of this compound and its epimers is closely related to the C-1 and C-8 carbons, depending on the oxidation states of the aldehyde substituents as well as the C-9 carbon, observing a lower activity, for example, in botryendial and botrydienal (**36** and **37**) with respect to botrydial (**35**). Likewise, the

configuration (*S*) at the C-1 carbon has been observed to be critical in the substrate–receptor role [95].

2.3. Phytotoxic Compounds Produced by Macrophomina *spp.*

Several species of *Macrophomina* cause a disease called "charcoal rot" on different hosts [96,97]. Here, we will only deal with *M. phaseolina* causing charcoal rot on soybean, although it can also affect other legume crops such as cowpea, common bean [98], and other plant species as strawberry [99] or sunflower [100]. The pathogen is widely distributed in different parts of the world, indicating its omnipresence in varied soil types. Disease symptoms are more severe under dry and warm (28–35ºC) growing conditions, with soil being the principal source of inoculum. Infection is started by the spores that survive in the soil or in the remains of infected plants and develops in response to root exudates of the host plants. Infection causes an abnormal development of the plant and chlorosis in the leaves, ending in the total wilting of the plants [96].

The management of this disease is quite complex, since an integrated approach is necessary to reduce the number of viable spores in the soil or in the material that is used, sowing clean seeds or adopting crop rotations with resistant material, because fungicides are not fully effective against the pathogen [101,102]. A recent meta-analysis on biological control methods highlighted that *Trichoderma gamsii*, *Gliocladium virens*, *Trichoderma viride*, and *Pseudomonas fluorescence* have a higher control efficiency [103]. Nevertheless, the search for genetic resistance in the crop is still scarce [104].

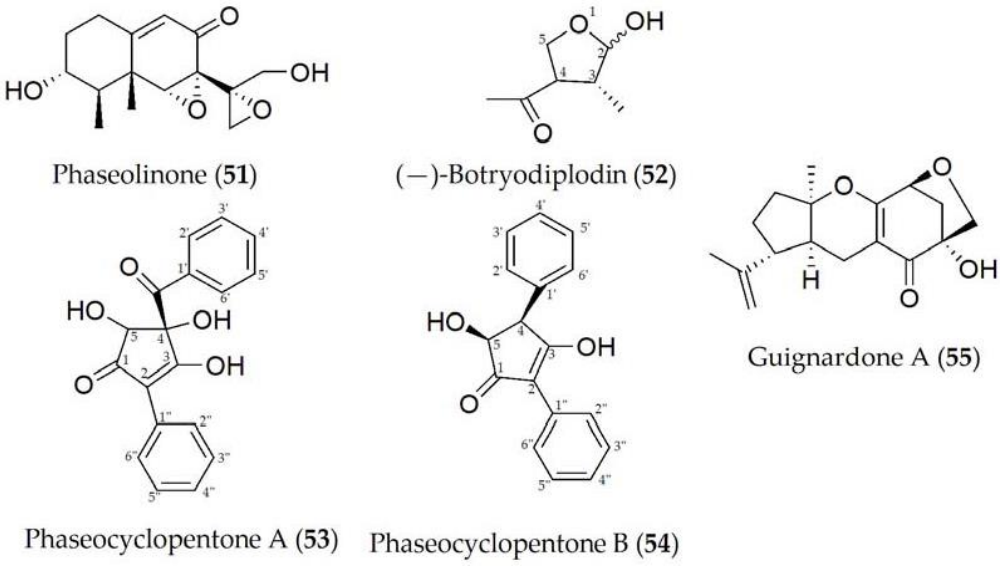


Figure 8. Phytotoxins isolated from *Macrophomina phaseolina*.

Phytotoxic metabolites produced by *M. phaseolina* have been described including phaseolinone, (-)-botryodiplodin, phaseocyclopentenones A and B, and guignardone A [55–57] (**51–55**, Figure 8). Phaseolinone (**51**) and (-)-botryodiplodin (**52**) are believed to play a role in the initial stages of infection, causing the wilting of seedlings and the formation of necrotic lesions on the leaves and roots [55]. This increases the virulence of *M. phaseolina* and may help to explain the highly efficient mechanism to infect different hosts and tissues. Likewise, although it has not yet been specified in a concrete way, it is speculated that the variation in the production of (**51**) and (**52**) between different isolates may be due to the geographical variation in the isolates due to different environmental conditions or the production and interaction with other phytotoxins [56].

Phaseocyclopentenones A and B (**53** and **54**) were recently described together with guignardone A (**56**), extracted from a strain of *M. phaseolina* isolated in infected soybean tissues from Argentina. Compounds **53–55** showed phytotoxic activity assayed on tomato plants, used as a non-host control by the leaf puncture assay, while only **53** and **54** were toxic when tested on cuttings of the same plant. No antifungal activity was detected for the three metabolites against some fungal pathogens such as *Cercospora nicotianae* and *Colletotrichum truncatum*, which are two severe pathogens both isolated from infected soybean plants in Argentina [57].

Structure–Toxicity Relationship Studies of Phytotoxins Produced by *Macrophomina* spp.

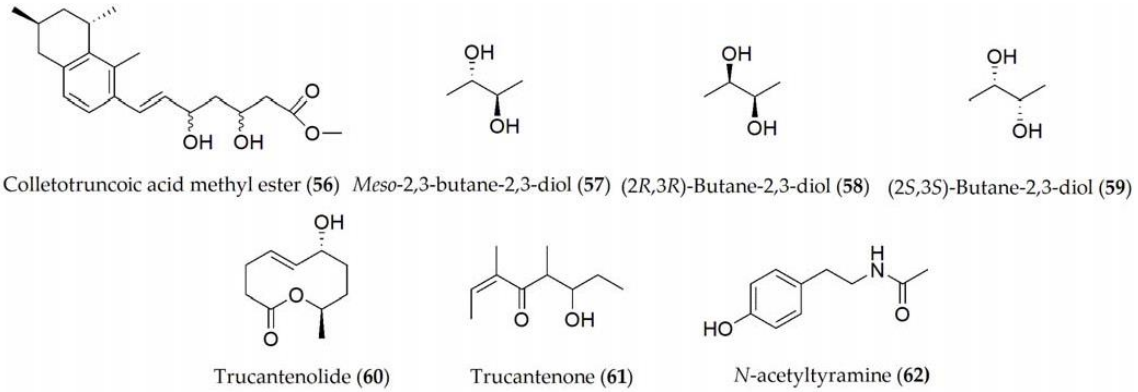
Phaseolinone (**51**) is considered a mutagenic compound due to its primary and sec- ondary alcoholic groups. When these hydroxyl groups are modified, a reduction in mu- tagenic activity is observed when one of them is replaced (with a ketone group), and the complete loss of this activity when a complete substitution of the hydroxyl groups is performed. In the same way, a reduction in the toxicity of the molecule was also observed as more hydroxyl groups are substituted, suggesting that side-chain epoxide and alcoholic groups are essential for its activity [105]. Additionally, (-)-botryodiplodin (**52**), which is a natural analogue of ribose, interferes with different cellular mechanisms such as trans- porters or enzymes, although it is not entirely clear which. An example of this interference may be the absence of a hydroxyl group at C-5, causing it not to be an optimal substrate

for the ribose 5-kinase enzyme, or it may exert another function when present in the cell cytoplasm in its phosphorylated form [106].

Phytotoxicity assays have shown that the functionalization of C-4 and C-5 in phaseo- cyclopentenones A (**53**) and B (**54**) are important for phytotoxicity. In addition, these compounds showed a different mechanism of action depending on the bioassay condition [57]. The complete phytotoxicity assays of guignardone A (**55**) in legumes have not been carried out yet.

2.4. Phytotoxic Compounds Produced by Colletotrichum *spp.*

Species of the anamorphic genus *Colletotrichum* (teleomorph *Glomerella*) are implicated in plant diseases, generally referred to as anthracnoses, which are found throughout the world. The various *Colletotrichum* species include some of the most destructive post-harvest pathogens that can affect a multitude of hosts including cereals, legumes, fruits, and vegetables [107]. *Colletotrichum* spp. can survive for several years on plant debris that remains in the field after harvest [108]. The pathogen requires more than 16 h of leaf wetness in combination with temperatures between 20 and 30 °C to infect the host plant [107]. Initial symptoms on leaves are small yellow spots that enlarge into brown-colored lesions with a distinct dark margin. This might result in premature leaf drop. In the stem, the first lesions appear in its base from where they progress upwards [109]. Large stem lesions can surround the whole stems and penetrate the vascular tissue, causing wilting with subsequent plant death. In susceptible genotypes, more than 20% of the harvested seeds could show necrotic lesions, affecting their quality and market sale. During the growing season, the inoculum is primarily spread by rain splash and secondarily by windblown infected debris or during the harvesting process [109].



Colletotruncoic acid methyl ester (**56**) *Meso*-2,3-butane-2,3-diol (**57**) (2*R*,3*R*)-Butane-2,3-diol (**58**) (2*S*,3*S*)-Butane-2,3-diol (**59**)

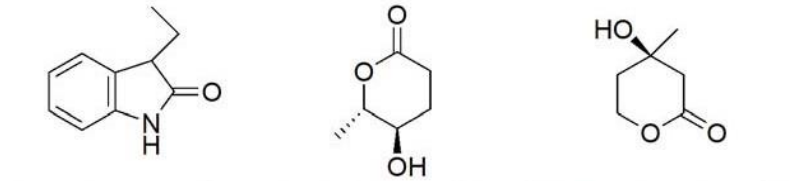
Trucantenolide (**60**) Trucantenone (**61**) *N*-acetyltyramine (**62**)

Figure 9. Phytotoxins isolated from *Colletotrichum truncatum*.

Chemical control through the application of fungicides is normally used, but this can generate some resistance in the fungus and can lose effectiveness over time. Biological control is also an alternative method, which uses various antagonistic microorganisms such as *Bacillus subtilis* [110]. In legumes, genetic sources of resistance have been developed in common bean, soybean, and lentil [111–113], where some varieties with partial resistance to the disease have been identified.

C. truncatum is the main causal agent of soybean anthracnose, which is characterized by pre- and postemergence damage on cotyledons, pods, petioles, and stems. The metabolites produced by *C. truncatum* are colletruncoic acid methyl ester and *meso*-2,3-butane-2,3-diol (**56–57**, Figure 9), together with the isomers of **57**, which are (2*R*,3*R*)-butane-2,3-diol and (2*S*,3*S*)-butane-2,3-diol (**58–59**, Figure 9), although no phytotoxic activity has yet been determined [58,59]. Recently, a bioactive disubstituted nonenolide, named truncatenolide (**60**), and a new trisubstituted oct-2-en-4-one, named truncatenone (**61**), and the well-known tyrosol and *N*-acetyltyramine (**9** and **62**) have also been described (Figure 9).

Truncatenolide (**60**) showed the strongest phytotoxic activity when tested on soybean seeds while tyrosol and *N*-acetyltyramine (**9** and **62**) exhibited phytotoxicity to a lesser extent. Furthermore, truncatenone (**61**) weakly stimulated the growth of the seed root in the condition tested [60]. When the same metabolites were assayed against *M. phaseolina* and *C. nicotianae*, truncatenolide (**60**) showed significant antifungal activity against *M. phaseolina* and the total inhibition of *C. nicotianae*. Thus, some other fungal nonenolides and their derivatives were assayed for their antifungal activity against both fungi in comparison with truncatenolide for a structure–activity relationship study.



Lupindolinone (**63**) Lupinlactone (**64**) (3*R*)-Mevalolactone (**65**)

Figure 10. Phytotoxins isolated from *Colletotrichum lupini*.

Lupindolinone, lupinlactone, (3*R*)-mevalonolactone, and tyrosol (**63–65** and **9**, Figure 10) were isolated from *Colletotrichum lupini*, which is the causal agent of anthracnose in lupin (*Lupinus albus*) [61]. When these metabolites were tested for their toxicity through different experiments including the effect on root elongation in cress (*Nasturtium officinale*), lupine and duckweed (*Lemma minor*) leaves, or on the seed

germination of parasitic plants such as broomrape (*Phelipanche ramosa*), only lupinlactone (**64**) and tyrosol (**9**) showed the greatest activity out of all of them [61].

Colletotrichin and colletopyrone (**66** and **67**, Figure 11) have been isolated from *Colletotrichum lindemuthianum*, the causal agent of anthracnose on common bean (*Phaselous vulgaris*). The exudate filtrates from the fungal culture have been shown to cause necrotic spots on common bean leaves [114] and to inhibit the seed germination of cowpea (*Vigna unguiculata*), soybean (*Glycine max*), maize (*Zea mays*), sorghum (*Sorghum* spp.), and millet (*Panicum miliaceum*) [62].

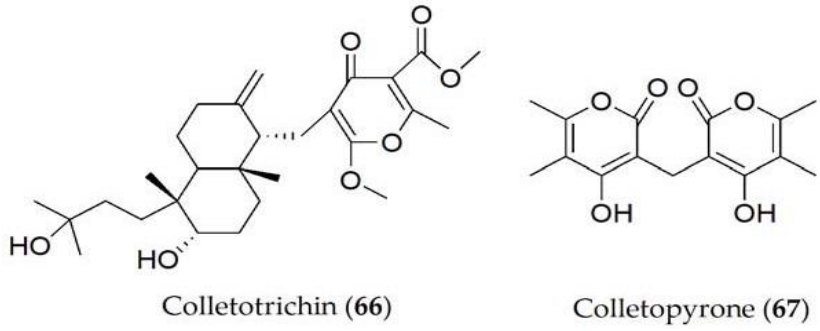


Figure 11. Phytotoxins isolated from *Colletotrichum lindemuthianum*.

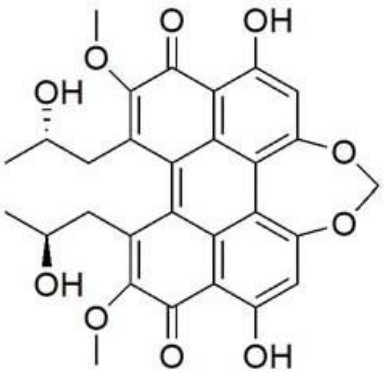
Structure–Toxicity Relationship Studies of Phytotoxins Produced by *Colletotrichum* spp.

A structure–activity relationship study was carried out using truncatenolide and pinolidoxin, 7-*epi*-pinolidoxin, 7,8-O,O′-diacetylpinolidoxin [42], stagonolide C [115], modiolide A, and stagonolide H [116]. The last three nonenolides were obtained from *Stagonospora cirsii* and were previously proposed as a mycoherbicide to the biocontrol of *Cirsium arvense* and *Sonchus arvensis*, which are two common weeds limiting the growth of several cereal cultures. Among all of the tested nonenolides, pinolidoxin (11) showed low antifun- gal activity against both fungi, while modiolide A selectively and totally inhibited only the growth of *C. nicotianae*. These results show that their activity could be linked to the nonenolide ring [60].

2.5. Phytotoxic Compounds Produced by Cercospora *spp.*

Cercospora is a genus of fungi that causes pink-violet spots on the seeds and spreads as the plant develops [117], penetrating through the stomata of the leaf surface

and colonizing the intercellular spaces. Initially, the necrotic red-violet lesions mainly affect the leaves, expanding rapidly to coalesce with adjacent lesions, resulting in severe blighting of the leaves, and conidia protrude in fasciculate bundles in moist conditions from the center. The symptoms were often confused with those developed from the fungi of the genus *Ascochyta* [118]. The spores can be dispersed by environmental agents such as rain or wind, although these can flourish in later or nearby crops if the infected remains are not removed. Environmental conditions such as high humidity and warm temperature are required for spore germination and fungal development [117]. Crop rotation, the usage of resistant varieties, and seeds treated to suppress spore development are useful practices applied to control the pathogen.



Cercosporin (**68**)

Figure 12. Phytotoxins isolated from *Cercospora kikuchii*.

Cercosporin (**68**, Figure 12) is the only compound elucidated by *Cercospora kikuchii* [119]. Cercosporin (**68**) is a non-specific phytotoxin, which is reported to have a role in the pathogenicity of the fungus, as it has been tested on different hosts (such as *Ricinus communis* or *Phaseolus vulgaris* among others), causing chlorosis and necrosis in most of them [63]. Additionally, the production of cercosporin (**68**) varied between different species or strains, being produced through a polyketide pathway and regulated by the calcium/calmodulin complex or mitogen-activated protein kinase signaling pathways (MAPKs). Likewise, this production is also affected by many other physiological and environmental factors such as the availability of nutrients, the ratio between C:N, and the amount of light or temperature [120].

Structure–Toxicity Relationship Studies of Phytotoxins Produced by *Cercospora* spp.

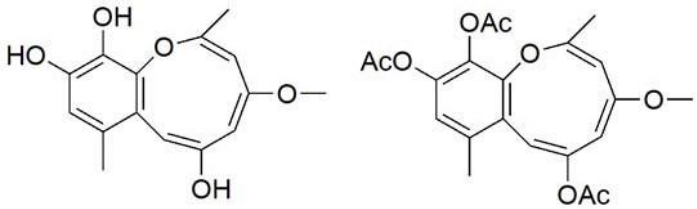
Cercosporin (**68**) has been classified as a photosensitizer, since the phytotoxicity of this compound depends on the intensity of light. This compound reacts with light,

producing free radicals and active oxygen species, particularly singlet oxygen. These reactive compounds are those that induce degradation in the acids of the cell wall of the host, thus increasing the virulence of the disease [121].

2.6. Phytotoxic Compounds Produced by Pleiochaeta setosa

Brown spot disease induced by *Pleiochaeta setosa* can affect legumes in general, although its most common host is lupine (*L. albus*), producing a disease known as brown spot disease, characterized by the appearance of brown spots on the aerial parts of plants such as the leaves and stems and can even affect the root, causing leaf necrosis and finally total wilt [122]. Although there are chemical methods of control including fungicide application, their use has not been proven effective yet. Instead, there are effective physical methods such as the use of heat and low humidity to sterilize seeds [123].

Setosol (**69**, Figure 13) is the only compound produced by *P. setosa*, although its triacetylated derivative (**70**, Figure 13) has also been isolated in lesser quantity [64]. Setosol (**69**) was tested on four lupine variants against an unpurified fungal extract of *P. setosa* and it was observed that the same lesions occurred as an infection caused by the fungus, which led to the conclusion that this is the compound responsible for pathogenicity in the host [64]. However, when setosol is acetylated (**70**), the molecule significantly loses its effectiveness [124].



Setosol (**69**) Triacetylsetosol (**70**)

Figure 13. Phytotoxins isolated from *Pleiochaeta setosa*.

Structure–Toxicity Relationship Studies of Phytotoxins Produced by *Pleichaeta* spp.

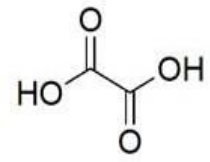
It has been shown that the toxicity of setosol (**69**) is related to the hydroxyl groups of the carbons in positions 6, 10, and 11. Setosol (**69**) has been shown to be an unstable molecule, so natural acetylation increases its stability. However, as this acetylation increases, its phytotoxic activity decreases. After studying the phytotoxicity of these

compounds and their structure, it has been hypothesized that the introduction of chlorine and bromine at these sites may increase the activity of the molecule [124].

2.7. Phytotoxic Compounds Produced by Scletotinia *spp.*

Sclerotinia disease can cause serious yield loss and seed quality problems. This genus of fungi is characterized by the formation of an apothecium in which ascospores are formed, thus differing in the regulation of sexual reproduction [125]. Symptoms are similar to those induced by *Botrytis cinerea*, starting with white and hairy mycelium developing in the aerial parts of the plant that later darken and harden, being more common during the inflorescence period. Later, when the wilted parts fall to the ground or are handled during the farming operations, the spores spread through the soil, favoring the beginning of a new infection cycle [126]. The effective control of this disease depends on various factors such as irrigation, avoiding an excess of water, and the application of fungicides, which are more effective when applied in the full bloom of primary inflorescences. Among the tested fungicides, benomyl, thiophanate methyl, and vinclozolin prevented the appearance of symptoms in leaf tissue on greenhouse-grown soybean plants. Furthermore, vinclozolin was also effective in reducing the mycelium growth of the pathogen *Botrytis cinerea* when added to PDA culture medium [127,128].

Ethanedioic acid, better known as oxalic acid (**71**, Figure 14), is the only metabolite reported for *Sclerotinia sclerotiorum* [65]. It acts during the pathogenesis process of the fungus by breaking the host cell wall, maximizing the efficacy of the different enzymes produced by the pathogen [129]. Its cytotoxic activity has been tested on sunflower and tomato, showing its involvement during the process, but its role against other crops still needs to be evaluated [130].



Oxalic acid (**71**)

Figure 14. Phytotoxin isolated from *Sclerotinia sclerotiorum*.

In studies related to oxalic acid (71), it has been observed that it is a determining compound in the pathogenicity of the *Sclerotinia* fungus, seeing that increasing production of this compound causes greater damage to the host [65]. Likewise, it has

been seen that this compound is metabolized with oxygen and carbon dioxide from the medium at the time of pathogenicity. However, the pathogenicity of oxalic acid (72), generated through the glyoxylate acid pathway [131], is closely related to its ability to manipulate enzymes involved in the plant defense mechanism. Alteration of these processes reduces the pathogenicity because the fungus is not able to extract nutrients involved in plant colonization [132].

3. Potential Application of Phytotoxins Isolated from Legume Fungal Pathogens

Some of the metabolites here reviewed have been shown to possess various biological activities including antibiotic, antifungal, antiviral, but also herbicidal activity. For this reason, they have been tested in other fields, and some of them possess valuable alternative uses, as summarized in Table 2.

Table 2. Discovered biological activities of some of the reported phytotoxins

Metabolites	Biological activity	Literature
Lentiquinone A (**1**)	Fungicidal activity	[72]
Lentiquinone B (**2**)	Antibiotic activity	[72]
Lentiquinone C (**3**)	Antibiotic activity	[72]
	Fungicidal activity	[133]
Lentisone (**4**)	Antibiotic activity	[21]
Pachybasin (**8**)	Fungicidal activity	[133]
Tyrosol (**9**)	Antioxidant effect	[134]
	Anti-bacterial activity	
Pseurotin A (**10**)	Anti-tumoral activity	[135]
Pinolidoxin (**11**)	Cytotoxic activity	[79]
	Fungicidal activity	[60]
Ascochitine (**19**)	Antibiotic activity	[136]
Lathyroxin A (**20**)	Herbicidal activity	[137]
Lathyroxin B (**21**)	Herbicidal activity	[137]
Solanapyrone A (**24**)	Fungicidal activity	[138]
Regiolone (**29**)	Anti-bacterial activity	[139]
	Fungicidal activity	
Botrydial (**35**)	Anti-bacterial activity	[140]
Botrylactone (**47**)	Anti-bacterial activity	[141]
Truncatenolide (**60**)	Fungicidal activity	[60]
Cercosporin (**68**)	Anti-tumoral activity	[142]
Setosol (**69**)	Anti-bacterial activity	[122]
	Fungicidal activity	
Oxalic acid (**71**)	Pesticide	[143]

3.1. Pharmacological Activity

Phytotoxins have been used for human benefit, playing an important role in the field of medicine, especially in forensic medicine, clinical toxicology, pharmacy, pharmacology, and veterinary medicines [59]. Most phytotoxins are known to be poisonous and toxic to humans [144]. However, some of them display antimicrobial and antitumor activity, which can be used in drug development and new discoveries. Phytotoxins play a vital role in cell-cycle regulation, DNA disruption, cytotoxicity, and anticancer effects. Several phytochemicals have been proposed as potential antimicrobial and antitumor agents that could serve as alternatives to traditional medicine and have been investigated as potential antibiotics due to the current problem of the appearance of strains resistant to common antibiotics. One of these cases studied was tyrosol (**9**), which has been shown to have antioxidant properties and to act as an antimicrobial agent against *Staphylococcus aureus* and *Escherichia coli* [134], and it has also been shown to be a quorum sensing molecule of *Candida albicans* [145]. In the case of ascochitine (**19**), it has been shown to have antibiotic activity against certain lines of fungi and bacteria such as *S. aureus* or *C. albicans*, although this type of study needs to be further developed in this field [136].

In the same way, the search for antitumor agents is on the rise in an attempt to avoid more invasive techniques that harm the state of health. One of these cases involves the metabolite pseurotin A (**10**), which after carrying out in vitro and in vivo tests, was shown to act as an antitumor agent. [129]. Concerning cercosporin (**68**), it has been shown that it can act as a photosensitizer for the activation of certain drugs in therapy against various types of tumors [140].

Finally, with respect to pinolidoxin (**11**), other studies have been carried out to assist in further research. This compound caused a clearly detectable actin microfilament disruption in the NIH/3T3 fibroblast cells, being less toxic than its homologue latrunculin A (which is the most commonly used drug in this area), and also accommodates significant structural changes without engendering a loss of bioactivity [146], thus improving the experiments carried out for the study of the cytoskeleton.

3.2. Crop Protection

A great amount of work has been conducted to test and describe the utility of phyto- toxin applications in agriculture, enhancing crops development as (1) natural herbicidal agents, and (2) pest or disease management. In fact, phytotoxins can be effectively used as weed control agents due to characteristics such as selectivity, low persistence in the environment, and environmental safety compared with synthetic herbicides [147]. The huge structural variety and advanced biological activity of these natural phytotoxins make them promising candidates for the development of natural herbicides that use new modes of action in both natural and well-controlled environments. One of the most important features is that they can aim at new sites of action, thereby reducing any form of herbicide resistance.

Phytotoxins produced by plant pathogens can be extracted both from damaged plant tissues or from an artificial culture medium and then used as defensive agents in other crops against a variety of viruses, bacteria, fungi, and insects. Lentiquinone A (**1**) recently showed some fungicidal activity against certain fungi such as *Verticillium dahlia*, *Penicillium allii*, *Rhizoctonia* spp., and *Phoma exigua* [72]. Lentiquinone B, lentiquinone C, and lentisone (**2**–**4**) exhibited antibiotic properties against the bacteria *Bacillus subtilis*, lentiquinone B (**2**) being the one with the lowest proportion of antibiosis of the three [72]. Pachybasin (**8**) and lentiquinone C (**3**), isolated from *A. lentis*, have been tested against rust and powdery mildew fungi of legumes and cereals of agronomic importance and showed significant preventive and curative effects [132]. Pinolidoxin (**11**) selectively inhibits cell growth (tested *in vitro* in a culture of cells extracted from a *Populus trichocarpa* canker), inhibiting phenylalanine ammonia-lyase, which suggests that it can possibly inhibit the defense system of the host plant [79]. This compound was also tested to verify its potential as a fungicide and it was shown that it is capable of inhibiting the growth of *C. nicotianae* in the same way as truncatenolide (**60**) [60].

Lathyroxin A and lathyroxin B (**20** and **21**) inhibited the germination of the seed of the parasitic plant *Phelipanche ramosa* [136]. Regiolone (**29**) inhibited pathogens such as *Bacillus subtilis*, *Colletotrichum gloeosporioides*, and *Magnaporthe oryzae* [139]. Botrydial (**35**) can act against bacteria of the genus *Bacillus* [140], while botrylactone (**47**) also has antibacterial activity against *Bacillus mycoides* and *Bacillus subtilis* [141].

Solanapyrone A (**24**) has fungicidal activity, since it is capable of inhibiting the competitors of *A. rabiei* such as other fungi of the genus *Alternaria*, *Epicoccum*, or

Ulocladium when the fungus has been established and has begun its infective process [139]. Setosol (**69**) mainly has two types of activities: one as a fungicide against fungi from families such as *Colletotrichum*, *Drechslera*, *Gerlachia*, and *Pyricularia* and also acts against yeasts of the genus *Cryptococcus* and against bacteria of the genus *Staphylococcus* [124].

Oxalic acid (**71**) is proposed as a pesticide against varroa mites (*Varroa destructor*), which is a very widespread parasite of honeybees (*Apis mellifera*) throughout the world, being able to eliminate the parasite from a whole hive in few months [148]. Oxalic acid (**71**) is also effective in periods without rearing, but has a very limited effect when the parasite is in its rearing period [143].

4. General Conclusions and Perspectives

Necrotrophic fungi are pathogens responsible for a wide variety of severe fungal diseases that can cause enormous losses in crops, and therefore cause great economic losses [149]. These fungi, among the different compounds that they are capable of secreting, produce phytotoxins, which have been determined to play a closely related role in the plant infection process, and only approximately 25% of these secondary compounds have been characterized in fungi [150]. Although only phytotoxins from necrotrophic fungi have been discussed in this review, it is also interesting to study and broaden the knowledge of other secondary metabolites that fungi are capable of exuding, since they also participate in cell regulation and in the development of the disease by modifying the metabolism of the plant.

Although a great variety of compounds produced by different fungi have been described over the years, this research field is still thriving. Therefore, for future projects, one of the main perspectives is to expand the knowledge of these fungi, investigating the metabolic route for secondary metabolite production [151]. In addition, multidisciplinary investigation groups worldwide have started to work on secondary metabolites produced by less common fungal species such as the necrotrophic fungus *Stemphylium*. Indeed, a recent metabolomic study was carried out by identifying already known metabolites; nevertheless, several features remain unknown due to the absence of satisfactory matches in the databases. Moreover, the complete biological characterization of the identified compound has not yet been carried out [152].

Fungal phytotoxins have potential applications in chemotaxonomy due to their species-specific production. The presence or absence of these compounds can be used to distinguish between closely related fungal species [153]. Indeed, this can be combined with molecular biology techniques such as DNA sequencing and fingerprinting, enhancing the accuracy and reliability of chemotaxonomic studies. This approach can also provide insights into the evolution and ecology of fungal species.

Studying the fungal secondary metabolites can also be useful for investigating plant-pathogen interactions. Several analytical techniques that allow a little manipulation and sample preparation have been developed for this kind of study. One of the most advanced analytical methods is desorption electrospray ionization mass spectrometry (DESI-MS). The technique is particularly useful for studying plant-pathogen interactions as it allows for the detection of a wide range of metabolites that could be involved in the interaction. The detectable metabolites include both volatile compounds that are emitted by plants in response to infection and non-volatile compounds such as lipids and proteins, which can provide information about the structural changes that occur in the plant because of infection. Another significant application of DESI-MS in plant-pathogen interaction is its ability to identify specific metabolites produced by the pathogen, favoring the chemotaxonomy [154,155]. The results of these studies might be employed to develop more effective strategies for controlling the pathogen and protecting the plant.

Indeed, fungal phytotoxins have the potential to serve as biomarkers for plant diseases due to their specificity. Recent studies have demonstrated that phytotoxins are produced during infection and can be detected in plant tissues. For instance, studies have been carried out for Fusarium head blight in wheat and corn or for Alternaria leaf blight [156,157]. They are ideal candidates for early disease diagnosis, and recent advances in techniques such as the already cited mass spectrometry or ELISA assay, further support their potential use as reliable and cost-effective markers. Moreover, investigating fungal phytotoxins could also be helpful in biocontrol. There is a worldwide paradigm shift toward reducing the usage of chemical pesticides. In this framework, fungal antagonists play a significant role as biocontrol agents (BAs) [158]. Thus, investigating phytotoxins, or more in general, bioactive secondary metabolites, can also help understand the BA-pathogen interaction. Co-culture studies applying high throughput screening techniques have already highlighted how the microbial community interacts [159]. However, extensive in planta studies in controlled conditions or field studies have yet to be reported. Understanding the role of fungal phytotoxins in interacting with other life forms is critical

for developing effective disease management strategies. Subsequently, following complete chemical and biological characterization including ecotoxicological studies, phytotoxins could be useful lead compounds in several scientific fields, either in medicine [144] or in agriculture [132,160]. Indeed, they could be applied to the crop as a spray or incorporated into the soil to provide long-term protection, being a safe and effective alternative to traditional chemical pesticides and can be used to reduce the risk of resistance development and environmental pollution.

In the past decades, metabolomics has emerged as a powerful tool for studying fungal phytotoxins. Targeted and non-targeted metabolomics approaches have been used to identify and quantify bioactive known secondary metabolites including phytotoxins. Metabolomics can help identify the metabolic changes in plants following exposure to fungal phytotoxins and understand the mechanisms by which these compounds cause disease symptoms [161]. Moreover, it can be used in combination with genomics and transcriptomics. For instance, combining metabolomics and genomics can provide insights into the genetic basis of phytotoxin production. Similarly, its integration with transcriptomics might reveal the regulatory mechanisms that govern phytotoxin biosynthesis and help to identify potential molecular targets in the host plants. Nevertheless, there is still room for improvement, particularly in protocols and method standardization [162].

Phytotoxins are also used in strategies finalized to induce resistance against fungal pathogen producers and responsible for the disease. The use of resistance inducers represents a helpful alternative for management of the plant disease, which can give good results when included in integrated biocontrol programs. Some examples of satisfactory fungal disease control achieved by the use of phytotoxins produced by pathogens have already been described. Thaxtomin A, the main phytotoxin produced by *Phakopsora pachyrhizi*, was successfully used in the induction of resistance of Asian soybean rust (ASR), caused by this pathogen [163]. *Magnaporthe grisea* is a ubiquitous fungus responsible for finger millet blast, the most devastating disease affecting this cereal. Their fungal toxins have been useful for the development of resistant varieties and their screening procedures [164]. In recent years, tissue culture based on in vitro selection has emerged as a feasible and cost-effective tool for developing stress-tolerant plants. Plants tolerant to biotic stresses can be acquired by applying selecting agents such as pathogen culture filtrate, phytotoxins, or the pathogen itself (for disease resistance) in the culture [165].

Finally, due to changes in environmental conditions, mainly the result of climate change, fungal pathogens are expanding their host range [166]. The World Health Organization (WHO) has observed an increase in fungal diseases in which humans act as a transmission vector. The spread of fungal diseases is mainly harmful to immunocompromised patients. The diagnosis of this type of disease is complicated due to the scarcity of data in medicine. Additionally, their treatment is complicated by the absence of antifungal drugs [167]. For this reason, the WHO has recently proposed lines of action for their control and study, starting from regional surveillance activities to an improvement in its diagnosis.

Finally, the commission also suggested the development of a multidisciplinary platform that facilitates the transfer of knowledge between different investigation fields to facilitate the understanding of pathophysiology for the development of antifungal drugs for the treatment of this type of disease [167]. Therefore, the experience of plant pathologists, biologists, and chemists who have worked in the frame of plant protection might be fundamental to develop scientific projects with a One-Health perspective.

Author Contributions: F.J.A.-J. drafted the manuscript; E.B., P.R., A.E. and D.R. deeply revised and contributed across the various sections. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Agencia Estatal Investigación (AEI), grant PID2020-114668RB-I00.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cubero, J.I. Legumes, the poorman's bread. *Arbor* **2016**, *192*, a312. [CrossRef]
2. Vaz Patto, M.C.; Amarowicz, R.; Aryee, A.N.; Boye, J.I.; Chung, H.J.; Martin-Cabrejas, M.A.; Domoney, C. Achievements and challenges in improving the nutritional quality of food legumes. *Crit. Rev. Plant. Sci.* **2015**, *34*, 105–143. [CrossRef]

3. Boelt, B.; Julier, B.; Karagic´, Đ.; Hampton, J. Legume seed production meeting market requirements and economic impacts. *Crit. Rev. Plant. Sci.* **2015**, *34*, 412–427. [CrossRef]
4. Arnoldi, A.; Zanoni, C.; Lammi, C.; Boschin, G. The role of grain legumes in the prevention of hypercholesterolemia and hypertension. *Crit. Rev. Plant. Sci.* **2015**, *34*, 144–168. [CrossRef]
5. Bähr, M.; Fechner, A.; Krämer, J.; Kiehntopf, M.; Jahreis, G. Lupin protein positively affects plasma LDL cholesterol and LDL: HDL cholesterol ratio in hypercholesterolemic adults after four weeks of supplementation: A randomized, controlled crossover study. *Nutr. J.* **2013**, *12*, 107. [CrossRef] [PubMed]
6. Ramírez-Bahena, M.H.; Peix, A.; Velázques, E.; Berdmar, E. Historia de la investigación en la simbiosis leguminosa-bacteria: Una perspectiva didáctica. *Arbor* **2016**, *192*, a319. [CrossRef]
7. Rubiales, D.; Annicchiarico, P.; Vaz Patto, M.C.; Julier, B. Legume breeding for the agroecological transition of global agri-food systems: A European perspective. *Front. Plant Sci.* **2021**, *12*, 78257448. [CrossRef] [PubMed]
8. Rubiales, D.; Fondevilla, S.; Chen, W.; Gentzbittel, L.; Higgins, T.J.V.; Castillejo, M.A.; Singh, K.B.; Rispail, N. Achievements and challenges in legume breeding for pest and disease resistance. *Crit. Rev. Plant. Sci.* **2015**, *34*, 195–236. [CrossRef]
9. Doehlemann, G.; Ökmen, B.; Zhu, W.; Sharon, A. Plant pathogenic fungi. *Microbiol. Spectr.* **2017**, *5*, 5–1. [CrossRef]
10. Lo Presti, L.; Lanver, D.; Schweizer, G.; Tanaka, S.; Liang, L.; Tollot, M.; Zuccaro, A.; Reismann, S.; Kahmann, R. Fungal effectors and plant susceptibility. *Annu. Rev. Plant Biol.* **2015**, *66*, 513–545. [CrossRef]
11. Buzi, A.; Chilosi, G.; Timperio, A.M.; Zolla, L.; Rossall, S.; Magro, P. Polygalacturonase produced by *Botrytis fabae* as elicitor of two furanoacetylenic phytoalexins in *Vicia faba* pods. *J. Plant Pathol.* **2003**, *85*, 111–116.
12. Dodds, P.N.; Rathjen, J.P. Plant immunity: Towards an integrated view of plant–pathogen interactions. *Nat. Rev. Genet.* **2010**,*11*, 539–548. [CrossRef] [PubMed]
13. Zhao, P.; Lu, G.H.; Yang, Y.H. Salicylic acid signalling and its role in responses to stress. Mechanism of plant hormone signaling under stress. In *Mechanism of Plant Hormone Signaling under Stress*, 1st ed.; Pandey, G.K., Ed.; Wiley Online Library: Hoboken, NJ, USA, 2007; Volume 1, pp. 413–441. [CrossRef]

14. Uma, B.; Rani, T.S.; Podile, A.R. Warriors at the gate that never sleep: Non-host resistance in plants. *J. Plant Physiol.* **2011**,*168*, 2141–2152. [CrossRef] [PubMed]
15. Biemelt, S.; Sonnewald, U. Plant–microbe interactions to probe regulation of plant carbon metabolism. *J. Plant Physiol.* **2006**,*163*, 307–318. [CrossRef] [PubMed]
16. Agrios, G.N. Enfermedades Ocasionadas Por Hongos. In *Fitopatología*, 2nd ed.; de Limusa, S.A.C.V., Ed.; Grupo Noriega: Cuauhtémoc, Ciudad de México, Mexico, 1997; Volume 1, pp. 273–301. Available online: http://www.noriega.com.mx (accessed on 30 January 2023).
17. Mendgen, K.; Hahn, M. Plant infection and the establishment of fungal biotrophy. *Trends Plant Sci.* **2002**, *7*, 352–356. [CrossRef] [PubMed]
18. Mengiste, T. Plant immunity to necrotrophs. *Annu. Rev. Phytopathol.* **2012**, *50*, 267–294. [CrossRef]
19. Meena, M.; Gupta, S.K.; Swapnil, P.; Zehra, A.; Dubey, M.K.; Upadhyay, R.S. Alternaria toxins: Potential virulence factors and genes related to pathogenesis. *Front. Microbiol.* **2017**, *8*, 1451. [CrossRef]
20. Meena, M.; Samal, S. Alternaria host-specific (HSTs) toxins: An overview of chemical characterization, target sites, regulation and their toxic effects. *Toxicol. Rep.* **2019**, *6*, 745–758. [CrossRef]
21. Masi, M.; Cimmino, A.; Reveglia, P.; Mugnai, L.; Surico, G.; Evidente, A. Advances on fungal phytotoxins and their role in grapevine trunk diseases. *J. Agric. Food Chem.* **2018**, *66*, 5948–5958. [CrossRef]
22. Masi, M.; Maddau, L.; Linaldeddu, B.T.; Scanu, B.; Evidente, A.; Cimmino, A. Bioactive metabolites from pathogenic and endophytic fungi of forest trees. *Curr. Med. Chem.* **2018**, *25*, 208–252. [CrossRef]
23. Evidente, A. Fungal bioactive macrolides. *Nat. Prod. Rep.* **2022**, *39*, 1591–1621. [CrossRef] [PubMed]
24. Evidente, A. Bioactive lipodepsipeptides produced by bacteria and fungi. *Int. J. Mol. Sci.* **2022**, *23*, 12342. [CrossRef] [PubMed]
25. Corsaro, M.M.; De Castro, C.; Evidente, A.; Lanzetta, R.; Molinaro, A.; Parrilli, M.; Sparapano, L. Phytotoxic extracellular polysaccharide fractions from *Cryphonectria parasitica* (Murr.) Barr strains. *Carbohydr. Polym.* **1998**, *37*, 167–172. [CrossRef]

26. Corsaro, M.M.; De Castro, C.; Evidente, A.; Lanzetta, R.; Molinaro, A.; Mugnai, L.; Parrilli, M.; Surico, G. Chemical structure of two phytotoxic exopolysaccharides produced by *Phomopsis foeniculi*. *Carbohydr. Res.* **1998**, *30*, 349–357. [CrossRef]
27. Cimmino, A.; Cinelli, T.; Evidente, M.; Masi, M.; Mugnai, L.; Silva, M.A.; Michereff, S.; Surico, G.; Evidente, A. Phytotoxic fungal exopolysaccharides produced by fungi involved in grapevine trunk diseases. *Nat. Prod. Commun.* **2016**, *11*, 349–357. [CrossRef]
28. Hatta, R.; Ito, K.; Hosaki, Y.; Tanaka, T.; Tanaka, A.; Yamamoto, M.; Akimitsu, K.; Tsuge, T. A conditionally dispensable chromosome controls host-specific pathogenicity in the fungal plant pathogen *Alternaria alternata*. *Genetics* **2002**, *161*, 59–70. [CrossRef]
29. Otani, H.; Kohmoto, K.; Kodama, M. Alternaria toxins and their effects on host plants. *Canad. J. Bot.* **1995**, *73*, 453–458. [CrossRef]
30. Walton, J.D. Host-selective toxins: Agents of compatibility. *Plant Cell* **1996**, *8*, 1723. [CrossRef]
31. Mitchell, R.E. The relevance of non-host-specific toxins in the expression of virulence by pathogens. *Annu. Rev. Phytopathol.* **1984**,*22*, 215–245. [CrossRef]
32. Pusztahelyi, T.; Holb, I.J.; Pócsi, I. Secondary metabolites in fungus-plant interactions. *Front. Plant Sci.* **2015**, *6*, 573. [CrossRef]
33. Möbius, N.; Hertweck, C. Fungal phytotoxins as mediators of virulence. *Curr. Opin. Plant Biol.* **2009**, *12*, 390–398. [CrossRef] [PubMed]
34. Samperna, S.; Masi, M.; Vurro, M.; Evidente, A.; Marra, M. Cyclopaldic acid, the main phytotoxic metabolite of *Diplodia cupressi*, induces programmed cell death and autophagy in *Arabidopsis thaliana*. *Toxins* **2022**, *14*, 474. [CrossRef] [PubMed]
35. Burns, D.C.; Mazzola, E.P.; Reynolds, W.F. The role of computer-assisted structure elucidation (CASE) programs in the structure elucidation of complex natural products. *Nat. Prod. Rep.* **2019**, *36*, 919–933. [CrossRef]
36. Manier, S.K.; Keller, A.; Schäper, J.; Meyer, M.R. Untargeted metabolomics by high resolution mass spectrometry coupled to normal and reversed phase liquid chromatography as a tool to study the in vitro biotransformation of new psychoactive substances. *Sci. Rep.* **2019**, *9*, 2741. [CrossRef] [PubMed]
37. Oberlies, N.H.; Knowles, S.L.; Amrine, C.S.M.; Kao, D.; Kertesz, V.; Raja, H.A. Droplet probe: Coupling chromatography to the in-situ evaluation of the chemistry of nature. *Nat. Prod. Rep.* **2019**, *36*, 944–959. [CrossRef]

38. Walsby-Tickle, J.; Gannon, J.; Hvinden, I.; Bardella, C.; Abboud, M.I.; Nazeer, A.; Hauton, D.; Pires, E.; Cadoux-Hudson, T.; Schofield, C.J.; et al. Anion-exchange chromatography mass spectrometry provides extensive coverage of primary metabolic pathways revealing altered metabolism in IDH1 mutant cells. *Commun. Biol.* **2020**, *3*, 247. [CrossRef] [PubMed]
39. Murray, G.M.; Brennan, J.P. *The Current and Potential Costs from Diseases of Wheat in Australia*; Grains Research and Development Corporation: Canberra, Australia, 2009; p. 70. Available online: https://www.grdc.com.au/DiseasesCostsWheat (accessed on 30 January 2023).
40. Tivoli, B.; Baranger, A.; Avila, C.M.; Banniza, S.; Barbetti, M.; Chen, W.; Davidson, J.; Lindeck, K.; Kharrat, M.; Rubiales, D.; et al. Screening techniques and sources of resistance to foliar diseases caused by major necrotrophic fungi in grain legumes. *Euphytica* **2006, *147*, 223–253. [CrossRef]**
41. Andolfi, A.; Cimmino, A.; Villegas-Fernández, A.M.; Tuzi, A.; Santini, A.; Melck, D.; Rubiales, D.; Evidente, A. Lentisone, a new phytotoxic anthraquinone produced by *Ascochyta lentis*, the causal agent of Ascochyta Blight in *Lens culinaris*. *J. Agric. Food Chem.* **2013**, *61*, 7301–7308. [CrossRef]
42. Evidente, A.; Lanzetta, R.; Capasso, R.; Vurro, M.; Botralico, A. Pinolidoxin, a phytotoxic nonenolide from *Ascochyta pinodes*. *Phytochemistry* **1993**, *3*, 999–1003. [CrossRef]
43. Evidente, A.; Capasso, R.; Abouzeid, M.A.; Lanzetta, R.; Vurro, M.; Bottalico, A. Three new toxic pinolidoxins from *Ascochyta pinodes*. *J. Nat. Prod.* **1993**, *56*, 1937–1943. [CrossRef]
44. Cimmino, A.; Andolfi, A.; Fondevilla, S.; Abouzeid, M.A.; Rubiales, D.; Evidente, A. Pinolide, a new nonenolide produced by *Didymella pinodes*, the causal agent of Ascochyta blight on *Pisum sativum*. *J. Agric. Food Chem.* **2012**, *60*, 5273–5278. [CrossRef] [PubMed]
45. Beed, F.D.; Strange, R.N.; Onfroy, C.; Tivoli, B. Virulence for faba bean and production of ascochitine by *Ascochyta fabae*. *Plant Pathol.* **1994**, *43*, 987–997. [CrossRef]
46. Iwai, I.; Mishima, H. Constitution of ascochitine. *Chem. Ind.* **1965**, *186*, 987–997.
47. Alam, S.S.; Bilton, J.N.; Slawin, A.M.; Williams, D.J.; Sheppard, R.N.; Strange, R.N. Chickpea blight: Production of the phytotoxins solanapyrones A and C by *Ascochyta rabiei*. *Phytochemistry* **1989**, *28*, 2627–2630. [CrossRef]

48. Hamid, K.; Strange, R.N. Phytotoxicity of solanapyrones A and B produced by the chickpea pathogen *Ascochyta rabiei* (Pass.) Labr. and the apparent metabolism of solanapyrone A by chickpea tissues. *Physiol. Mol. Plant Pathol.* **2000**, *56*, 235–244. [CrossRef]
49. Latif, Z.; Strange, R.N.; Bilton, J.; Riazuddin, S. Production of the phytotoxins, solanapyrones A and C and cytochalasin D among nine isolates of *Ascochyta rabiei*. *Plant Pathol.* **1993**, *42*, 172–180. [CrossRef]
50. Cimmino, A.; Villegas-Fernández, A.M.; Andolfi, A.; Melck, D.; Rubiales, D.; Evidente, A. Botrytone, a new naphthalenone pentaketide produced by *Botrytis fabae*, the causal agent of chocolate spot disease on *Vicia faba*. *J. Agric. Food Chem.* **2011**, *59*, 9201–9206. [CrossRef]
51. Deighton, N.; Muckenschnabel, I.; Colmenares, A.J.; Collado, I.G.; Williamson, B. Botrydial is produced in plant tissues infected by *Botrytis cinerea*. *Phytochemistry* **2001**, *57*, 689–692. [CrossRef]
52. Colmenares, A.J.; Aleu, J.; Duran-Patron, R.; Collado, I.G.; Hernandez-Galan, R. The putative role of botrydial and related metabolites in the infection mechanism of *Botrytis cinerea*. *J. Chem. Ecol.* **2002**, *28*, 997–1005. [CrossRef]
53. Colmenares, A.J.; Durán-Patrón, R.M.; Hernández-Galán, R.; Collado, I.G. Four new lactones from *Botrytis cinerea*. *J. Nat. Prod.***2002**, *65*, 1724–1726. [CrossRef]
54. Dalmais, B.; Schumacher, J.; Moraga, J.; Le Pecheur, P.; Tudzynski, B.; Collado, I.G.; Viaud, M. The *Botrytis cinerea* phytotoxin botcinic acid requires two polyketide synthases for production and has a redundant role in virulence with botrydial. *Molecular Plant Pathol.* **2011**, *12*, 564–579. [CrossRef] [PubMed]
55. Siddiqui, K.A.I.; Gupta, A.K.; Paul, A.K.; Banerjee, A.K. Purification and properties of a heat-resistant exotoxin produced by *Macrophomina phaseolina* (Tassi) Goid in culture. *Experientia* **1979**, *35*, 1222–1223. [CrossRef] [PubMed]
56. Ramezani, M.; Shier, W.T.; Abbas, H.K.; Tonos, J.L.; Baird, R.E.; Sciumbato, G.L. Soybean charcoal rot disease fungus *Macrophomina phaseolina* in Mississippi produces the phytotoxin (-)-botryodiplodin but no detectable phaseolinone. *J. Nat. Prod.* **2007**, *70*, 128–129. [CrossRef] [PubMed]
57. Masi, M.; Sautua, F.; Zatout, R.; Castaldi, S.; Arrico, L.; Isticato, R.; Pescitelli, G.; Carmona, M.A.; Evidente, A. Phaseocyclopen- tenones A and B, phytotoxic penta- and tetrasubstituted cyclopentenones produced by *Macrophomina phaseolina*, the

causal agent of charcoal rot of soybean in Argentina. *J. Nat. Prod.* **2021**, *84*, 459–465. [CrossRef] [PubMed]

58. Stoessl, A.; Stothers, J.B. Colletruncoic acid methyl ester, a unique meroterpenoid from *Colletotrichum truncatum*. *Z. Naturforsch., C, J. Biosci.* **1986**, *41*, 677–680. [CrossRef]
59. Evidente, A.; Cimmino, A.; Masi, M. Phytotoxins produced by pathogenic fungi of agrarian plants. *Phytochem. Rev.* **2019**, *18*, 843–870. [CrossRef]
60. Masi, M.; Castaldi, S.; Sautua, F.; Pescitelli, G.; Carmona, M.A.; Evidente, A. Truncatenolide, a bioactive disubstituted nonenolide produced by *Colletotrichum truncatum*, the causal agent of anthracnose of soybean in Argentina: Fungal antagonism and SAR Studies. *J. Agric. Food Chem.* **2022**, *70*, 9834–9844. [CrossRef]
61. Masi, M.; Nocera, P.; Boari, A.; Zonno, M.C.; Pescitelli, G.; Sarrocco, S.; Barroncelli, R.; Vannacci, G.; Vurro, M.; Evidente, A. Secondary metabolites produced by *Colletotrichum lupini*, the causal agent of anthachnose of lupin (*Lupinus* spp.). *Mycologia* **2020**, *112*, 533–542. [CrossRef]
62. Amusa, N.A. Production, partial purification and bioassay of toxic metabolites of three plant pathogenic species of *Colletotrichum* in Nigeria. *Mycopathologia* **1994**, *128*, 161–166. [CrossRef]
63. Fajola, A.O. Cercosporin, a phytotoxin from *Cercospora* spp. *Physiol. Plant Pathol.* **1978**, *13*, 157–164. [CrossRef]
64. Okeke, B.; Kaouadji, M.; Seigle-Murandi, F.; Steiman, R. Setosol, a biologically active heptaketide-like metabolite from the *Pleiochaeta setosa* phytopathogen. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 734–736. [CrossRef]
65. Godoy, G.; Steadman, J.R.; Dickman, M.B.; Dam, R. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiol. Mol. Plant Pathol.* **1990**, *37*, 179–191. [CrossRef]
66. Shahid, A.A.; Husnain, T.; Riazuddin, S. Ascochyta blight of chickpea: Production of phytotoxins and disease management. *Biotechnol. Adv.* **2008**, *26*, 511–515. [CrossRef] [PubMed]
67. Rubiales, D.; Fondevilla, S. Future prospects for ascochyta blight resistance breeding in cool season food legumes. *Front. Plant Sci.* **2012**, *3*, 27. [CrossRef] [PubMed]

68. Tivoli, B.; Banniza, S. Comparison of the epidemiology of ascochyta blights on grain legumes. *Eur. J. Plant. Pathol.* **2007**, *119*, 59–76. [CrossRef]
69. Gan, Y.T.; Siddique, K.H.M.; MacLeod, W.J.; Jayakumar, P. Management options for minimizing the damage by ascochyta blight (*Ascochyta rabiei*) in chickpea (*Cicer arietinum* L.). *Field Crops Res.* **2006**, *97*, 121–134. [CrossRef]
70. Stoddard, F.L.; Nicholas, A.H.; Rubiales, D.; Thomas, J.; Villegas-Fernández, A.M. Integrated pest management in faba bean. *Field Crops Res.* **2010**, *115*, 308–318. [CrossRef]
71. Khan, T.N.; Timmerman-Vaughan, G.M.; Rubiales, D.; Warkentin, T.D.; Siddique, K.H.M.; Erskine, W.; Barbetti, M.J. *Didymella pinodes* and its management in field pea: Challenges and opportunities. *Field Crops Res.* **2013**, *148*, 61–77. [CrossRef]
72. Masi, M.; Nocera, P.; Zonno, M.C.; Tuzi, A.; Pescitelli, G.; Cimmino, A.; Boari, A.; Infantino, A.; Vurro, M.; Evidente, A. Lentiquinones A, B, and C, phytotoxic anthraquinone derivatives isolated from *Ascochyta lentis*, a pathogen of lentil. *J. Nat. Prod.* **2018**, *81*, 2700–2709. [CrossRef]
73. Duval, J.; Pecher, V.; Poujol, M.; Lesellier, E. Research advances for the extraction, analysis and uses of anthraquinones: A review. *Ind. Crops Prod.* **2016**, *94*, 812–833. [CrossRef]
74. Fouillaud, M.; Venkatachalam, M.; Girard-Valenciennes, E.; Caro, Y.; Dufossé, L. Anthraquinones and derivatives from marine-derived fungi: Structural diversity and selected biological activities. *Mar. Drugs* **2016**, *14*, 64. [CrossRef] [PubMed]
75. Evidente, A.; Capasso, R.; Vurro, M.; Bottalico, A. Ascosalitoxin, a phytotoxic trisubstituted salicylic aldehyde from *Ascochyta pisi*. *Phytochemistry* **1993**, *34*, 995–998. [CrossRef]
76. Bertini, S. Su di un composto ad azione antibiotica prodotto da *Ascochyta pisi* Lib. *Ann. Stazione Chim. Agrar. Sper. Roma* **1956**, *11*, 545–556.
77. Oku, H.; Nakanishi, T. A toxic metabolite from *Ascochyta fabae* having antibiotic activity. *Phytopathology* **1963**, *53*, 1321–1325.
78. Kim, W.; Chen, W. Phytotoxic metabolites produced by legume-associated *Ascochyta* and its related genera in the *Dothideomycetes*. *Toxins* **2019**, *11*, 627. [CrossRef] [PubMed]
79. Türkkan, M.; Dolar, F.S.; S¸enyuva, H.Z.; Özcan, S. Determination and identification of the solanapyrones and new metabolites from *Ascochyta rabiei*. *Žemdirbyste˙ (Agric.)* **2011**, *98*, 439–444.

80. Kaur, S. Phytotoxicity of solanapyrones produced by the fungus *Ascochyta rabiei* and their possible role in blight of chickpea (*Cicer arietinum*). *Plant Sci.* **1995**, *109*, 23–29. [CrossRef]
81. Evidente, A.; Capasso, R.; Andolfi, A.; Vurro, M.; Zonno, M.C. Structure–activity relationship studies of putaminoxins and pinolidoxins: Phytotoxic nonenolides produced by phytopathogenic *Phoma* and *Ascochyta* species. *Nat. Toxins* **1998**, *6*, 183–188. [CrossRef]
82. Vurro, M.; Ellis, B.E. Effect of fungal toxins on induction of phenylalanine ammonia-lyase activity in elicited cultures of hybrid poplar. *Plant Sci.* **1997**, *126*, 29–38. [CrossRef]
83. Wang, X.Z.; Luo, X.H.; Xiao, J.; Zhai, M.M.; Yuan, Y.; Zhu, Y.; Crews, P.; Yuang, C.; Wu, Q.X. Pyrone derivatives from the endophytic fungus *Alternaria tenuissima* SP-07 of Chinese herbal medicine *Salvia przewalskii*. *Fitoterapia* **2014**, *99*, 184–190. [CrossRef]
84. Jenkins, K.M.; Toske, S.G.; Jensen, P.R.; Fenical, W. Solanapyrones EG, antialgal metabolites produced by a marine fungus. *Phytochem.* **1998**, *49*, 2299–2304. [CrossRef]
85. Mizushina, Y.; Kamisuki, S.; Kasai, N.; Shimazaki, N.; Takemura, M.; Asahara, H.; Lin, S.; Yoshida, S.; Matsukage, A.; Koiwai, O. et al. A plant phytotoxin, solanapyrone A, is an inhibitor of DNA polymerase β and λ. *J. Biol. Chem.* **2002**, *277*, 630–638. [CrossRef] [PubMed]
86. García-Díaz, M.; Domínguez, O.; López-Fernández, L.A.; de Lera, L.T.; Saníger, M.L.; Ruiz, J.F.; Párraga, M.; García-Ortiz, M.J.; Kirchhoff, T.; del Mazo, J.; et al. DNA polymerase lambda (Pol λ), a novel eukaryotic DNA polymerase with a potential role in meiosis. *J. Mol. Biol.* **2000**, *301*, 851–867. [CrossRef]
87. Yamtich, J.; Sweasy, J.B. DNA polymerase family X: Function, structure, and cellular roles. *Biochim. Biophys. Acta Proteins Proteom.* **2010**, *1804*, 1136–1150. [CrossRef] [PubMed]
88. Schmidt, L.E.; Gloer, J.B.; Wicklow, D.T. Solanapyrone analogues from a *Hawaiian fungicolous* fungus. *J. Nat. Prod.* **2007**, *70*, 1317–1320. [CrossRef]
89. Villegas-Fernández, A.M.; Sillero, J.C.; Emeran, A.A.; Winkler, J.; Raffiot, B.; Tay, J.; Flores, F.; Rubiales, D. Identification and multi-environment validation of resistance to *Botrytis fabae* in *Vicia faba*. *Field Crops Res.* **2009**, *114*, 84–90. [CrossRef]

90. Sahile, S.; Ahmed, S.; Fininsa, C.; Abang, M.M.; Sakhuja, P.K. Survey of chocolate spot (*Botrytis fabae*) disease of faba bean (*Vicia faba* L.) and assessment of factors influencing disease epidemics in northern Ethiopia. *J. Crop Prot.* **2008**, *27*, 1457–1463. [CrossRef]
91. Hua, L.; Yong, C.; Zhanquan, Z.; Boqiang, L.; Guozheng, Q.; Shiping, T. Pathogenic mechanisms and control strategies of *Botrytis cinerea* causing post-harvest decay in fruits and vegetables. *Food Qual. Saf.* **2018**, *2*, 111–119. [CrossRef]
92. Davidson, J.A.; Kimber, R. Integrated disease management of Ascochyta blight in pulse crops. In *Ascochyta Blights of Grain Legumes*; Tivoli, B., Baranger, A., Muehlbauer, F.J., Cooke, B.M., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 99–110. [CrossRef]
93. Rubiales, D.; Khazaei, H. Advances in disease and pest resistance in faba bean. *Theor. Appl. Genet.* **2022**, *135*, 3735–3756. [CrossRef]
94. Ascari, J.; Boaventura, M.A.D.; Takahashi, J.A.; Duran-Patron, R.; Hernandez-Galan, R.; Macias-Sanchez, A.J.; Collado, I.G. Phytotoxic activity and metabolism of *Botrytis cinerea* and structure–activity relationships of isocaryolane derivatives. *J. Nat. Prod.* **2013**, *76*, 1016–1024. [CrossRef]
95. Durán-Patrón, R.; Hernández-Galán, R.; Rebordinos, L.G.; Cantoral, J.M.; Collado, I.G. Structure-activity relationships of new phytotoxic metabolites with the botryane skeleton from *Botrytis cinerea*. *Tetrahedron* **1999**, *55*, 2389–2400. [CrossRef]
96. Su, G.; Suh, S.O.; Schneider, R.W.; Russin, J.S. Host specialization in the charcoal rot fungus, *Macrophomina phaseolina*. *Phytopathol- ogy* **2001**, *91*, 120–126. [CrossRef]
97. Naseri, B. Charcoal rot of bean in diverse cropping systems and soil environments. *J. Plant. Dis. Prot.* **2014**, *121*, 20–25. [CrossRef]
98. Naseri, B. Root rot of common bean in Zanjan, Iran: Major pathogens and yield loss estimates. *Australas. Plant Pathol.* **2008**, *37*, 546–551. [CrossRef]
99. Capote, N.; Pastrana, A.M.; Aguado, A.; Ureba, M.B.; Akdi, K. Control biológico de las enfermedades ocasionadas por *Macrophomina phaseolina* y *Fusarium solani* en el cultivo de la fresa. *Phytoma* **2016**, *280*, 36–42.
100. Khan, S.N. *Macrophomina phaseolina* as causal agent for charcoal rot of sunflower. *Mycopath* **2007**, *5*, 111–118.

101. Lodha, S.; Mawar, R. Population dynamics of *Macrophomina phaseolina* in relation to disease management: A review. *J. Phytopathol.* **2020**, *168*, 1–17. [CrossRef]
102. Naseri, B.; Veisi, M.; Khaledi, N. Towards a better understanding of agronomic and soil basis for possible charcoal root rot control and production improvement in bean. *Arch. Phytopathol. Pflanzenschutz* **2018**, *51*, 349–358. [CrossRef]
103. Naseri, B.; Younesi, H. Beneficial microbes in biocontrol of root rots in bean crops: A meta-analysis (1990–2020). *Physiol. Mol. Plant Pathol.* **2021**, *116*, 101712. [CrossRef]
104. Coser, S.M.; Chowda Reddy, R.V.; Zhang, J.; Mueller, D.S.; Mengistu, A.; Wise, K.A.; Allen, T.W.; Singh, A.; Singh, A.K. Genetic architecture of charcoal rot (*Macrophomina phaseolina*) resistance in soybean revealed using a diverse panel. *Front. Plant Sci.* **2017**, *8*, 1626. [CrossRef]
105. Bhattacharya, G.; Dhar, T.K.; Bhattacharya, F.K.; Siddiqui, K.A. Mutagenic action of phaseolinone, a mycotoxin isolated from *Macrophomina phaseolina*. *Aust. J. Biol. Sci.* **1987**, *40*, 349–354. [CrossRef] [PubMed]
106. Shier, W.T.; Abbas, H.K.; Baird, R.E.; Ramezani, M.; Sciumbato, G.L. (-)-Botryodiplodin, a unique ribose-analog toxin. *Toxin Rev.* **2007**, *26*, 343–386. [CrossRef]
107. García-Pajón, C.M.; Collado, I.G. Secondary metabolites isolated from *Colletotrichum* species. *Nat. Prod. Rep.* **2003**, *20*, 426–431. [CrossRef] [PubMed]
108. Chongo, G.; Bernier, C.C. Effects of host, inoculum concentration, wetness duration, growth stage, and temperature on anthracnose of lentil. *Plant Dis.* **2000**, *84*, 544–548. [CrossRef]
109. Cannon, P.F.; Damm, U.; Johnston, P.R.; Weir, B.S. *Colletotrichum*: Current status and future directions. *Stud. Mycol.* **2012**, *73*, 181–213. [CrossRef]
110. Janisiewicz, W.J.; Korsten, L. Biological control of postharvest diseases of fruits. *Annu. Rev. Phytopathol.* **2002**, *40*, 411–441. [CrossRef]
111. Yang, H.C.; Hartman, G.L. Methods and evaluation of soybean genotypes for resistance to *Colletotrichum truncatum*. *Plant Dis.* **2015**, *99*, 143–148. [CrossRef]
112. Geffroy, V.; Sévignac, M.; Billant, P.; Dron, M.; Langin, T. Resistance to *Colletotrichum lindemuthianum* in *Phaseolus vulgaris*: A case study for mapping two independent genes. *Theor. Appl. Genet.* **2008**, *116*, 407–415. [CrossRef]

113. Barilli, E.; Moral, J.; Aznar-Fernández, T.; Rubiales, D. Resistance to anthracnose (*Colletotrichum lentis*, race 0) in *Lens* spp. germplasm. *Agronomy* **2020**, *10*, 1799. [CrossRef]
114. Fernández, M.T.; Fernandez, M.; Centeno, M.L.; Cañal, M.J.; Rodriguez, R. Reaction of common bean callus to culture filtrate of *Colletotrichum lindemuthianum*: Differences in the composition and toxic activity of fungal culture filtrates. *Plant Cell Tissue Organ Cult.* **2000**, *6*, 41–49. [CrossRef]
115. Evidente, A.; Cimmino, A.; Berestetskiy, A.; Mitina, G.; Andolfi, A.; Motta, A. Stagonolides B F, nonenolides produced by *Stagonospora cirsii*, a potential mycoherbicide of *Cirsium arvense*. *J. Nat. Prod.* **2008**, *71*, 31–34. [CrossRef] [PubMed]
116. Evidente, A.; Cimmino, A.; Berestetskiy, A.; Andolfi, A.; Motta, A. Stagonolides G I and modiolide A, nonenolides produced by *Stagonospora cirsii*, a potential mycoherbicide for *Cirsium arvense*. *J. Nat. Prod.* **2008**, *71*, 1897–1901. [CrossRef] [PubMed]
117. Albu, S.; Schneider, R.W.; Price, P.P.; Doyle, V.P. *Cercospora* cf. *flagellaris* and *Cercospora* cf. *sigesbeckiae* are associated with Cercospora leaf blight and purple seed stain on soybean in North America. *Phytopathology* **2016**, *106*, 1376–1385. [CrossRef] [PubMed]
118. Kimber, R.B.E.; Paull, J.G. Identification and genetics of resistance to cercospora leaf spot (*Cercospora zonata*) in faba bean (*Vicia faba*). *Euphytica* **2011**, *177*, 419–429. [CrossRef]
119. Newman, A.G.; Townsend, C.A. Molecular characterization of the cercosporin biosynthetic pathway in the fungal plant pathogen *Cercospora nicotianae*. *J. Am. Chem. Soc.* **2016**, *138*, 4219–4228. [CrossRef]
120. You, B.J.; Lee, M.H.; Chung, K.R. Production of cercosporin toxin by the phytopathogenic *Cercospora* fungi is affected by diverse environmental signals. *Can. J. Microbiol.* **2008**, *54*, 259–269. [CrossRef]
121. Daub, M.E.; Ehrenshaft, M. The photoactivated *Cercospora* toxin cercosporin: Contributions to plant disease and fundamental biology. *Annu. Rev. Phytopathol.* **2000**, *38*, 461–490. [CrossRef]
122. Gan, M.J.; Ash, G.J.; Cowley, R.B.; Savocchia, S.; Luckett, D.J. Genetic variation of *Pleiochaeta setosa* from *Lupinus albus*. *Australasian Plant Pathol.* **2009**, *38*, 518–524. [CrossRef]

123. Del Moral, J.; Arias, A.; De Arcos, R. Aparición en España del moteado *Pleiochaeta setosa* (Kichn) Hughes del altramuz. *Bol. Serv. Plagas* **1981**, *7*, 141–145.
124. Okeke, B.; Seigle-Murandi, F.; Steiman, R. Biological activity of a heptaketide metabolite from *Pleiochaeta setosa*. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 173–175. [CrossRef]
125. Ellis, M.B.; Waller, J.M. *Sclerotinia fuckeliana* (conidial state: *Botrytis cinerea*). *CMI Descr. Pathog. Fungi Bact.* **1974**, *1974*, 431.
126. Purdy, L. *Sclerotinia sclerotiorum*: History, diseases and symptomatology, host range, geographic distribution, and impact. *Phytopathology* **1979**, *69*, 875–880. [CrossRef]
127. Steadman, J.R. White mold-a serious yield-limiting disease of bean. *Plant Dis.* **1983**, *67*, 346–350. [CrossRef]
128. Mueller, D.S.; Dorrance, A.E.; Derksen, R.C.; Ozkan, E.; Kurle, J.E.; Grau, C.R.; Gaska, J.M.; Hartman, G.L.; Pedersen, W.L. Efficacy of fungicides on *Sclerotinia sclerotiorum* and their potential for control of Sclerotinia stem rot on soybean. *Plant Dis.* **2002**, *86*, 26–31. [CrossRef] [PubMed]
129. Noyes, R.D.; Hancock, J.G. Role of oxalic acid in the *Sclerotinia* wilt of sunflower. *Physiol.Plant Pathol.* **1981**, *18*, 123–132. [CrossRef]
130. Magro, P.; Marciano, P.; Di Lenna, P. Oxalic acid production and its role in pathogenesis of *Sclerotinia sclerotiorum*. *FEMS Microbiol. Lett.* **1984**, *24*, 9–12. [CrossRef]
131. Gadd, G.M. Fungal production of citric and oxalic acid: Importance in metal speciation, physiology and biogeochemical processes. *Adv. Microb. Physiol.* **1999**, *41*, 47–92. [CrossRef]
132. Ziman, L.; Je˛dryczka, M.; Šrobárová, A. Relationship between morphological and biochemical characteristics of *Sclerotinia sclerotiorum* isolates and their aggressivity/Beziehung zwischen morphologischen und biochemischen Eigenschaften von *Sclerotinia sclerotiorum Stämmen und ihrer Aggressivität*. *Z. Pflanzenkrankh. Pflanzenschutz/ JPDP* **1998**, *105*, 283–288.
133. Barilli, E.; Agudo, F.J.; Masi, M.; Nocera, P.; Evidente, A.; Rubiales, D. Anthraquinones and their analogues as potential biocontrol agents of rust and powdery mildew diseases of field crops. *Pest. Manag. Sci.* **2022**, *78*, 3489–3497. [CrossRef]

134. Casadey, R.; Challier, C.; Altamirano, M.; Spesia, M.; Criado, S. Antioxidant and antimicrobial properties of tyrosol and derivative-compounds in the presence of vitamin B2. Assays of synergistic antioxidant effect with commercial food additives. *Food Chem.* **2020**, *335*, 127576. [CrossRef]
135. Helal, G.A.; Ahmed, F.A.; Askora, A.; Saber, T.M.; Rady, S.M. Pseurotin a from *Aspergillus fumigatus* Fr. AUMC 8002 exhibits anticancer activity against hepatocellular carcinoma in vitro and in vivo. *Slov. Vet. Zb.* **2019**, *56*, 59–74. [CrossRef]
136. Foremska, E.; Kostecki, M.; Chełkowski, J. Biosynthesis, preparation and properties of ascochitine. *Acta Biotechnol.* **1992**, *12*, 461–465. [CrossRef]
137. Masi, M.; Nocera, P.; Boari, A.; Cimmino, A.; Zonno, M.C.; Infantino, A.; Vurro, M.; Evidente, A. Lathyroxins A and B, phytotoxic monosubstituted phenols isolated from *Ascochyta lentis* var. *lathyri*, a fungal pathogen of grass pea (*Lathyrus sativus*). *J. Nat. Prod.* **2018**, *81*, 1093–1097. [CrossRef] [PubMed]
138. Kim, W.; Park, J.J.; Dugan, F.M.; Peever, T.L.; Gang, D.R.; Vandemark, G.; Chen, W. Production of the antibiotic secondary metabolite solanapyrone A by the fungal plant pathogen *Ascochyta rabiei* during fruiting body formation in saprobic growth. *Environ. Microbiol.* **2017**, *19*, 1822–1835. [CrossRef]
139. Deng, Q.; Li, G.; Sun, M.; Yang, X.; Xu, J. A new antimicrobial sesquiterpene isolated from endophytic fungus *Cytospora* sp. from the Chinese mangrove plant *Ceriops tagal*. *Nat. Prod. Res.* **2020**, *34*, 1404–1408. [CrossRef]
140. Vignatti, P.; Gonzalez, M.E.; Jofré, E.C.; Bolívar-Anillo, H.J.; Moraga, J.; Viaud, M.; Collado, I.G.; Pieckenstain, F.L. Botrydial confers *Botrytis cinerea* the ability to antagonize soil and phyllospheric bacteria. *Fungal Biol.* **2020**, *124*, 54–64. [CrossRef]
141. Welmar, K.; Tschesche, R.; Breitmaier, E. Botrylacton, ein neuer wirkstoff aus der nährlösung des pilzes *Botrytis cinerea*, 2. *Chem. Ber.* **1979**, *112*, 3598–3602. [CrossRef]
142. Grigalavicius, M.; Mastrangelopoulou, M.; Berg, K.; Arous, D.; Ménard, M.; Raabe-Henriksen, T.; Brondz, E.; Siem, S.; Görgen, A.; Edin, N.; et al. Proton-dynamic therapy following photosensitiser activation by accelerated protons demonstrated through fluorescence and singlet oxygen production. *Nat. Commun.* **2019**, *10*, 3986. [CrossRef]

143. Jack, C.J.; van Santen, E.; Ellis, J.D. Evaluating the efficacy of oxalic Acid vaporization and brood interruption in controlling the honey bee pest *Varroa destructor* (Acari: *Varroidae*). *J. Econ. Entomol.* **2020**, *113*, 582–588. [CrossRef]
144. Balde, E.S.; Andolfi, A.; Bruyere, C.; Cimmino, A.; Lamoral-Theys, D.; Vurro, M.; Van Damme, M.; Altomare, C.; Mathieu, V.; Kiss, R.; et al. Investigations of fungal secondary metabolites with potential anticancer activity. *J. Nat. Prod.* **2010**, *73*, 969–971. [CrossRef]
145. Chen, H.; Fujita, M.; Feng, Q.; Clardy, J.; Fink, G.R. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5048–5052. [CrossRef] [PubMed]
146. Fürstner, A.; Nagano, T.; Müller, C.; Seidel, G.; Müller, O. Total synthesis and evaluation of the actin-binding properties of microcarpalide and a focused library of analogues. *Chem. Eur. J* **2007**, *13*, 1452–1462. [CrossRef] [PubMed]
147. Cimmino, A.; Masi, M.; Evidente, M.; Superchi, S.; Evidente, A. Fungal phytotoxins with potential herbicidal activity: Chemical and biological characterization. *Nat. Prod. Rep.* **2015**, *32*, 1629–1653. [CrossRef] [PubMed]
148. Gregorc, A.; Planinc, I. Acaricidal effect of oxalic acid in honeybee (*Apis mellifera*) colonies. *Apidologie* **2001**, *32*, 333–340. [CrossRef]
149. Wang, X.; Jiang, N.; Liu, J.; Liu, W.; Wang, G.L. The role of effectors and host immunity in plant–necrotrophic fungal interactions. *Virulence* **2014**, *5*, 722–732. [CrossRef]
150. Montes-Belmont, R. Chemical diversity in plants against phytopathogenic fungi. *Rev. Mex. Micol.* **2009**, *29*, 73–82.
151. Wojakowska, A.; Muth, D.; Naroz˙na, D.; Ma̧drzak, C.; Stobiecki, M.; Kachlicki, P. Changes of phenolic secondary metabolite profiles in the reaction of narrow leaf lupin (*Lupinus angustifolius*) plants to infections with *Colletotrichum lupini* fungus or treatment with its toxin. *Metabolomics* **2013**, *9*, 575–589. [CrossRef]
152. Olsen, K.J.K.; Rossman, A.; Andersen, B. Metabolite production by species of *Stemphylium*. *Fungal Biol.* **2018**, *122*, 172–181. [CrossRef]
153. Frisvad, J.C.; Andersen, B.; Thrane, U. The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. *Mycol. Res.* **2008**, *112*, 231–240. [CrossRef]

154. Tata, A.; Perez, C.J.; Hamid, T.S.; Bayfield, M.A.; Ifa, D.R. Analysis of metabolic changes in plant pathosystems by imprint imaging DESI-MS. *J. Am. Soc. Mass Spectrom.* **2014**, *26*, 641–648. [CrossRef]
155. Hemalatha, R.G.; Pradeep, T. Understanding the molecular signatures in leaves and flowers by desorption electrospray ionization mass spectrometry (DESI MS) imaging. *J. Agric. Food Chem.* **2013**, *61*, 7477–7487. [CrossRef] [PubMed]
156. Ferrigo, D.; Raiola, A.; Causin, R. *Fusarium* toxins in cereals: Occurrence, legislation, factors promoting the appearance and their management. *Molecules* **2016**, *21*, 627. [CrossRef] [PubMed]
157. Logrieco, A.; Moretti, A.; Solfrizzo, M. *Alternaria* toxins and plant diseases: An overview of origin, occurrence and risks. *World Mycotoxin J.* **2009**, *2*, 129–140. [CrossRef]
158. Thambugala, K.M.; Daranagama, D.A.; Phillips, A.J.; Kannangara, S.D.; Promputtha, I. Fungi vs. fungi in biocontrol: An overview of fungal antagonists applied against fungal plant pathogens. *Front. Cell. Infect. Microbiol.* **2020**, *10*, 604923. [CrossRef] [PubMed]
159. Zhang, X.; Elliot, M.A. Unlocking the trove of metabolic treasures: Activating silent biosynthetic gene clusters in bacteria and fungi. *Curr. Opin. Microbiol.* **2019**, *51*, 9–15. [CrossRef]
160. Barilli, E.; González-Bernal, M.J.; Cimmino, A.; Agudo-Jurado, F.J.; Masi, M.; Rubiales, D.; Evidente, A. Impact of fungal and plant metabolites application on early development stages of pea powdery mildew. *Pest Manag. Sci.* **2019**, *75*, 2464–2473. [CrossRef]
161. O'Banion, B.S.; O'Neal, L.; Alexandre, G.; Lebeis, S.L. Bridging the gap between single-strain and community-level plant-microbe chemical interactions. *Mol. Plant Microbe Interact.* **2020**, *33*, 124–134. [CrossRef]
162. Ellis, S.R.; Bruinen, A.L.; Heeren, R. A critical evaluation of the current state-of-the-art in quantitative imaging mass spectrometry. *Anal. Bioanal. Chem.* **2014**, *406*, 1275–1289. [CrossRef]
163. De Paula, S.; Dalio, R.J.D.; Maximo, H.J.; Pino, L.E.; Amorim, D.J.; Da Paz, S.M.R.; Ribeiro Brandão, D.F.; Garcia Borges, C.; Demétrio Pascholati, S.F. Potential of thaxtomin A for the control of the Asian soybean rust. *Can. J. Plant Pathol.* **2022**, *44*, 56–65. [CrossRef]

164. Shanmugapackiam, S.; Parthasarathy, S.; Raguchander, T. Detection of phytotoxin produced from leaf, neck and finger blast disease causing *Magnaporthe grisea* through GCMS analysis. *Int. J. Biochem. Res. Rev.* **2017**, *19*, 1–10. [CrossRef]
165. Zeleke, K. Application of tissue culture on biotic stress tolerance in plants. *Int. J. Curr. Res. Acad. Rev.* **2022**, *10*, 49–57. [CrossRef]
166. West, J.S.; Townsend, J.A.; Stevens, M.; Fitt, B.D. Comparative biology of different plant pathogens to estimate effects of climate change on crop diseases in Europe. *Eur. J. Plant Pathol.* **2012**, *133*, 315–331. [CrossRef]
167. World Health Organization. Antimicrobial Resistance Division. In *WHO Fungal Priority Pathogens List to Guide Research, Development and Public Health Action*; World Health Organization: Geneva, Switzerland, 2022; Available online: http://hdl.handle.net/20.500.12105/15113 (accessed on 30 January 2023).

CHAPTER II

Comparative analysis of secondary metabolites produced by *Ascochyta fabae* under in vitro conditions and their phytotoxicity on the primary host, *Vicia faba*, and related legume crops

Comparative analysis of secondary metabolites produced by *Ascochyta fabae* under in vitro conditions and their phytotoxicity on the primary host, *Vicia faba*, and related legume crops

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This chapter is being considered in **Toxins**

Paper accepted

Abstract: Ascochyta blight, caused by *Ascochyta fabae*, poses a significant threat to faba bean and other legumes worldwide. Necrotic lesions on stems, leaves and pods characterize the disease. Given the economic impact of this pathogen and the potential involvement of secondary metabolites in symptom development, a study was conducted to investigate the fungus's ability to produce bioactive metabolites that might contribute to its pathogenicity. For this investigation the fungus was cultured in three substrates (Czapek-Dox, PDB, and rice). Metabolites produced were analysed by NMR and LC-HRMS methods resulting in dereplication of seven metabolites, which varied with the cultural substrates. Ascochlorin, ascofuranol, and (*R*)-mevalonolactone were isolated from the Czapek-Dox extract; ascosalipyrone, benzoic acid, and tyrosol from the PDB extract; and ascosalitoxin and ascosalipyrone from the rice extract. The phytotoxicity of the pure metabolites was assessed at different concentrations on their primary hosts and related legumes. The fungal exudates displayed varying degrees of phytotoxicity, with the Czapek-Dox medium's exudate exhibiting the highest activity across almost all legumes tested. Species belonging to the genus *Vicia* spp. were the most susceptible, with faba bean being susceptible to all metabolites, at least at the highest concentration tested, as

expected. In particular, ascosalitoxin and benzoic acid were the most phytotoxic in the tested condition and, as a consequence, expected to play and important role on necrosis's appearance.

Keywords: Fungal metabolites; Ascochyta blight; legumes; phytotoxins

Key Contribution: First report from *Ascochyta fabae* of ascochlorin; ascofuranol; (R)-mevalonolactone ascosalipyrone; benzoic acid; tyrosol; ascosalitoxin and ascosalipyrone as phytotoxic metabolites affecting *Vicia* species.

1.Introduction

Cold-weather legumes are a valuable source of premium plant-based protein suitable for human consumption and livestock feed. They play an essential role in crop rotation on arable lands, helping to minimize the requirement for fertilizer usage and acting as effective interim crops [1-6]. However, as for any crop, legumes can be affected by a number of diseases, out of which Ascochyta blights are one of the most important group of necrotic fungal diseases globally present in all legume cultivation areas [7]. Different *Ascochyta* species cause the Ascochyta blight diseases in a host-specific manner in many instances: *Ascochyta fabae* Speg., *Ascochyta lentis* Vassiljevsky, *Ascochyta pisi* Lib., *Ascochyta pinodes* (Berk. & Blox.) Jones, *Ascochyta rabiei* (Pass) Labr., and *Ascochyta viciae-villosae* Ondrej are pathogens of faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.), pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), and hairy vetch (*Vicia villosa* Roth), respectively [7-11].

Ascochyta blight management remains problematic, mainly due to the reduced levels of plant resistance available and also because the use of fungicides is uneconomic, forcing the integration of genetic resistance with cultural practices [12,13]. Symptoms generally emerge in the above-ground sections of the plants when exposed to high percentage of humidity and moderate temperature, resulting in necrotic lesions on both leaves and stems [14,15]. Leaves with multiple lesions tend to fade prematurely, particularly in the lower sections of the plants. On diseased stems, these fungi induce extensive necrotic lesions, which can result in stem breakage and the demise of plant portions situated above the affected area. The infection can also spread through contaminated grains and pods, posing a risk to subsequent crops, as their use can have detrimental effects on the growth of emerging plants. In this study we focus on Ascochyta blight of faba bean which is widespread and can cause significant damage by breaking

stems, by leaf lesions and seed depreciation. Disease control through crop rotation, clean seed, and chemical treatment is not wholly effective [4] and only moderate levels of genetic resistance are available [16,17], reinforcing the need to understand pathogenicity factors as targets both for resistance breeding and for designing alternative management strategies.

A histological examination revealed that cellular damage and collapse occurred prior to direct fungal contact aimed at breaking down host tissues for nutrient acquisition [18]. To achieve this, necrotrophic fungi can suppress plant defences by releasing harmful substances, primarily enzymes that catalyze the breakdown of structural components and other vital compounds, as well as phytotoxins that induce cell damage and modifications. Nevertheless, the phytotoxic compounds produced by *Ascochyta* associated with legumes are often host-specific or exhibit toxicity toward various plants, including their respective hosts. The precise roles of these compounds in the pathogenic process remain unknown [19].

Several metabolites with cytotoxic capacity involved in the pathogenesis process have been found in different *Ascochyta* species. In detail, a range of polyketide-derived secondary metabolites were isolated from the organic extracts of *A. lentis*, *A. pinodes* and *A. pisi* and chemically characterized by 1D and 2D NMR spectroscopy and mass spectrometry.

Previously, only one metabolite named ascochitine, an *o*-quinone methide, has been identified in *A. fabae* [20]. However, pathogenicity studies have shown that ascochitine is not crucial for causing disease in faba beans, and there is no apparent correlation between the amount of ascochitine and the aggressiveness of *A. fabae* isolates [21]. This finding suggests that other phytotoxic metabolites may be produced by *A. fabae* that play a role in pathogenicity.

To get new insight on the interaction between *A. fabae* with its host plants and to get new insights into the role of secondary metabolites involved in pathogenicity, we conducted a comprehensive study to isolate and characterize the phytotoxic secondary metabolites produced by *A. fabae* under *in vitro* conditions. *A. fabae* was grown here in three common culture media to explore their influence on secondary metabolite production. Crude organic extracts from the cultures were subjected to bioassays on the primary host (faba bean) and related legumes of the genera *Vicia*, *Lens* and *Pisum*. Following this, the organic extracts were purified using chromatographic methods, and spectroscopic techniques, essentially NMR, were employed to dereplicate and

characterize the most abundant secondary metabolites fully. Finally, the phytotoxicity of the purified compounds was assessed to gain valuable insights into their roles in fungal pathogenesis.

2. Results

Ascochyta fabae isolate Af-CO99-01 was grown in vitro in three different substrates (two liquid media, Czapek-Dox and PDB, and one solid rice culture, as detailed in the Materials and Methods section) to explore the production of secondary metabolites. After extraction, the phytotoxicity of the three corresponding organic residues on the primary host, faba bean, and other related legumes of economic importance was assayed at different concentrations.

2.1. Bioassays of fungal organic extract

The three organic extracts exhibited varying degrees of phytotoxicity, which depended on the fungal growth medium, the applied concentration, and the specific plant species. All three fungal extracts displayed significantly higher phytotoxicity (assessed as

Figure 1. Symptoms developed on detached leaves of several legume hosts as a consequence of the following treatments: (a) uninoculated, (b) water, (c) methanol (MeOH 5%), (d) *A. fabae* extract at 2 mg/mL from Czapek-Dox medium, (e) *A. fabae* extract at 2 mg/mL from PDB medium and, (f) *A. fabae* extract at 2 mg/mL from rice substrate.

Table 1. Differences by host specie on disease severity (necrotic area, mm²) caused by *Ascochyta fabae* fungal exudates from different culture media (Czapek-Dox, PDB and Rice). Values are the general average of all concentrations tested. Negative controls (blank untreated, water and MeOH 5%) were also included. The experiment was repeated four times.

	Host plant									
Treatment	**Faba bean**		**Narbon vetch**		**Common vetch**		**Lentil**		**Pea**	
Blank	0 ± 0	d	0 ± 0	c	0 ± 0	c	0 ± 0	c	0 ± 0	c
Water	0 ± 0	d	1.8 ± 1.6	c	0.2 ± 0.1	c	0 ± 0	c	0.3 ± 0.2	c
MeOH	3.4 ± 1.5	c	7.3 ± 3.3	bc	0.2 ± 0.05	c	0.5 ± 0.3	c	0.4 ± 0.2	c
Czapek-Dox	110.6 ± 3.9	a	96.4 ± 8.8	a	27.9 ± 5.5	ab	19.9 ± 5.2	a	29.2 ± 3.2	a
PDB	95.6 ± 5.4	b	45.2 ± 4.7	b	30.2 ± 7.3	a	1.0 ± 0.6	c	7.5 ± 2.6	b
Rice	81.6 ± 2.6	b	38.9 ± 5.1	b	17.1 ± 5.1	b	9.1 ± 3.4	b	9.9 ± 2.1	b

Values, per column and treatment, followed by different letters differ significantly at $P < 0.01$.

foliar damaged area, mm²) compared to the controls. The faba bean was the legume crop with the most significant damage, regardless of the culture media employed. Both narbon and common vetches were also significantly damaged, while disease symptoms were generally lower on lentil and pea leaves (Figure 1, Table 1, Supplementary Table 1).

Regardless of the host species, the fungal exudate from the Czapek-Dox medium caused higher disease symptoms, followed by exudates from PDB and rice culture (Figure

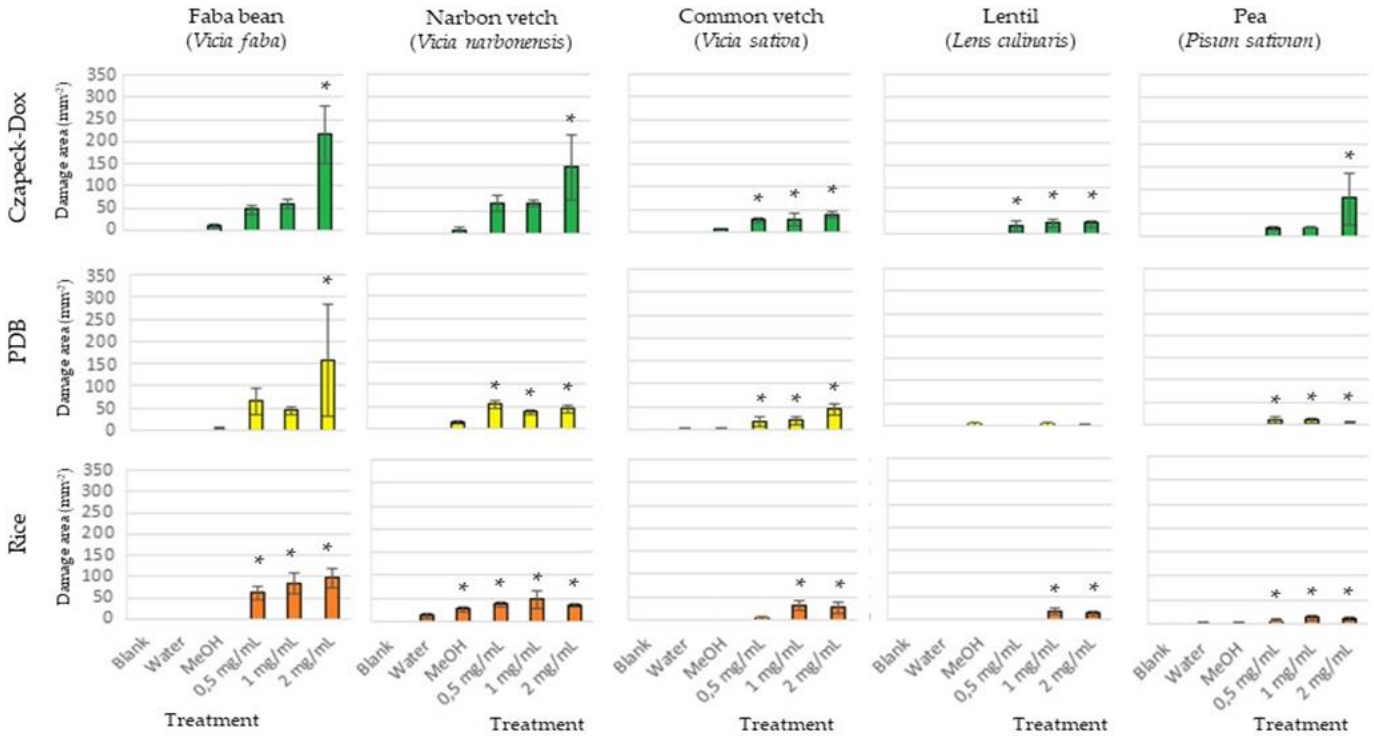


Figure 2. Diseased area (mm²) measured on detached leaves of 5 legume crops treated with exudates from the fungus *Ascochyta faba* growth in vitro on 3 different culture media as: Czapek-Dox (green), Potato Dextrose Broth = PDB (yellow) and Rice (orange) at concentrations of 0.5, 1 and 2 mg/mL. Negative controls (blank untreated, water and MeOH 5%) were also included. The experiment was repeated four times. Asterisk (*) indicates values significantly different from control MeOH 5%.

2, Table 1, Supplementary Table 1). In addition, dose-dependent differences were also observed, especially for the exudate from Czapek-Dox medium on faba bean, narbon vetch and pea, as well as for exudate from PDB on faba bean, being the higher dose applied (2 mg/mL) the most phytotoxic. Other treatments did not show a dose-dependent effect.

Given the phytotoxicity exhibited by all three fungal exudates, being the Czapek-Dox extract the most active among all legume species tested, a comprehensive analysis was conducted to determine the specific metabolite composition displayed by each exudate. The objective of this analysis was to elucidate the distinct metabolic profiles inherent to each exudate. This investigation was undertaken to elucidate potential commonalities or disparities within the exudates, shedding light on the underlying factors contributing to the different phytotoxic effects observed.

2.2. Identification of secondary metabolites from culture filtrates of A. fabae *cultures*

Due to the different phytotoxicity displayed by the organic extracts derived from the Czapek-Dox, PDB, and rice cultures observed on both host and no-host legume crops, their purification was carried out using chromatographic techniques, as detailed in the materials and methods section. The predominant metabolites synthesized by the fungus

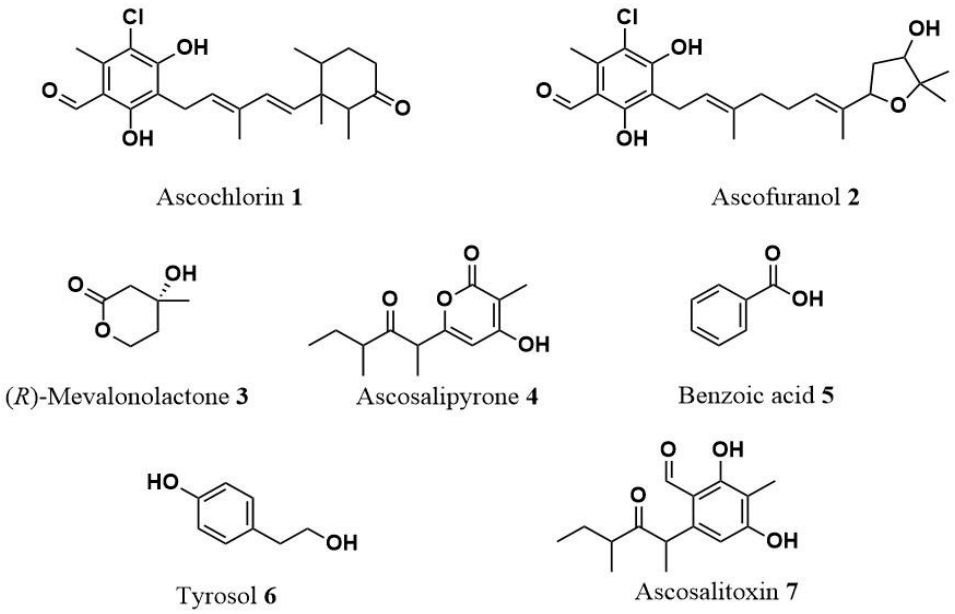


Figure 3. Structure of ascochlorin (1), ascofuranol (2), (R)-mevalonolactone (3), ascosalipyrone (4), benzoic acid (5), tyrosol (6), ascosalitoxin (7).

in each culture medium were identified through a comprehensive analysis of NMR spectroscopy and high-resolution LC/MS spectra.

From the Czapek-Dox culture, three distinct metabolites were purified: ascochlorin (**1**), ascofuranol (**2**), and (*R*)-mevalonolactone (**3**). Figure 3 illustrates their respective structures. By comparing their spectroscopic properties to existing literature data, compounds **1**-**3** were successfully identified and dereplicated. Ascochlorin (**1**) was determined to have a molecular formula of $C_{23}H_{29}ClO_4$ based on its HR-ESIMS, revealing two identifiable mass adducts: $[M+H]^+$ and $[M+Na]^+$. The structural assignment was further confirmed by ^{1}H-NMR spectra, which exhibited diagnostic peaks such as the aldehyde proton at δ 10.14, chelated OH at δ 12.72, one aromatic methyl group signal at δ 2.57, and the other four methyl groups signals at δ 1.92, 0.83, 0.81, and 0.70, respectively. Similarly, ascofuranol (**2**) was found to possess a molecular formula of $C_{23}H_{31}ClO_5$ based on HR-ESIMS, with two identifiable mass adducts: $[M+H]^+$ and $[M+Na]^+$. The structural assignment was supported by ^{1}H-NMR spectra, highlighting diagnostic peaks such as the aldehyde proton at δ 10.14, chelated OH at δ 12.70, one aromatic methyl signal at δ 2.60, geminal dimethyl groups at δ 1.28 and 1.21, and olefinic protons resonating at δ 5.49 and δ 5.15. (*R*)-Mevalonolactone (**3**) had a molecular formula of $C_6H_{10}O_3$ as deduced from its HR-ESIMS from $[M+K]^+$ adduct and the dimer $[2M+Na]^+$. ^{1}H-NMR spectra confirm the structural assignment for the presence of diagnostic peaks: singlet methyl group at δ 1.28 and the two diastereotopic protons at δ 4.62 and δ 4.41, respectively. All the spectra are reported in supplementary Figures 1-6.

The PDB culture yielded three unique metabolites: ascosalipyrone (**4**), benzoic acid (**5**), and tyrosol (**6**), that were dereplicated according to their spectroscopic properties reported in literature (illustrated in Supplementary Figures 7-12 provide the ^{1}H-NMR and ESI/MS spectra). Ascosalipyrone (**4**) was determined to have a molecular formula of $C_{13}H_{18}O_4$ based on its HR-ESIMS, revealing two identifiable mass adducts: $[M+H]^+$ and the dimer $[2M+Na]^+$. The structural assignment was further confirmed by ^{1}H-NMR spectra, which exhibited diagnostic peaks such as the deshielded olefinic proton at δ 5.95, two multiplets at δ 2.69 and δ 1.68, and the four signals of methyl groups at δ 1.94, 1.38, 1.05, and 0.82, respectively.

The two simple aromatic compounds benzoic acid (5) and tyrosol (6) were determined to have molecular formulas $C_7H_6O_2$ and C8H10O2, respectively. The HR-ESIMS of compound 5 revealed two adducts $[M-H2O+H]^+$ and $[M+H]^+$, while for compound 6 again the adduct [M-H2O+H]+ was detected and the dimer $[2M+H]^+$

(Supplementary Figures 10, 12). The structural assignment of benzoic acid (5) was further confirmed by 1H-NMR spectra, which reveal the typical pattern of monosubstituted benzene with one doublet at δ 8.10 of the two ortho-equivalent protons and two triplets at δ 7.62 and δ 7.48 The ^{1}H NMR spectrum of tyrosol (6) showed the typical signal pattern of a para-disubstituted benzene with two doublets, each for two equivalent protons, at δ: 7.10 and 6.79 and the two triplet of the two methylene of the 2-hydroxy ethyl residue at δ 3.82 and 2.8.

Lastly, the rice culture produced two distinct metabolites: ascosalipyrone (**4**) and ascosalitoxin (**7**) (illustrated in Figure 3). Also, compound **7** was dereplicated by comparing the spectroscopic properties with those reported in the previous study. Supplementary Figures 13 and 14 display its ^{1}H-NMR and ESI/MS spectra. Ascosalitoxin (**7**) was determined to have a molecular formula of $C_{13}H_{18}O_4$ based on its HR-ESIMS, revealed by the identifiable mass adduct $[M+H]^+$. The structural assignment was further confirmed by ^{1}H-NMR spectra, which exhibited diagnostic peaks such as the aldehyde proton at δ 10.23, chelated OH at δ 12.68, one aromatic proton signal at δ 6.23, and the four methyl groups signals at δ 2.11, 1.43, 1.08, and 0.72, respectively.

All the dereplicated metabolites have been previously reported as fungal metabolites. Nevertheless, their presence in *A. fabae* in vitro cultures is being reported here for the first time.

2.3. Bioassay of pure compounds

The degree of phytotoxicity caused by pure metabolites from *A. fabae* grown on Czapek-Dox, PDB, and rice substrates varied according to the host species and the applied concentration. In general terms, faba bean and narbon vetch were the most susceptible hosts to all the metabolites applied and at any concentrations, followed by common vetch (Figure 4, Supplementary Table 2, Supplementary Figure 15). By contrast, low or no phytotoxicity was induced in both lentil and pea leaves, not significantly different from the negative controls (Supplementary Table 2, Supplementary Figure 15).

Observed by the legume host, species belonging to the genus *Vicia* were the most susceptible (faba bean, narbon vetch and common vetch), while in pea and lentil hosts, poor or no phytotoxicity was observed. As expected, Faba bean was susceptible to all metabolites, at least at the highest concentration tested. In particular, ascosalitoxin (**7**) and

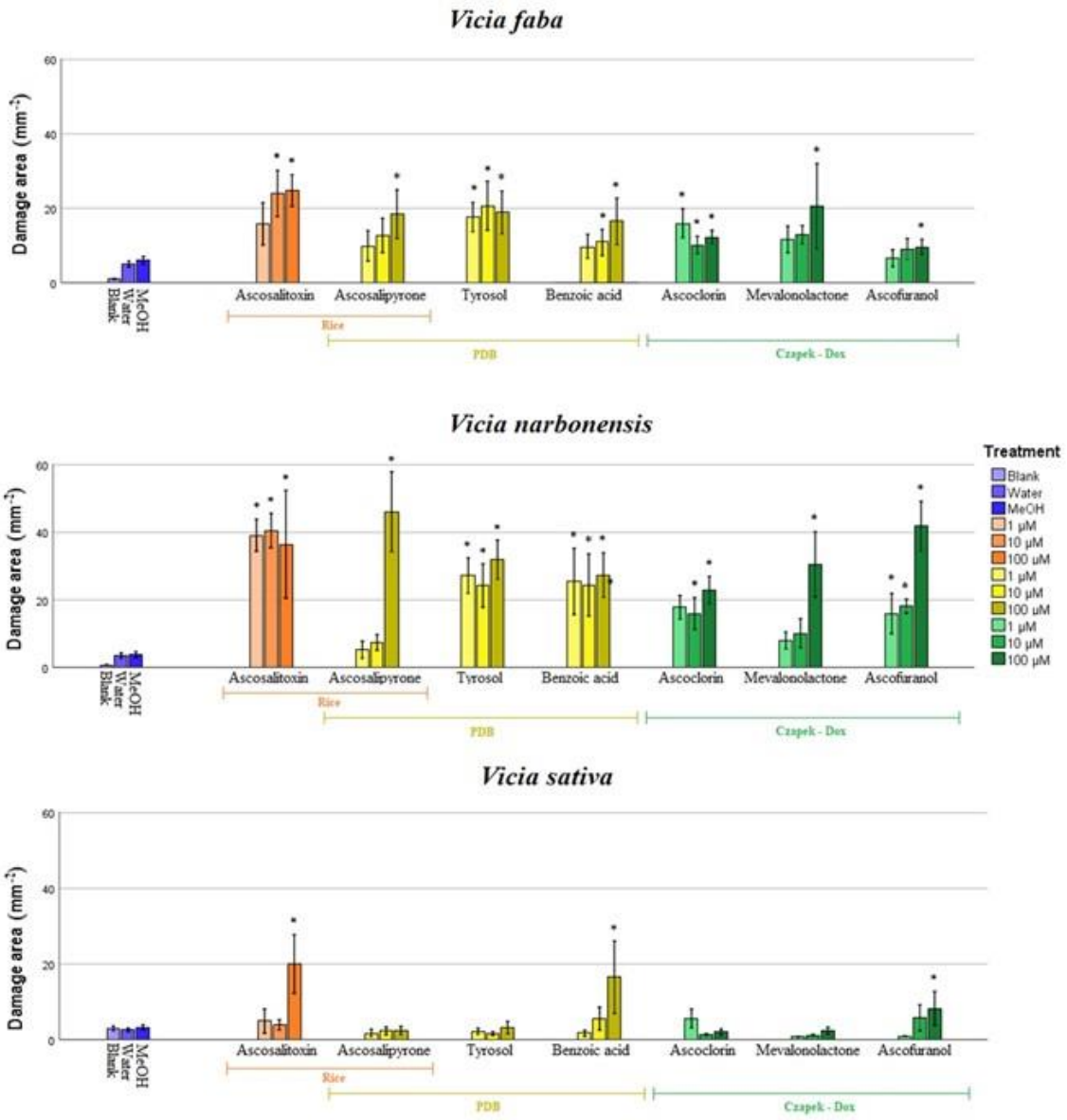


Figure 4. Necrotic area (mm2) incited by metabolites 1-7 at different concentrations (1, 10 and 100 µM showed as increased colour intensity) on leaves of *Vicia faba, V. narbonensis, V. sativa* (LSD test, P < 0.01). In orange metabolites isolated from rice substrate, in yellow, metabolites isolated from PDB culture; in green, metabolites isolated from Czapek-dox; in blue, negative control: blank uninoculated, water and MeOH 5% controls. Asterisk (*) indicates values significantly different from control MeOH 5%.

benzoic acid (**5**) cause damaged areas of 29.8 and 30.8 mm2 and 15 and 16 mm^2 at applied concentrations of 10 and 100 µM, respectively. Tyrosol (**6**) was phytotoxic at any concentration tested, with damaged areas higher than 17 mm^2. The other metabolites tested were also phytotoxic, especially at 100 µM. From our results, narbon vetch was the most susceptible legume species tested here, susceptible to all the pure metabolites but with resulting necrotic lesions bigger than those measured in faba bean (Figure 4; Supplementary Table 2; Supplementary Figure 15). Phytotoxicity from ascochlorin (**1**),

ascofuranol (**2**), benzoic acid (**5**), tyrosol (**6**), and ascosalitoxin (**7**) was not dose-dependent, showing activity at any concentration tested. By contrast, (*R*)-mevalonolactone (**3**) and ascosalipyrone (**4**) showed phytotoxicity only at the higher concentration tested (values higher than 30 and 46 mm^2, respectively). Common vetch was less affected by metabolite's application, showing significant necrotic areas only with ascofuranol (**2**), benzoic acid (**5**) and ascosalitoxin (**7**) at the highest concentration rate.

3. Discussion

Among the diseases affecting legumes, ascochyta blight, incited from the fungal pathogen *Ascochyta fabae*, is one of the most critical necrotic diseases globally present in all legume cultivation areas [17]. Numerous studies suggest that symptoms associated with Ascochyta blight disease seem to be triggered when there is a shift in host physiology, particularly during periods of plant tissue stress [19]. In fact, various chemical and physical factors, whether directly or indirectly, play a role in activating metabolic pathways, which may include the phytotoxic secondary metabolites generated by the fungus. The legume-associated *Ascochyta* spp. produce different metabolites with pathogenesis-determining cytotoxic capacity, many of which display significant toxicity to plants [19,22]. To shed light on the interaction between *A. fabae* and its host plants and to get new insights into the role of secondary metabolites involved in pathogenicity, we conducted a comprehensive study to isolate and characterize the most abundant phytotoxic secondary metabolites produced by this pathogen under *in vitro* conditions. Due to difference found in the bibliography concerning the culture media described for isolation of phytotoxins produced by *Ascochyta* spp. [19,22-24], our study was conducted growing *A. fabae* on three commonly used growth media: PDB, Czapek-Dox and rice substrate. Despite variations in culture media and substrates employed, the mycelial growth and spore production performed well. The mycelium initially displayed a pale cream colour, transitioning into shades of greyish white, dark greenish, and creamy white, aligning with expectations. However, the subsequent investigation into the metabolic profile revealed the significant impact of cultural conditions on the production of secondary metabolites. This outcome is consistent with prior research involving the One Strain Many Compounds Strategy (OSMAC) applied to other fungal and bacteria species [25]. Still, this is the first time that this strategy is applied to an isolate of *A. fabae*. Exploring diverse cultural conditions is essential for comprehensively exploring the

selected microorganism's chemical space and biosynthetic pathways, effectively simulating *in vivo* conditions. This is particularly relevant in chemical ecology studies aimed at elucidating the role of specialized metabolites in host-pathogen interactions and identifying chemical biomarkers for early disease detection [25,26].

After filtration of the mycelium and extraction of the cultures, the phytotoxicity of each resulting fungal extracts was tested on the host plant and related legumes. The bioassays revealed differences among the extracts, with that obtained from the Czapek-Dox medium being the most active, followed by that from PDB, and finally, from rice substrate. Regarding the host susceptibility, species belonging to the genus *Vicia* spp. were the most susceptible to all the extracts, with faba bean showing the most damage, as expected, since it is the primary host, followed by narbon vetch and common vetch. Lentils and peas, on the other hand, displayed lower susceptibility to the phytotoxic activity of the extracts. These results align with previous findings from cross-inoculation studies with different *Ascochyta* spp. isolates on a panel of legume hosts [11]. The authors found that *A. fabae* infected beans (common bean, faba bean, and soybean) and common vetch principally, while negligible damages were observed in both peas and lentils. These results suggested that the specificity observed might be attributed to bioactive secondary metabolites in the extracts, which could play a role in specific host interactions.

Indeed, when chemical investigation was carried out on the three organic extracts, differences in metabolic composition were appreciated. The prevalent metabolic constituents produced during the *in vitro* growth by the Ascochyta blight pathogen were isolated by chromatographic methods and dereplicated by extensive spectroscopic studies. As a result, the main compounds identified were ascochlorin, ascofuranol, (*R*)-mevalonolactone, ascosalipyron, benzoic acid, tyrosol, and ascosalitoxin. This was the first time where these metabolites had been purified and dereplicated from an *A. fabae* isolate. Still, their isolation and amount highly depended on the selected cultural condition. In detail, we isolated and dereplicated ascochlorin and ascofuranol together with (*R*)-mevalonolactone in the Czapek-Dox medium. In contrast, ascosalipyrone, benzoic acid and tyrosol were isolated and dereplicated in the PDB medium. Finally, when *A. fabae* was grown on rice substrate, the main constituents of the extract were ascosalipyrone and ascosalitoxin.

Ascochlorin and ascofuranol are the class natural compounds of meroterpenoids of polyketide–terpene hybrid origin. Ascochlorin was originally isolated from a culture extract of *A. viciae* [27] and later from the entomopathogenic fungus *Verticillium*

hemipterigenum [28]. This compound bears a structural resemblance to ubiquinol, an integral component of the respiratory chain for ATP synthesis, exerting inhibitory effects on protozoan alternate oxidase at the ubiquinol binding site [29]. Notably, ascochlorin has demonstrated diverse biological activities, including antiviral and antibiotic properties, as evident in studies targeting *Candida albicans* [27]. Additionally, it hinders the respiratory chain of ascomycetes yeast *Pichia anomala* by affecting the coenzyme Q [30]. Furthermore, it functions as a non-toxic anticancer agent by inducing G1 cell cycle arrest through p21 induction in a c-Myc-dependent manner rather than p53-dependent [31]. Ascofuranol, a derivative of ascofuranone, was initially isolated from *A. viciae* [32] and was later identified in *A. rabiei* extracts [33]. Ascofuranol exerts its inhibitory action on the alternative oxidase of Trypanosoma by targeting the ubiquinol-binding domain [32]. Ascosalitoxin is a trisubstituted salicylic aldehyde derivative and a biosynthetic precursor of the ascochitine [21]. While this compound was initially isolated from *Ascochyta pisi* [23], it has also been discovered from an endophytic fungus isolated from the medicinal plant *Hintonia latiflora* [34]. Ascosalitoxin has demonstrated cytotoxic activity against human tumour cell lines, manifesting inhibitory effects on the HL-60 cell line [35]. Ascosalipyrone, a polyketide, was first isolated from *A. salicorniae* [36]. It displays potential biological activity as an inhibitor of protein phosphatases [37]. Additionally, it shows antiplasmodial activity against the K1 and NF 54 strains of *Plasmodium falciparum* in conjunction with antimicrobial activity and inhibition of the tyrosine kinase p56lck [36]. In our study, ascosalipyrone was dereplicated from PDB and rice substrate extracts. Notably, the former exhibited higher phytotoxic activity, possibly attributable to the lower production of ascosalipyrone on the rice substrate. In the PDB fungal extracts, two other metabolites were also found: tyrosol and benzoic acid, two simple phenolic metabolites synthesized via the shikimate and phenylpropanoid pathways. They could contribute to infection and phytotoxicity. Nevertheless, it is essential to acknowledge that these metabolites might also fulfill other roles in disease progression or fungal development. Tyrosol is a derivative of phenethyl alcohol. While it has previously been isolated from *A. lentis* and *A. lentis* var. *lathyri* [22], this study marks its first isolation from *A. fabae*. Tyrosol is recognized as a phytotoxic metabolite isolated from both plants and fungi [38]. It frequently appears in cultures of botryosphaeraceous fungi [38] and has been associated with "quorum sensing" in the human pathogenic fungus *Candida albicans* [39]. Tyrosol exhibits antioxidant and anti-inflammatory properties [40] and protects against oxidative stress in renal cells alongside hydroxytyrosol [41]. Furthermore, studies

have linked the cytotoxicity of tyrosol and its derivatives with the inhibition of DNA replication initiation [42]. Its potential suitability for stroke therapy in rats has also been explored [43]. On the other hand, benzoic acid has been identified in *Streptomyces lavandulae* [44,45], as well as in *Lactobacillus plantarum*, where it exhibited antimicrobial activity against Gram-negative bacteria such as *Pantoea agglomerans* (*Enterobacter agglomerans*) and *Fusarium avenaceum* (*Gibberella panacea*). In faba beans infected with Fusarium wilt, benzoic acid has been observed to reduce tissue and cell structure resistance, decrease photosynthesis, and increase cell wall degrading enzyme activity [46]. Additionally, it has been shown to inhibit primary root elongation in *Arabidopsis* seedlings, resulting in reduced sizes [47]. (*R*)-Mevalonolactone, a lactone belonging to the δ-valerolactone class, plays a pivotal role as an essential intermediate in the mevalonate biosynthetic pathway. This biosynthetic route is crucial for synthesizing isoprenoids, versatile compounds with diverse functions in various cellular processes [48,49]. Notably, (*R*)-mevalonolactone has been isolated from different pathogenic and non-pathogenic fungi cultivated *in vitro*, including *Colletotrichum lupini*, *Diaporthaceae* sp. PSU-SP2/4, *Alternaria euphorbicola*, *Pseudallescheria boydii*, and *Phomopsis archeri* [50-53]. (*R*)-Mevalonolactone was found to enhance chlorophyll content in Leman fronds and inhibit root elongation in cress. Additionally, it led to a considerable reduction in seed germination of *Pelipanche ramosa* [50]. Nevertheless, no phytotoxic effects have been reported for this metabolite.

All these metabolites underwent a phytotoxicity bioassay on the same panel of legume crops of the crude extracts. Notably, prior to this study, there was a lack of information available in the literature regarding the phytotoxic activity of some of the dereplicated metabolites. They all showed different degrees of phytotoxicity regarding legumes and concentration, with the phytotoxic effect more evident in *Vicia* species. In detail, ascochlorin displays intense phytotoxic activity as necrosis on leaves in both faba bean and narbon vetch. Surprisingly, it does not induce significant damage in common vetch despite being first isolated from *A. viciae*. Ascofuranol has a dose-dependent effect in faba beans and peas, while in narbon vetch, necrotic damages are high at all concentrations tested. Ascosalitoxin was the most active metabolite isolated, inducing necrosis in all legume crops tested except lentils. This agrees with previous studies where phytotoxic activity from ascosalitoxin on pea leaves and pods and on tomato seedlings was described [23]. Ascosalitoxin was extracted from rice extract. Although the fungal rice extract had the lowest activity in *Vicia* spp., the pure compound caused the most

damage. The difference in activity between the fungal extract and the pure compound may be due to its concentration in the organic extract. For ascosalipyrone this is the first evidence regarding its phytotoxic activity, as no previously available information was described. It was active on faba bean, narbon vetch and pea leaves, but only at the highest concentration tested (100 µM). Similar behavior was observed for (*R*)-mevalonolactone being the most phytotoxin on faba bean and narbon vetch, showing a dose-dependent effect. Tyrosol displayed no dose-dependent effect against faba bean and narbon vetch, causing significant damage even at the lowest concentration of 1µM. Nevertheless, contrary to previous results [19], lentil is only slightly susceptible, while no significantly diseased leaves were observed in treated vetch or pea. Since tyrosol is a ubiquitous metabolite in plants, this might exceed the plant's usual levels, leading to necrosis at even lower compound concentrations. Interestingly, benzoic acid is the only compound that exhibits activity in all leguminous plants, but its behavior varies. In narbon vetch, its activity does not depend on concentration. In contrast, in all others, it does, with faba beans showing activity from 10µM and lentils, peas, and beans only at their highest concentration applied.

The observed variations in bioassay results between crude extracts and pure metabolites may be ascribed to differences in the tested concentrations and production disparities within the various culture media and the potential synergistic or antagonistic effects that may exist among the metabolites generated in each medium. When dealing with complex mixtures of natural products, dereplicate and identify the specific components responsible for their activities and comprehending the mechanisms involved in their interactions remains a tricky challenge. Such mechanisms can be multifaceted and vary depending on cultivation methods, preparation, and processing of these compounds, as observed in previous research [54,55]. Modern analytical chemistry techniques, chemoinformatic tools, and metabolomics play a pivotal role in the dereplication of intricate organic extracts by identifying and cataloguing known metabolites and promoting the search for novel compounds. Additionally, it offers a powerful tool for quantifying minor components within these extracts, aiding in accurately assessing their abundance [56-60]. Furthermore, it is essential to highlight that the low amount and the high number of chiral carbons in ascochlorin ascofuranol, ascosalipyrone, and ascosalitoxin did not allow their complete stereochemical characterization using the spectroscopic method. Existing information on the stereochemistry of these metabolites is limited [23,61]. Future research should prioritize the assignment of chiral carbon

configurations to fully elucidate their role in the *Ascochyta*-legume interaction. The absolute assignment of fungal secondary metabolite configurations through advanced spectroscopic methods, including NMR and optical techniques, is indispensable for comprehensively understanding their biological relevance [62-64]. Accurate molecular structure determination provides insights into the compound's bioactivity and potential ecological roles.

Finally, it is essential to highlight that high-molecular-weight phytotoxins were not studied under the conditions of this research. In previous studies involving other fungal species, hydrophilic high-molecular-weight metabolites, such as polysaccharides peptides with phototoxic properties that could have a role as elicitors, have been detected and studied *in vitro* [65-67].

In conclusion, our research has provided insights into the phytotoxic secondary metabolites produced by *A. fabae* under varying *in vitro* conditions, unveiling a rich diversity of compounds with differential phytotoxic effects. Notably, prior to this study, only ascochitine was known to be produced by *A. fabae*. We have dereplicated a panel of seven metabolites belonging to different classes of natural compounds that could be used for targeted in-depth investigations. Nevertheless, the observed variations in bioassay results between crude extracts and pure metabolites underline the intricate nature of these interactions, influenced by synergistic or antagonistic effects among the metabolites and further studies using more sensitive techniques are also needed to identify the minor constituents of the fungal exudate. These findings pave the way for further research to elucidate these seven metabolites' underlying mechanisms and ecological implications in legume-plant interactions. Understanding these complex relationships is essential for advancing our knowledge of host-pathogen interactions and developing more specific strategies for Ascochyta blight management and early detection.

4. Materials and Methods

4.1. Fungal strain, culture medium and growth conditions

A previously well-characterized monoconidial strain of *Ascochyta fabae* (Af-CO99-01) [68], isolated from a diseased faba bean (*Vicia faba*) crop and belonging to the fungal collection maintained at the Institute for Sustainable Agriculture (IAS-CSIC, Córdoba, Spain) was selected for these experiments. The pathogen was preserved in

sterile cellulose filter papers. Before the experiment, inoculum was prepared by multiplying spores of the isolate on potato dextrose agar (PDA) (Sigma Aldrich, Saint-Quentin Fallavier, France) medium under controlled conditions as previously described [11]. Then, the isolate was differentially growth in two artificial media and one solid substrate as follow: i) 10 flasks containing 1 L of Czapek-Dox medium [69]. Each flask was inoculated with a 1-week-old mycelial plate of the isolate on PDA. The cultures were incubated at 24 °C (stirring conditions, 150 rpm), in absence of light for 21 days. The fungal mycelium was then removed by filtration through four layers of filter paper, centrifuged and kept at -20 °C until next analysis; ii) 10 flasks containing 1 L of potato dextrose broth (PDB) (BD Difco®, Crystal Lake, NJ, USA) medium. Each flask was inoculated with a 1-week-old mycelial plate of the isolate on PDA and kept at similar conditions than mentioned in point i); iii) 1 L flask containing 400 g of common rice. Water was added to the flask (45%, vol/vol) and allowed 24 h to be absorbed. Then, the material was sterilized at 121 °C for 30 min. Inoculation was carried out with a 1-week-old mycelial plate of the isolate on PDA. The culture was then incubated at conditions described by Reveglia *et al.* [70].

4.2. General Experimental Procedure for Chemical Analysis

Analytical and preparative TLCs were carried out on silica gel (Merck, Kieselgel 60, F254, 0.25 and 0.5 mm respectively) and reverse phase (Merck, Kieselgel 60 RP-18, F254, 0.20 mm) plates. The spots were visualized by exposure to UV radiation (254 and/or 312 nm) or by spraying first with 10% H_2SO_4 in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min on a hot plate. Column chromatography was performed using silica gel (Merck, Kieselgel 60, 0.063-0.200 mm). Solvents as *n*-hexane MeOH, *i*-PrOH, $CHCl_3$, CH_2Cl_2 were purchased from Panreac AppliChem (Barcelona, Spain). Unless otherwise noted, optical rotation was measured in MeOH on a Jasco (Tokyo, Japan) polarimeter, whereas the CD spectrum was recorded on a JASCO J-815 CD in MeOH. ^{1}H and ^{13}C NMR and 2D NMR spectra were recorded at 400 or 500, and 100 or 125 MHz in $CDCl_3$ on Bruker and Varian instruments. The same solvent was used as an internal standard. HR-ESIMS analyses were performed using the LC/MS TOF system (AGILENT 6230B, HPLC 1260 Infinity) column Phenomenex LUNA (C18 (2) 5 µm 150x 4.6 mm). ^{1}H-NMR and ESI/MS (+) spectra of the identified compounds are reported in the supplementary Figures 1-14.

4.3. Extraction and Purification of Secondary Metabolites Produced in Czapek-Dox Culture

The culture filtrates (10 L) of *A. fabae* were lyophilized and dissolved in 1/10 distilled water of the original volume. The solution was exhaustively extracted with EtOAc (3 × 300 mL). The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure. The corresponding residue (439 mg) was purified by silica gel column, eluted with $CHCl_3$-*i*-PrOH (95:5), yielding six homogeneous fraction groups. The residue of the first fraction (80 mg) was purified by TLC, eluted with $CHCl_3$ affording two white amorphous solids identified as ascochlorin (**1**, 1.7 mg) and ascofuranol (**2**, 2 mg). The residue of the fourth fraction (80 mg) was purified by TLC, eluted with $CHCl_3$-*i*-PrOH (97:3), affording a white amorphous solid identified as (*R*)-mevalonolactone (**3**, 1.9 mg).

- Ascochlorin (**1**): ^{1}H-NMR and ESI/MS (+) data agree with those previously reported [27,71].
- Ascofuranol (**2**): ^{1}H-NMR and ESI/MS (+) data agree with those previously reported [32].
- (*R*)-Mevalonolactone (**3**): ^{1}H-NMR and ESI/MS (+) data agree with those previously reported [50].

4.4. Extraction and Purification of Secondary Metabolites Produced in PDB Culture

The culture filtrates (10 L) of *A. fabae* were lyophilized and dissolved in 1/10 distilled water of original volume. The solution was exhaustively extracted with EtOAc (3 × 300 mL). The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure. The corresponding residue (441 mg) was purified by silica gel column, eluted with $CHCl_3$-*i*-PrOH (95:5), yielding seven homogeneous fraction groups. The residue of the second fraction (34 mg) was purified by TLC, eluted with $CHCl_3$-*i*-PrOH (97:3), affording two white amorphous solid identified as ascosalipyrone (**4**, 10 mg), and as benzoic acid (**5**, 8 mg). The residue of the third fraction (60 mg) was purified by TLC and eluted with $CHCl_3$-*i*-PrOH (95:5), affording a white amorphous solid identified as tyrosol (**6**, 1.6 mg).

- Ascosalipyrone (**4**): ^{1}H-NMR and ESI/MS (+) data agree with those previously reported [36].
- Benzoic acid (**5**): ^{1}H-NMR and ESI/MS (+) data agree with those previously reported. [44].
- Tyrosol (**6**): ^{1}H-NMR and ESI/MS (+) data agree with those previously reported [72].

4.5. Extraction and Purification of Secondary Metabolites Produced in Rice Culture

The solid culture of *A. fabae* (440 g) was subjected to air-drying at 27 °C for a minimum of two weeks before the extraction process. Subsequently, the dried material was finely ground using a laboratory mill and then subjected to extraction with a mixture of 500 mL of MeOH–H_2O (1% NaCl) in a 1:1 ratio. Afterward, the mixture underwent centrifugation at 10,000 rpm for 1 hour. The resulting pellet was subjected to a second round of extraction using the same solvent mixture under identical conditions. The two supernatants obtained were combined, treated with *n*-hexane (2 × 500 mL) for defatting, and further extracted with CH_2Cl_2 (3 × 500 mL). The organic extracts in CH_2Cl_2 were pooled, desiccated using Na_2SO_4, and subsequently concentrated under reduced pressure to produce a brown solid residue weighing 226 mg. The corresponding residue was purified by silica gel column, eluted with $CHCl_3$-*i*-PrOH (95:5), yielding six homogeneous fraction groups. The residue of the second fraction (4.7 mg) was purified by TLC eluted with $CHCl_3$-*i*-PrOH (97:3). This afforded a white amorphous solid identified as ascosalitoxin (**7**, 0.8 mg). The residue of fraction six (18 mg) was purified by TLC, eluted with $CHCl_3$-*i*-PrOH (95:5), yielding another amorphous solid identified as ascosalipyrone (**4**, 1 mg).

- Ascosalitoxin (**7**): ^{1}H-NMR and ESI/MS (+) data agree with those previously reported [23,34].

4.6. Bioassays

The phytotoxic effects of all the *A. fabae* organic extracts and those of pure compounds **1**-**7** were evaluated using a detached leaf method [73]. Several legume crops (listed in Table 2) were selected and grown in chamber as follow: seeds were sow in pots

Table 2. Legume species and genotypes grow under controlled conditions and are used in detached leaves assays.

Legume	Plant specie	Genotype
Faba bean	*Vicia faba*	Baraca
Narbon vetch	*V. narbonensis*	VN01
Common vetch	*V. sativa*	Buzza
Lentil	*Lens culinaris*	Pardina
Pea	*Pisum sativum*	Messire

(6 × 6 × 10 cm) filled with a potting mixture (sand/peat, 1:3 vol/vol), then were grown in a growth chamber at 20 ± 2 °C and 65% relative humidity under a photoperiod with 14 h light/10 h dark at light intensity of 200 µmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes until the fifth leaf stage was achieved [70]. Leaves from each legume specie were excised and placed, adaxial side up, on 4% technical agar in Petri dishes. The three organic fungal extracts were dissolved in MeOH (5%) and then added to the assay concentration with distilled water of 0.5, 1 and 2 mg/mL. Similarly, bioassays performed with pure compounds **1-7** were arranged in Petri dishes as described above and assayed at 1, 10 and 100 µM concentrations. For each legume specie, fungal extract, compound, and concentration assayed, cut leaves were arranged in a randomized design with three replicates per treatment, each replicate having four leaves.

4.7. Data analysis

A completely randomized design was used in all detached leaves essays. The presence of symptoms through the appearance of dark spots or discoloration of the plant tissue was monitored introducing a method of image acquisition by an android smartphone. The smartphone was equipped with CMOS image sensor and SMD LED background light illumination to provide a constant brightness for all the images captured and reduce the effect of ambient lighting condition. Samsung galaxy J2 smartphone (Samsung Engineering Co., Ltd, Seoul, Korea) was used to acquire 2 images per detached leaves and per plate to be analyzed. All the images collected were in RGB color space. Damage area (mm^2) was measured on the smartphone captured images, with the help of ImageJ (1.46 r) program (free license). The significance of the differences in leaf damage between plant species, treatments and concentrations was estimated by one-way analysis of variance (ANOVA). All statistical analyses were performed using the Statistix 9.0

package (Analytical Software, Tallahase, FL, USA). Significance of differences between means was determined by calculating least significant difference (LSD).

1D and 2D NRM data were analysed and interpreted by MNova software v. 14 (MestreLab Research S.L, Santiago de Compostela, Spain).

Supplementary Materials: Supplementary Figure 1. ^{1}H-NMR spectrum of ascochlorin (**1**) ($CDCl_3$, 400 MHz); **Supplementary Figure 2**. ESI/MS (+) spectrum of ascochlorin (**1**); **Supplementary Figure 3**. ^{1}H-NMR spectrum of ascofuranol (**2**) ($CDCl_3$, 400 MHz); **Supplementary Figure 4**. ESI/MS (+) spectrum of ascofuranol (**2**); **Supplementary Figure 5**. ^{1}H-NMR spectrum of (*R*)-mevalonolactone (**3**) ($CDCl_3$, 400 MHz); **Supplementary Figure 6**. ESI/MS (+) spectrum of (*R*)-mevalonolactone (**3**); **Supplementary Figure 7**. ^{1}H-NMR spectrum of ascosalipyrone (**4**) ($CDCl_3$, 400 MHz); **Supplementary Figure 8**. ESI/MS (+) spectrum of ascosalipyrone (**4**); **Supplementary Figure 9**. ^{1}H-NMR spectrum of benzoic acid (**5**) ($CDCl_3$, 400 MHz); **Supplementary Figure 10**. ESI/MS (+) spectrum of benzoic acid (**5**); **Supplementary Figure 11**. ^{1}H-NMR spectrum of tyrosol (**6**) ($CDCl_3$, 400 MHz); **Supplementary Figure 12**. ESI/MS (+) spectrum of tyrosol (**6**); **Supplementary Figure 13**. ^{1}H-NMR spectrum of ascosalitoxin (**7**) ($CDCl_3$, 400 MHz); **Supplementary Figure 14**. ESI/MS (+) spectrum of ascosalitoxin (**7**); **Supplementary Figure 15**. Images of the symptoms of each of the compounds. **Supplementary Table 1**. Diseased area (mm^2) measured on detached leaves of several legume crops with exudates from the fungus *A. fabae* growth in vitro on 3 different culture media (Czapek-Dox = CD, Potato Dextrose Broth = PDB and Rice) at concentrations of 0.5, 1 and 2 mg/mL. Negative (blank untreated, water and MeOH 5%) controls were also included. The experiment was repeated four times. **Supplementary Table 2.** Diseased area (mm^2) measured in leaves detached from various legume crops with metabolites produced by the exudate of the *A. fabae* fungus from the three-growth media at concentrations of 1, 10 and 100µM. Negative controls (untreated blank, water and MeOH 5%) were also included. *P*-value compared with value from MeOH 5% control.

Data Availability Statement: All the data that arose from this research are included in the manuscript and in the supplementary material.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rubiales, D.; Annicchiarico, P.; Vaz Patto, M.C.; Julier, B. Legume breeding for the agroecological transition of global agri-food systems: A European perspective. *Frontiers in Plant Science* **2021**, *12*, 782574.
2. Ferreira, H.; Pinto, E.; Vasconcelos, M.W. Legumes as a cornerstone of the transition toward more sustainable agri-food systems and diets in Europe. *Front. Sus. Food Sys.* **2021**, 5, 694121.
3. Rubiales, D.; Duc, G.; Stoddard, F. Faba beans in sustainable agriculture. *Field Crops Research* **2010**, *115*, 201-33.
4. Gharzeddin, K.; Maalouf, F.; Khoury, B.; Abou Khater, L.; Christmann, S.; Jamal El Dine, N.A. Efficiency of different breeding strategies in improving the faba bean productivity for sustainable agriculture. Euphytica, 2019, 215, 1-15.
5. Yitayih, G.; Fininsa, C.; Terefe, H.; Shibabaw, A. Integrated management approaches reduced yield loss, and increased productivity in faba bean, due to gall disease in northwestern Ethiopia. *Arc. Phytopathol. Plant Prot.* **2022**, 55, 1592-1610.
6. Karkanis, A.; Ntatsi, G.; Lepse, L.; Fernández, J. A.; Vågen, I. M.; Rewald, B.; Alsina, I.; Kronberga, A.; Balliu, A.; Olle, M.; Bodner, G.; Dubova, L.; Rosa, E.; Savvas, D. Faba bean cultivation-revealing novel managing practices for more sustainable and competitive European cropping systems. *Front. Plant Sci.***2018**, 9, 1115.
7. Tivoli, B.; Banniza, S. Comparison of the epidemiology of ascochyta blights on grain legumes; Springer: 2007.
8. Hernandez-Bello, M.; Chilvers, M.; Akamatsu, H.; Peever, T. Host specificity of *Ascochyta* spp. infecting legumes of the *Viciae* and *Cicerae* tribes and pathogenicity of an interspecific hybrid. *Phytopathology* **2006**, *96*, 1148-1156.
9. Zhang, C.; Chen, W.; Sankaran, S. High-throughput field phenotyping of Ascochyta blight disease severity in chickpea. Crop Prot. **2019**, 125, 104885.
10. Bretag, T. W.; Keane, P.J.; Price, T.V. The epidemiology and control of ascochyta blight in field peas: a review. *Aus. J. Agr. Res*. **2006**, 57, 883-902.
11. Barilli, E.; Cobos, M.J.; Rubiales, D. Clarification on host range of *Didymella pinodes* the causal agent of pea Ascochyta blight. *Frontiers in Plant Science* **2016**, *7*, 592.

12. Ahmed, S.; Abang, M.; Maalouf, F. Integrated management of Ascochyta blight (*Didymella fabae*) on faba bean under Mediterranean conditions. *Crop Protection* **2016**, *81*, 65-69.
13. Koder, S.B.; Nawale, R.; Katyayani, K.K.S.; Rana, M.; Srivastava, S. Symptoms, biology and management of ascochyta blight (Phoma exigua) of French beans: A review. *Agr. Sci. Digest-A Res. J.* **2022**, 42, 657-664.
14. Trapero-Casas, A.; Kaiser, W.J. Influence of temperature, wetness period, plant age, and inoculum concentration on infection and development of Ascochyta blight of chickpea. *Phytopathol.* **1992**, 82, 589-596.
15. Rubiales, D.; Fondevilla, S.; Chen, W.; Gentzbittel, L.; Higgins, T.J.; Castillejo, M.A.; Singh, K.B.; Rispail, N. Achievements and challenges in legume breeding for pest and disease resistance. *Critical Reviews in Plant Sciences* **2015**, *34*, 195-236.
16. Pratap, A.; Douglas, C.; Prajapati, U.; Kumari, G.; War, A.R.; Tomar, R.; Pandey, A.K.; Dubey, S. Breeding progress and future challenges: biotic stresses. The mungbean genome. Compendium of Plant Genomes. Springer, Cham. **2020**, pp. 55-80.
17. Rubiales, D.; Ávila, C.M.; Sillero, J.C.; Hybl, M.; Narits, L.; Sass, O.; Flores, F. Identification and multi-environment validation of resistance to *Ascochyta fabae* in faba bean (*Vicia faba*). *Field Crops Research* **2012**, *126*, 165-170.
18. Mengiste, T. Plant immunity to necrotrophs. *Annual Review of Phytopathology* **2012**, *50*, 267-294.
19. Kim, W.; Chen, W. Phytotoxic metabolites produced by legume-associated *Ascochyta* and its related genera in the Dothideomycetes. *Toxins* **2019**, *11*, 627.
20. Oku, H.; Nakanishi, T. A toxic metabolite from *Ascochyta fabae* having antibiotic activity. *Phytopathology* **1963**, *53*, 1321-1325.
21. Kim, W.; Lichtenzveig, J.; Syme, R.A.; Williams, A.H.; Peever, T.L.; Chen, W. Identification of a polyketide synthase gene responsible for Ascochitine biosynthesis in *Ascochyta fabae* and its abrogation in sister taxa. *Msphere* **2019**, *4*, e00622-00619.
22. Agudo-Jurado, F.J.; Reveglia, P.; Rubiales, D.; Evidente, A.; Barilli, E. Status of Phytotoxins Isolated from Necrotrophic Fungi Causing Diseases on Grain Legumes. *International Journal of Molecular Sciences* **2023**, *24*, 5116.

23. Evidente, A.; Capasso, R.; Vurro, M.; Bottalico, A. Ascosalitoxin, a phytotoxic trisubstituted salicylic aldehyde from *Ascochyta pisi*. *Phytochemistry* **1993**, *34*, 995-998
24. Beed, F.; Strange, R.; Onfroy, C.; Tivoli, B. Virulence for faba bean and production of ascochitine by *Ascochyta fabae*. *Plant pathology* **1994**, *43*, 987-997.
25. Pan, R.; Bai, X.; Chen, J.; Zhang, H.; Wang, H. Exploring structural diversity of microbe secondary metabolites using OSMAC strategy: A literature review. *Frontiers in Microbiology* **2019**, *10*, 294.
26. Farinella, V.F.; Kawafune, E.S.; Tangerina, M.M.; Domingos, H.V.; Costa-Lotufo, L.V.; Ferreira, M.J. OSMAC strategy integrated with molecular networking for accessing griseofulvin derivatives from endophytic fungi of *Moquiniastrum polymorphum* (Asteraceae). *Molecules* **2021**, *26*, 7316.
27. Tamura, G.; Suzuki, S.; Takatsuki, A.; Ando, K.; Arima, K. Ascochlorin, a new antibiotic, found by paper-disc agar-diffusion method. I isolation, biological and chemical properties of ascochlorin (studies on antiviral and antitumor antibiotics. I). *The Journal of Antibiotics* **1968**, *21*, 539-544.
28. Seephonkai, P.; Isaka, M.; Kittakoop, P.; Boonudomlap, U.; Thebtaranonth, Y. A novel ascochlorin glycoside from the insect pathogenic fungus *Verticillium hemipterigenum* BCC 2370. *The Journal of Antibiotics* **2004**, *57*, 10-16.
29. Mogi, T.; Miyoshi, H. Properties of cytochrome bd plastoquinol oxidase from the cyanobacterium *Synechocystis* sp. PCC 6803. *Journal of Biochemistry* **2009**, *145*, 395-401.
30. Berry, E.A.; Huang, L.S.; Lee, D.W.; Daldal, F.; Nagai, K.; Minagawa, N. Ascochlorin is a novel, specific inhibitor of the mitochondrial cytochrome bc1 complex. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* **2010**, *1797*, 360-370.
31. Jeong, J.H.; Jeong, Y.J.; Cho, H.J.; Shin, J.M.; Kang, J.H.; Park, K.K.; Park, Y.Y.; Chung, I.K.; Chang, H.W.; Magae, J. Ascochlorin inhibits growth factor-induced HIF-1α activation and tumor-angiogenesis through the suppression of EGFR/ERK/p70S6K signaling pathway in human cervical carcinoma cells. *Journal of Cellular Biochemistry* **2012**, *113*, 1302-1313.
32. Sasaki, H.; Hosokawa, T.; Sawada, M.; Ando, K. Isolation and structure of ascofuranone and ascofranol, antibiotics with hypolipidemic activity. *The Journal of Antibiotics* **1973**, *26*, 676-680.

33. Hamid, K. Separation and phytotoxicity of solanapyrone compounds produced by *Ascochyta rabiei* (Pass.) Labr. and their metabolism by chickpea *(Cicer arietinum* L.*)*; University of London, University College London (United Kingdom): 1999.
34. Leyte-Lugo, M.; González-Andrade, M.; González, M.a.d.C.; Glenn, A.E.; Cerda-García-Rojas, C.M.; Mata, R. (+)-Ascosalitoxin and vermelhotin, a calmodulin inhibitor, from an endophytic fungus isolated from *Hintonia latiflora*. *Journal of Natural Products* **2012**, *75*, 1571-1577.
35. Yu, C.; Nian, Y.; Chen, H.; Liang, S.; Sun, M.; Pei, Y.; Wang, H. Pyranone Derivatives with antitumor activities, from the endophytic fungus *Phoma* sp. YN02-P-3. *Frontiers in Chemistry* **2022**, *10*, 950726.
36. Osterhage, C.; Kaminsky, R.; König, G.M.; Wright, A.D. Ascosalipyrrolidinone a, an antimicrobial alkaloid, from the obligate marine fungus *Ascochyta salicorniae*. *The Journal of Organic Chemistry* **2000**, *65*, 6412-6417.
37. Seibert, S.F.; Eguereva, E.; Krick, A.; Kehraus, S.; Voloshina, E.; Raabe, G.; Fleischhauer, J.; Leistner, E.; Wiese, M.; Prinz, H. Polyketides from the marine-derived fungus *Ascochyta salicorniae* and their potential to inhibit protein phosphatases. *Organic & Biomolecular Chemistry* **2006**, *4*, 2233-2240.
38. Salvatore, M.M.; Alves, A.; Andolfi, A. Secondary metabolites of *Lasiodiplodia theobromae*: Distribution, chemical diversity, bioactivity, and implications of their occurrence. *Toxins* **2020**, *12*, 457.
39. Chen, H.; Fujita, M.; Feng, Q.; Clardy, J.; Fink, G.R. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proceedings of the National Academy of Sciences* **2004**, *101*, 5048-5052.
40. Muriana, F.J.; Montserrat-de la Paz, S.; Lucas, R.; Bermudez, B.; Jaramillo, S.; Morales, J.C.; Abia, R.; Lopez, S. Tyrosol and its metabolites as antioxidative and anti-inflammatory molecules in human endothelial cells. *Food & Function* **2017**, *8*, 2905-2914.
41. Loru, D.; Incani, A.; Deiana, M.; Corona, G.; Atzeri, A.; Melis, M.; Rosa, A.; Dessì, M. Protective effect of hydroxytyrosol and tyrosol against oxidative stress in kidney cells. *Toxicology and Industrial Health* **2009**, *25*, 301-310.
42. Karković Marković, A.; Torić, J.; Barbarić, M.; Jakobušić Brala, C. Hydroxytyrosol, tyrosol and derivatives and their potential effects on human health. *Molecules* **2019**, *24*, 2001.

43. Bu, Y.; Rho, S.; Kim, J.; Kim, M.Y.; Lee, D.H.; Kim, S.Y.; Choi, H.; Kim, H. Neuroprotective effect of tyrosol on transient focal cerebral ischemia in rats. *Neuroscience letters* **2007**, *414*, 218-221.
44. Ohtsuki, T.; Sato, K.; Sugimoto, N.; Akiyama, H.; Kawamura, Y. Absolute quantification for benzoic acid in processed foods using quantitative proton nuclear magnetic resonance spectroscopy. *Talanta* **2012**, *99*, 342-348.
45. Shibata, M.; UYEDA, M.; KIDO, Y.; TOYA, N.; NAKASHIMA, R.; TERAZUMI, R. A new antibiotic K-82 A and minor components, produced by *Streptomyces lavendulae*, strain no. K-82. *The Journal of Antibiotics* **1980**, *33*, 1231-1235.
46. Yang, W.; Guo, Y.; Li, Y.; Lv, J.; Dong, K.; Dong, Y. Benzoic acid phytotoxicity the structural resistance and photosynthetic physiology of Faba bean and promotes Fusarium wilt incidence. *SSRN Electronic Jounal* **2022**, https://doi.org/10.2139/ssrn.4000347.
47. Zhang, K.; Sun, P.; Khan, A.; Zhang, Y. Photochemistry of biochar during ageing process: Reactive oxygen species generation and benzoic acid degradation. *Science of the Total Environment* **2021**, *765*, 144630.
48. Buhaescu, I.; Izzedine, H. Mevalonate pathway: a review of clinical and therapeutical implications. *Clinical Biochemistry* **2007**, *40*, 575-584.
49. Lasunción, M.A.; Martinez-Botas, J.; Martin-Sanchez, C.; Busto, R.; Gómez-Coronado, D. Cell cycle dependence on the mevalonate pathway: Role of cholesterol and non-sterol isoprenoids. *Biochem. Pharm*. **2022**, 196, 114623.
50. Masi, M.; Nocera, P.; Boari, A.; Zonno, M.C.; Pescitelli, G.; Sarrocco, S.; Baroncelli, R.; Vannacci, G.; Vurro, M.; Evidente, A. Secondary metabolites produced by *Colletotrichum lupini*, the causal agent of anthachnose of lupin (*Lupinus* spp.). *Mycologia* **2020**, *112*, 533-542.
51. Khamthong, N.; Rukachaisirikul, V.; Phongpaichit, S.; Preedanon, S.; Sakayaroj, J. An antibacterial cytochalasin derivative from the marine-derived fungus *Diaporthaceae* sp. PSU-SP2/4. *Phytochemistry Letters* **2014**, *10*, 5-9.
52. Hemtasin, C.; Kanokmedhakul, S.; Kanokmedhakul, K.; Hahnvajanawong, C.; Soytong, K.; Prabpai, S.; Kongsaeree, P. Cytotoxic pentacyclic and tetracyclic aromatic sesquiterpenes from *Phomopsis archeri*. *Journal of Natural Products* **2011**, *74*, 609-613.

53. Varejão, E.V.V.; Demuner, A.J.; Barbosa, L.C.d.A.; Barreto, R.W. Phytotoxic effects of metabolites from *Alternaria euphorbiicola* against its host plant *Euphorbia heterophylla*. *Química Nova* **2013**, *36*, 1004-1007.
54. Junio, H.A.; Sy-Cordero, A.A.; Ettefagh, K.A.; Burns, J.T.; Micko, K.T.; Graf, T.N.; Richter, S.J.; Cannon, R.E.; Oberlies, N.H.; Cech, N.B. Synergy-directed fractionation of botanical medicines: a case study with goldenseal (*Hydrastis canadensis*). *Journal of Natural Products* **2011**, *74*, 1621-1629.
55. Caesar, L.K.; Cech, N.B. Synergy and antagonism in natural product extracts: when 1+ 1 does not equal 2. *Natural Product Reports* **2019**, *36*, 869-888.
56. Gauglitz, J.M.; West, K.A.; Bittremieux, W.; Williams, C.L.; Weldon, K.C.; Panitchpakdi, M.; Di Ottavio, F.; Aceves, C.M.; Brown, E.; Sikora, N.C. Enhancing untargeted metabolomics using metadata-based source annotation. *Nature Biotechnology* **2022**, *40*, 1-6.
57. Li, Y.; Fiehn, O. Flash entropy search to query all mass spectral libraries in real time. *Nature Methods* **2023**, *20*, 1-4.
58. Burns, D.C.; Mazzola, E.P.; Reynolds, W.F. The role of computer-assisted structure elucidation (CASE) programs in the structure elucidation of complex natural products. *Natural Product Reports* **2019**, *36*, 919-933.
59. Marcarino, M.O.; Zanardi, M.M.; Cicetti, S.; Sarotti, A.M. NMR calculations with quantum methods: development of new tools for structural elucidation and beyond. *Acc. Chem. Res*. **2020**, 53, 1922-1932.
60. Beniddir, M.A.; Kang, K.B.; Genta-Jouve, G.; Huber, F.; Rogers, S.; Van Der Hooft, J.J. Advances in decomposing complex metabolite mixtures using substructure-and network-based computational metabolomics approaches. *Nat. Prod. Rep*. **2021**, 38, 1967-1993.
61. Mori, K.; Takechi, S. Synthesis of the natural enantiomers of ascochlorin, ascofuranone and ascofuranol. *Tetrahedron* **1985**, *41*, 3049-3062.
62. Menna, M.; Imperatore, C.; Mangoni, A.; Della Sala, G.; Taglialatela-Scafati, O. Challenges in the configuration assignment of natural products. A case-selective perspective. *Natural Product Reports* **2019**, *36*, 476-489.
63. Bitchagno, G.T.M.; Nchiozem-Ngnitedem, V.-A.; Melchert, D.; Fobofou, S.A. Demystifying racemic natural products in the homochiral world. *Nature Reviews Chemistry* **2022**, *6*, 806-822.

64. Pereda-Miranda, R.; Bautista, E.; Martínez-Fructuoso, L.; Fragoso-Serrano, M. From relative to absolute stereochemistry of secondary metabolites: applications in plant chemistry. *Revista Brasileira de Farmacognosia* **2023**, *33*, 1-48.
65. Lo Presti, L.; Lanver, D.; Schweizer, G.; Tanaka, S.; Liang, L.; Tollot, M.; Zuccaro, A.; Reissmann, S.; Kahmann, R. Fungal effectors and plant susceptibility. *Annual Review of Plant Biology* **2015**, *66*, 513-545.
66. Chen, H.; Singh, H.; Bhardwaj, N.; Bhardwaj, S. K.; Khatri, M.; Kim, K. H.; Peng, W. An exploration on the toxicity mechanisms of phytotoxins and their potential utilities. *Crit. Rev. Env. Sci. Tech*. **2022**, 52, 395-435.
67. Guo, J.; Cheng, Y. Advances in fungal elicitor-triggered plant immunity. *International Journal of Molecular Sciences* **2022**, *23*, 12003.
68. Sillero, J.C.; Rojas-Molina, M.M.; Ávila, C.M.; Rubiales, D. Induction of systemic acquired resistance against rust, ascochyta blight and broomrape in faba bean by exogenous application of salicylic acid and benzothiadiazole. *Crop Protection* **2012**, *34*, 65-69.
69. Gulder, T.A.; Hong, H.; Correa, J.; Egereva, E.; Wiese, J.; Imhoff, J. F.; Gross, H. Isolation, structure elucidation and total synthesis of lajollamide A from the marine fungus *Asteromyces cruciatus*. *Marine Drugs*. **2012** ,10, 2912-2935.
70. Reveglia, P.; Agudo-Jurado, F. J.; Barilli, E.; Masi, M.; Evidente, A.; Rubiales, D. Uncovering phytotoxic compounds produced by *Colletotrichum* spp. involved in legume diseases using an OSMAC–metabolomics approach. *Journal of Fungi* **2023**, *9*, 610.
71. Sasaki, H.; Hosokawa, T.; Nawata, Y.; Ando, K. Isolation and structure of ascochlorin and its analogs. *Agricultural and Biological Chemistry* **1974**, *38*, 1463-1466.
72. Kimura, Y.; Tamura, S. Isolation of L-β-phenyllactic acid and tyrosol as plant growth regulators from *Gloeosporium laeticolor*. *Agricultural and Biological Chemistry* **1973**, *37*, 2925-2925.
73. Sillero, J.; Fondevilla, S.; Davidson, J.; Patto, M.V.; Warkentin, T.; Thomas, J.; Rubiales, D. Screening techniques and sources of resistance to rusts and mildews in grain legumes. *Euphytica* **2006**, *147*, 255-272.

Supplementary Materials:

Comparative analysis of secondary metabolites produced by *Ascochyta fabae* under *in vitro* conditions and their phytotoxicity on the primary host, *Vicia faba*, and related legume crops

Supporting Information List

Supplementary Figure 1. ^{1}H NMR spectrum of ascochlorin (**1**) ($CDCl_3$, 400 MHz).

Supplementary Figure 2. ESI/MS (+) spectrum of ascochlorin (**1**).

Supplementary Figure 3. ^{1}H NMR spectrum of ascofuranol (**2**) ($CDCl_3$, 400 MHz).

Supplementary Figure 4. ESI/MS (+) spectrum of ascofuranol (**2**).

Supplementary Figure 5. ^{1}H NMR spectrum of (*R*)-mevalonolactone (**3**) ($CDCl_3$, 400 MHz).

Supplementary Figure 6. ESI/MS (+) spectrum of (*R*)-mevalonolactone (**3**).

Supplementary Figure 7. ^{1}H NMR spectrum of ascosalipyrone (**4**) ($CDCl_3$, 400 MHz).

Supplementary Figure 8. ESI/MS (+) spectrum of ascosalipyrone (**4**).

Supplementary Figure 9. ^{1}H NMR spectrum of benzoic acid (**5**) ($CDCl_3$, 400 MHz).

Supplementary Figure 10. ESI/MS (+) spectrum of benzoic acid (**5**).

Supplementary Figure 11. ^{1}H NMR spectrum of tyrosol (**6**) ($CDCl_3$, 400 MHz).

Supplementary Figure 12. ESI/MS (+) spectrum of tyrosol (**6**).

Supplementary Figure 13. ^{1}H NMR spectrum of ascosalitoxin (**7**) ($CDCl_3$, 400 MHz).

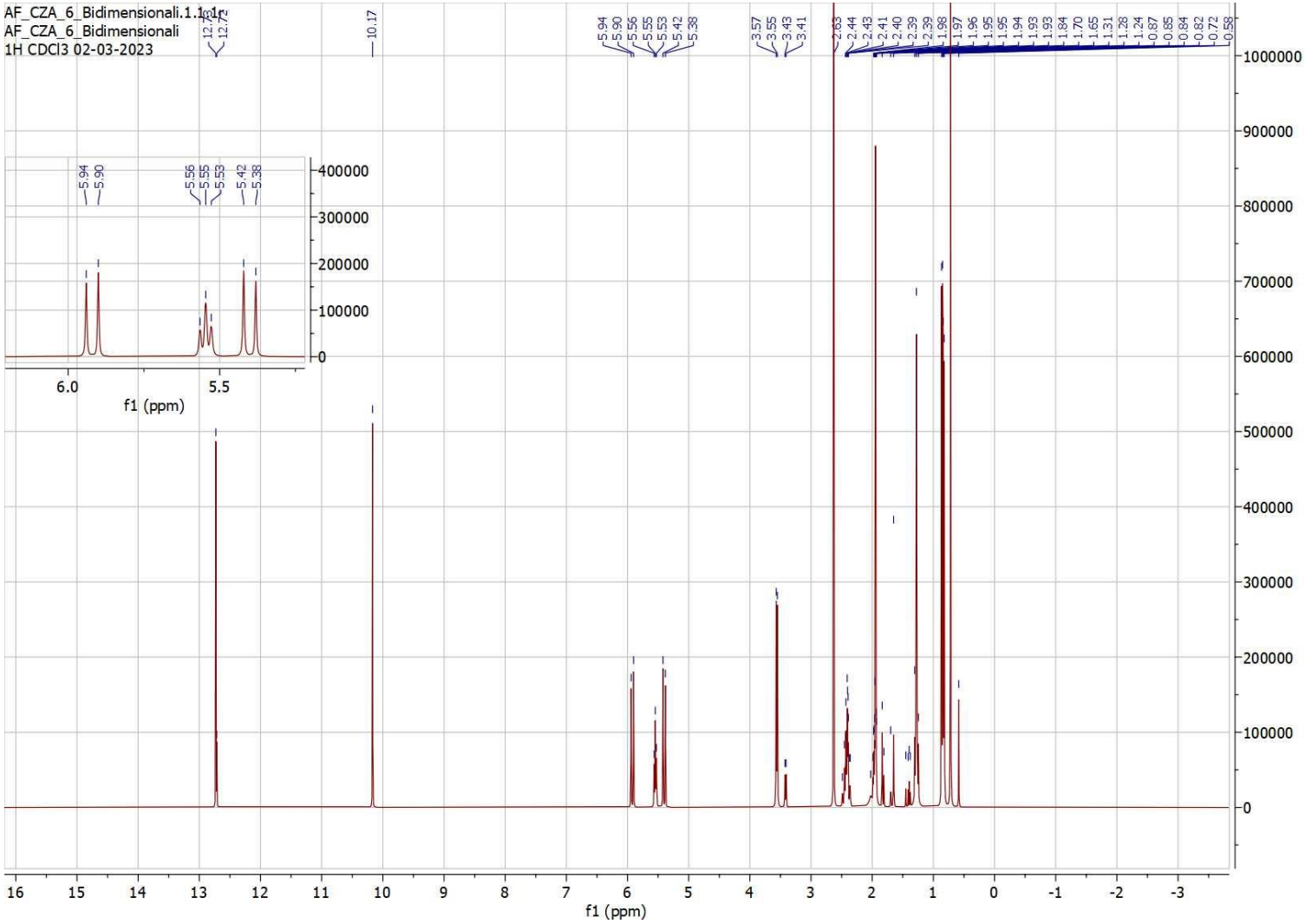
Supplementary Figure 14. ESI/MS (+) spectrum of ascosalitoxin (**7**).

Supplementary Figure 15. Symptoms developed on detached leaves of several legume hosts treated as follow: (a) uninoculated, (b) water, (c) methanol (MeOH 5%), (d) ascoclorin, (e) ascofuranol, (f) (*R*)-mevalonolactone, (g) ascosalipyrone, (h) benzoic acid, (i) tyrosol and (j) ascosalitoxin.

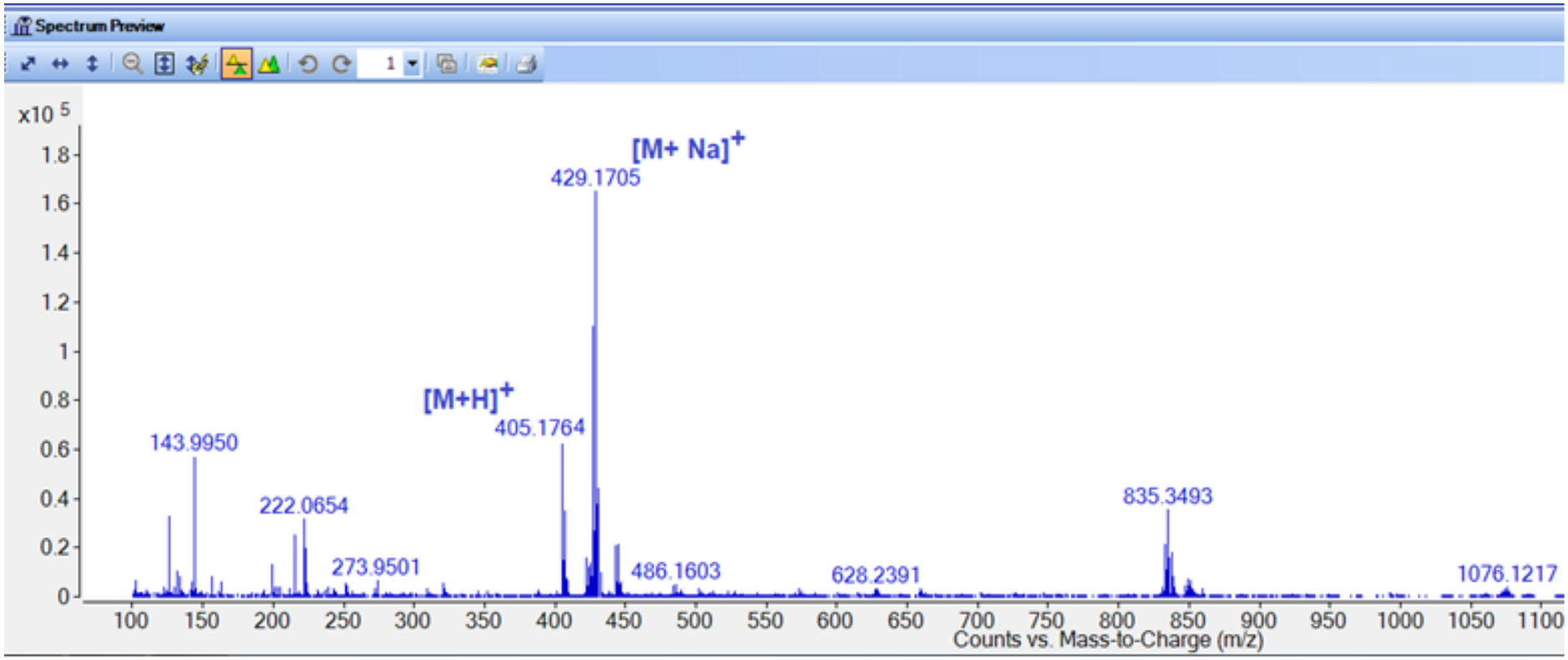
Supplementary Table 1. Diseased area (mm^2) measured on detached leaves of several legume crops with exudates from the fungus *Ascochyta fabae* growth *in vitro* on 3 different culture media (Czapek-Dox = CD, Potato Dextrose Broth = PDB and rice) at concentrations of 0.5, 1 and 2 mg/mL. Negative (blank untreated, water and MeOH 5%) controls were also included. The experiment was repeated four times.

Supplementary Table 2. Diseased area (mm^2) measured in leaves detached from various legume crops with metabolites produced by the exudate of the *Ascochyta fabae* fungus from the three-growth media at concentrations of 1, 10 and 100µM. Negative controls (untreated blank, water and MeOH 5%) were also included. *P*-value compared with value from MeOH 5% control.

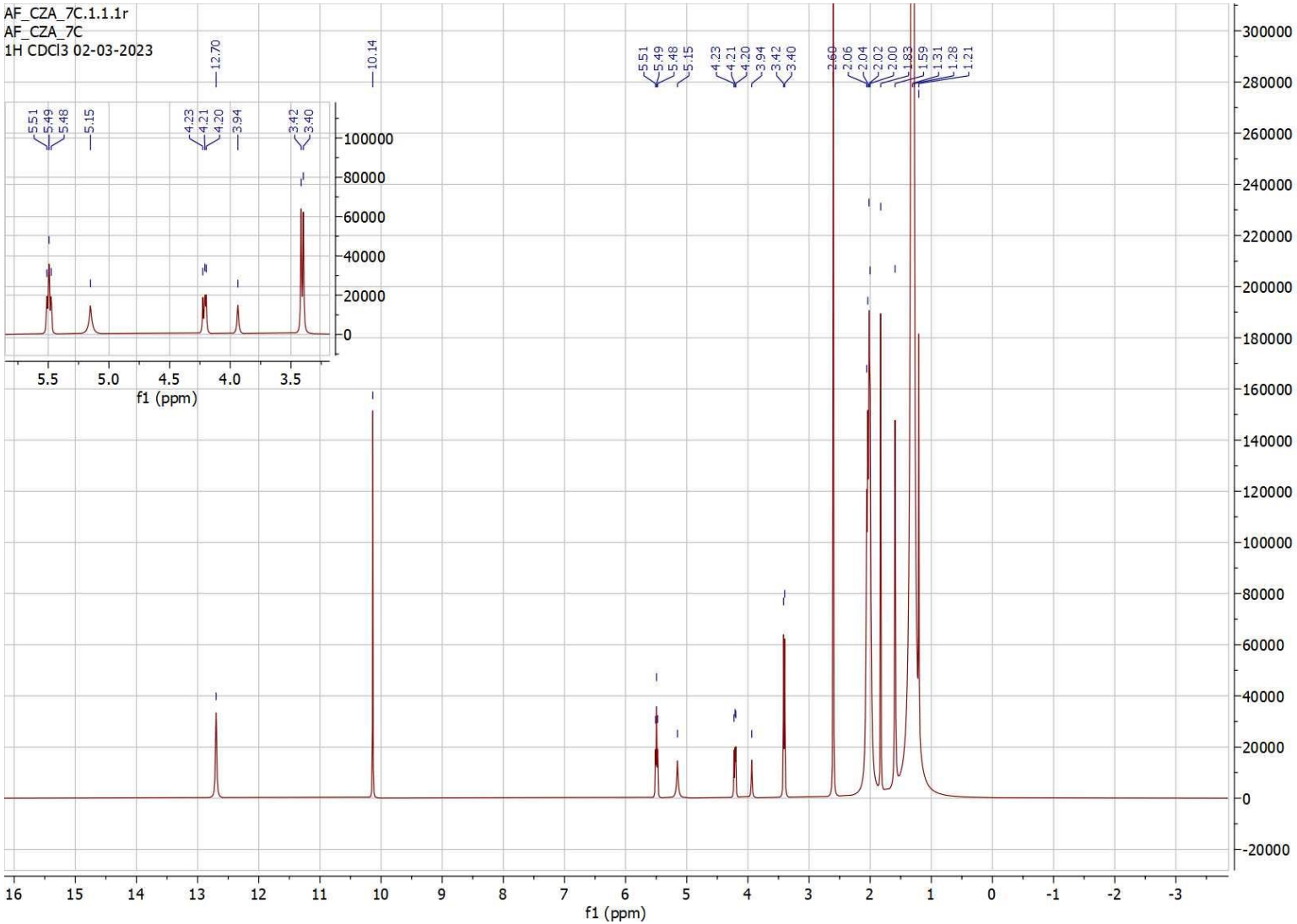
Supplementary Figure 1. ^{1}H NMR spectrum of ascochlorin (**1**) ($CDCl_3$, 400 MHz).



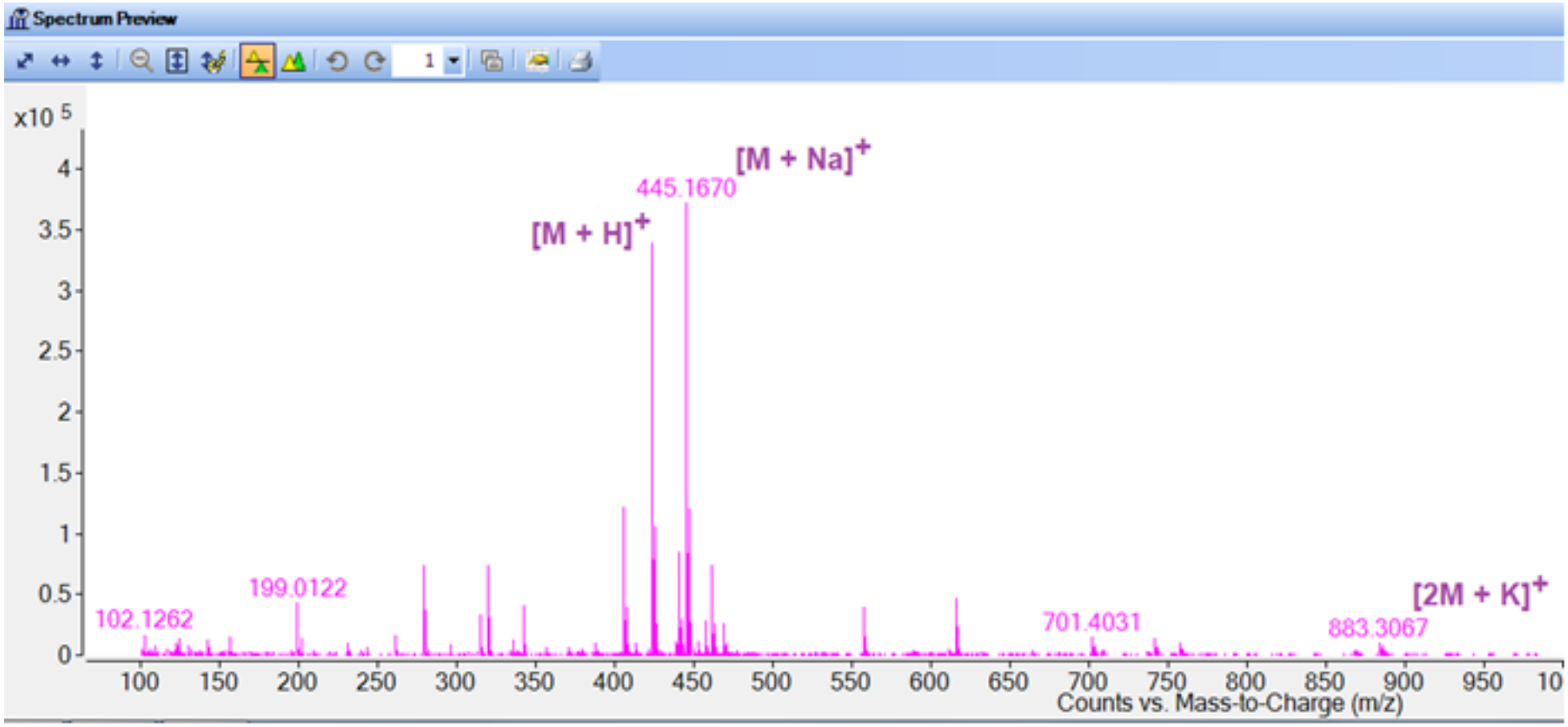
Supplementary Figure 2. ESI/MS (+) spectrum of ascochlorin (**1**).



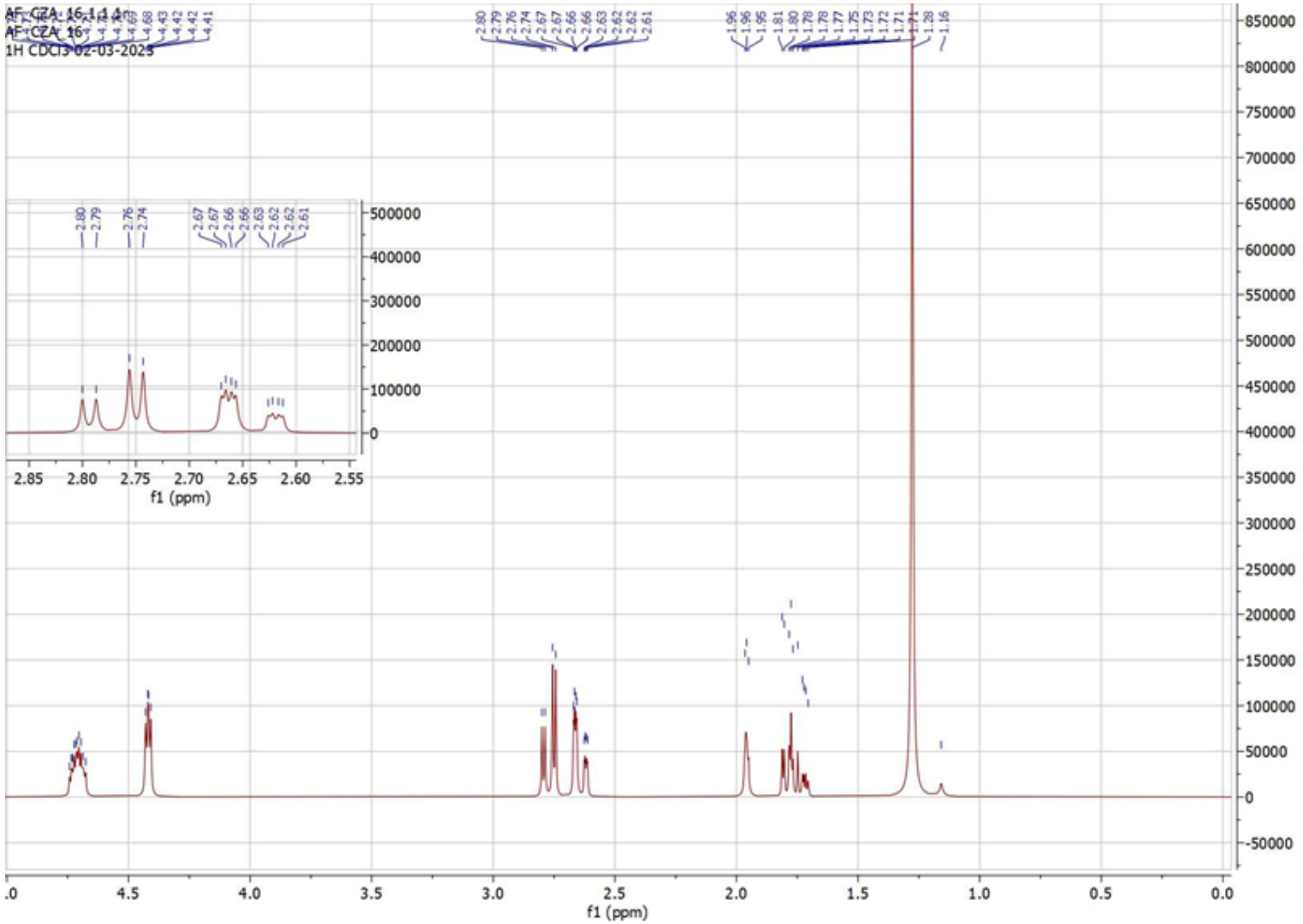
Supplementary Figure 3. ^{1}H NMR spectrum of ascofuranol (**2**) ($CDCl_3$, 400 MHz).



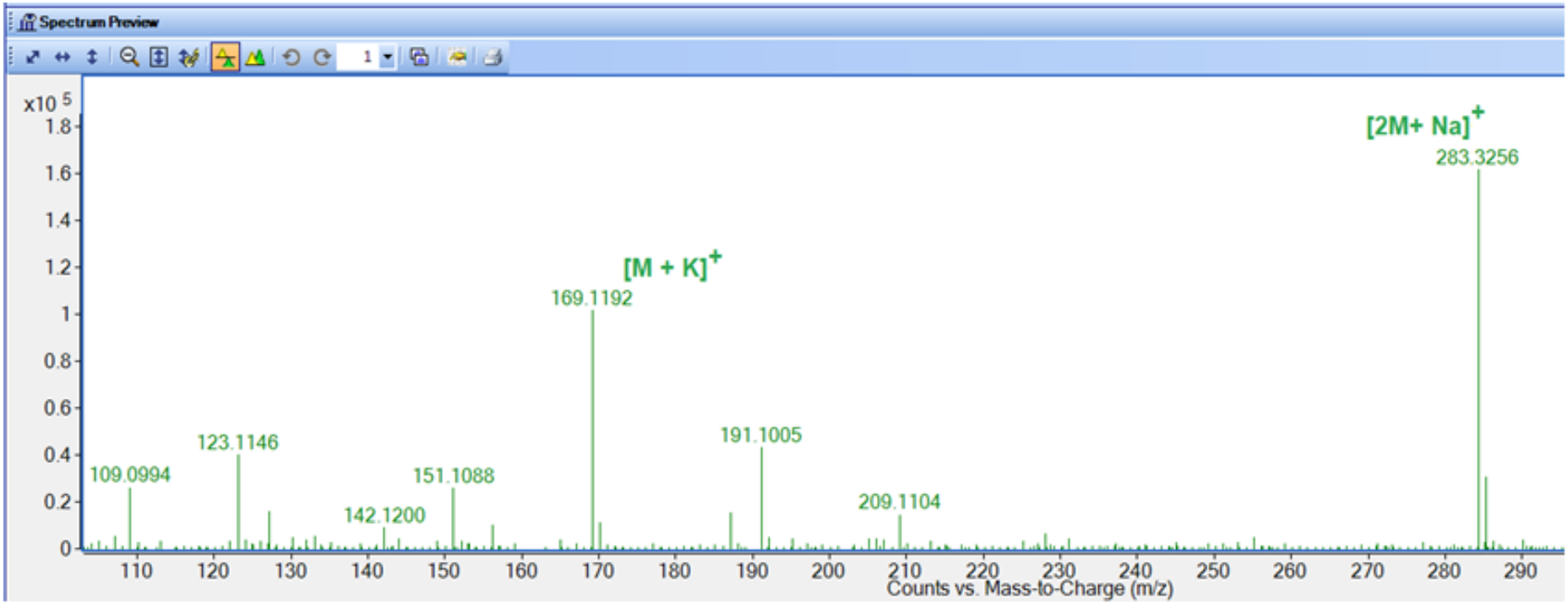
Supplementary Figure 4. ESI/MS (+) spectrum of ascofuranol (**2**).



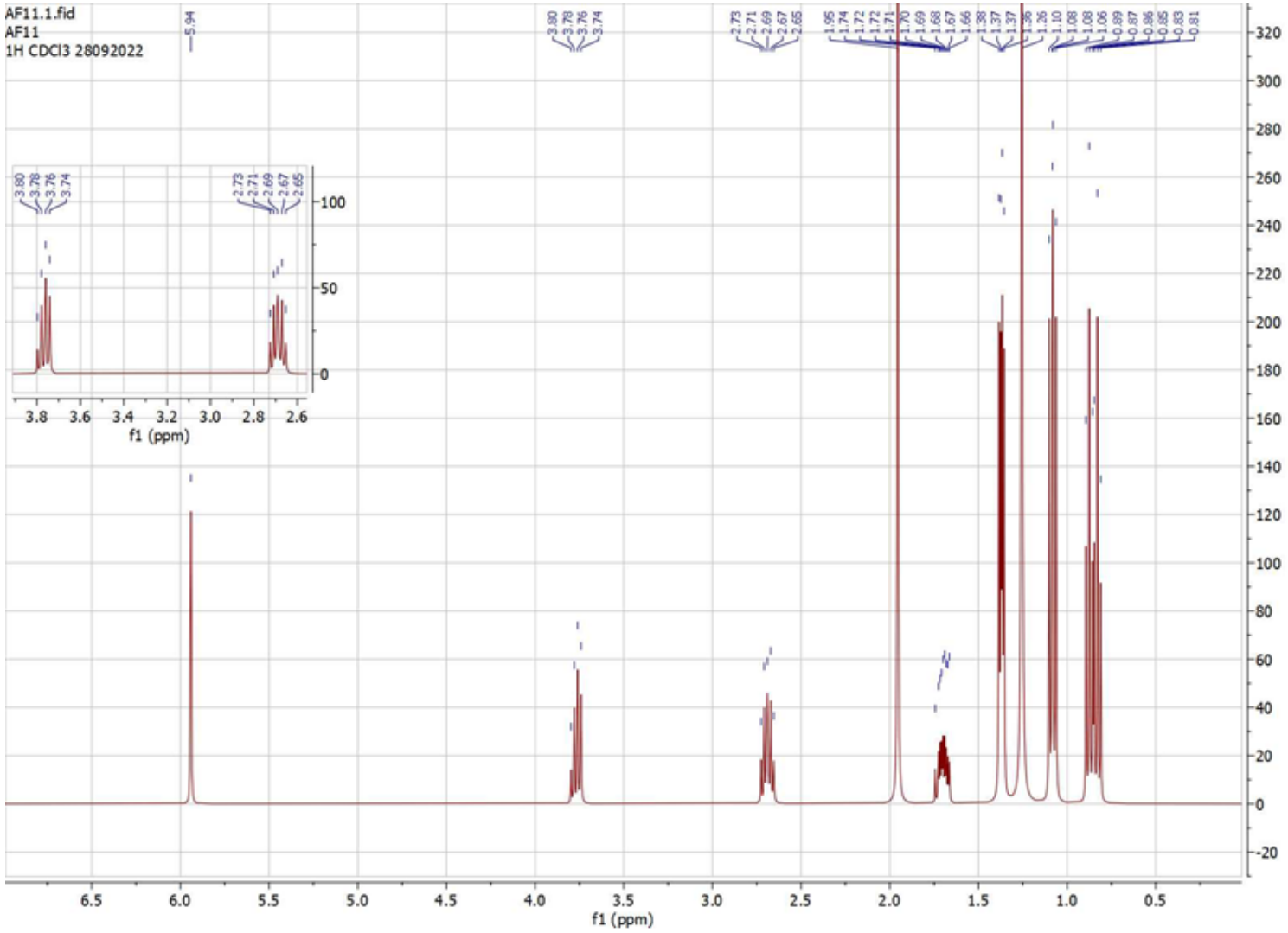
Supplementary Figure 5. ^{1}H NMR spectrum of (*R*)-mevalonolactone (**3**) ($CDCl_3$, 400 MHz).



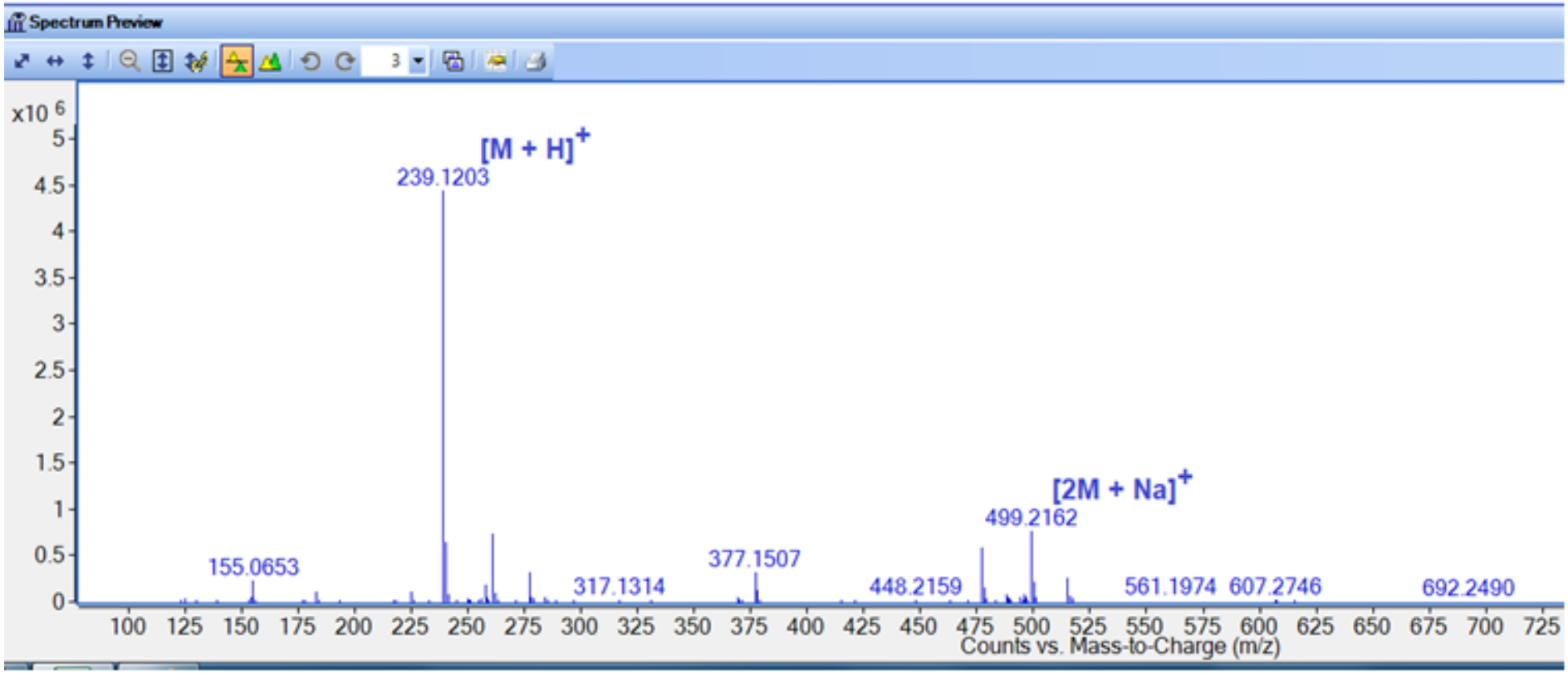
Supplementary Figure 6. ESI/MS (+) spectrum of (*R*)-mevalonolactone (**3**).



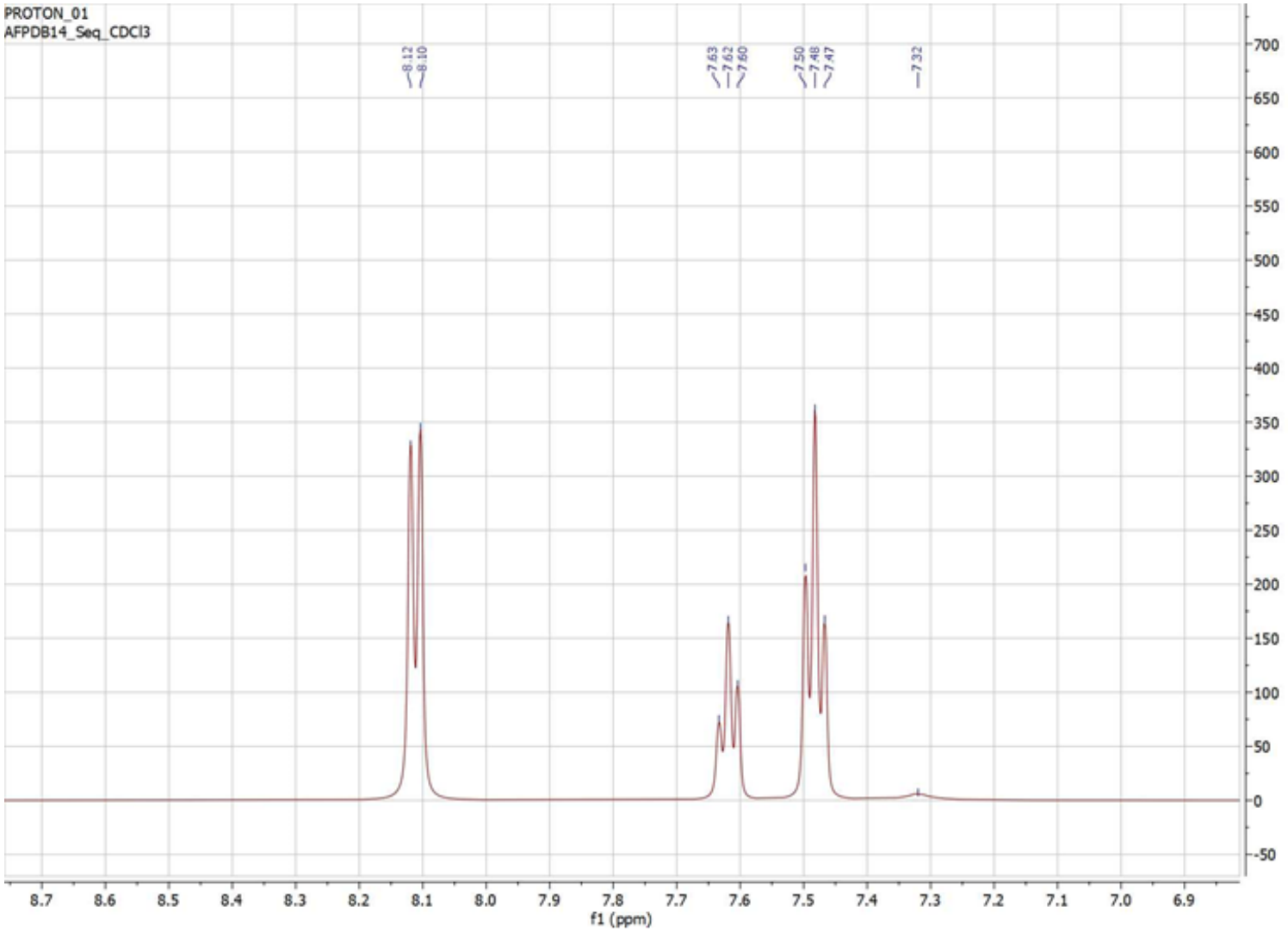
Supplementary Figure 7. ^{1}H NMR spectrum of ascosalipyrone (**4**) ($CDCl_3$, 400 MHz).



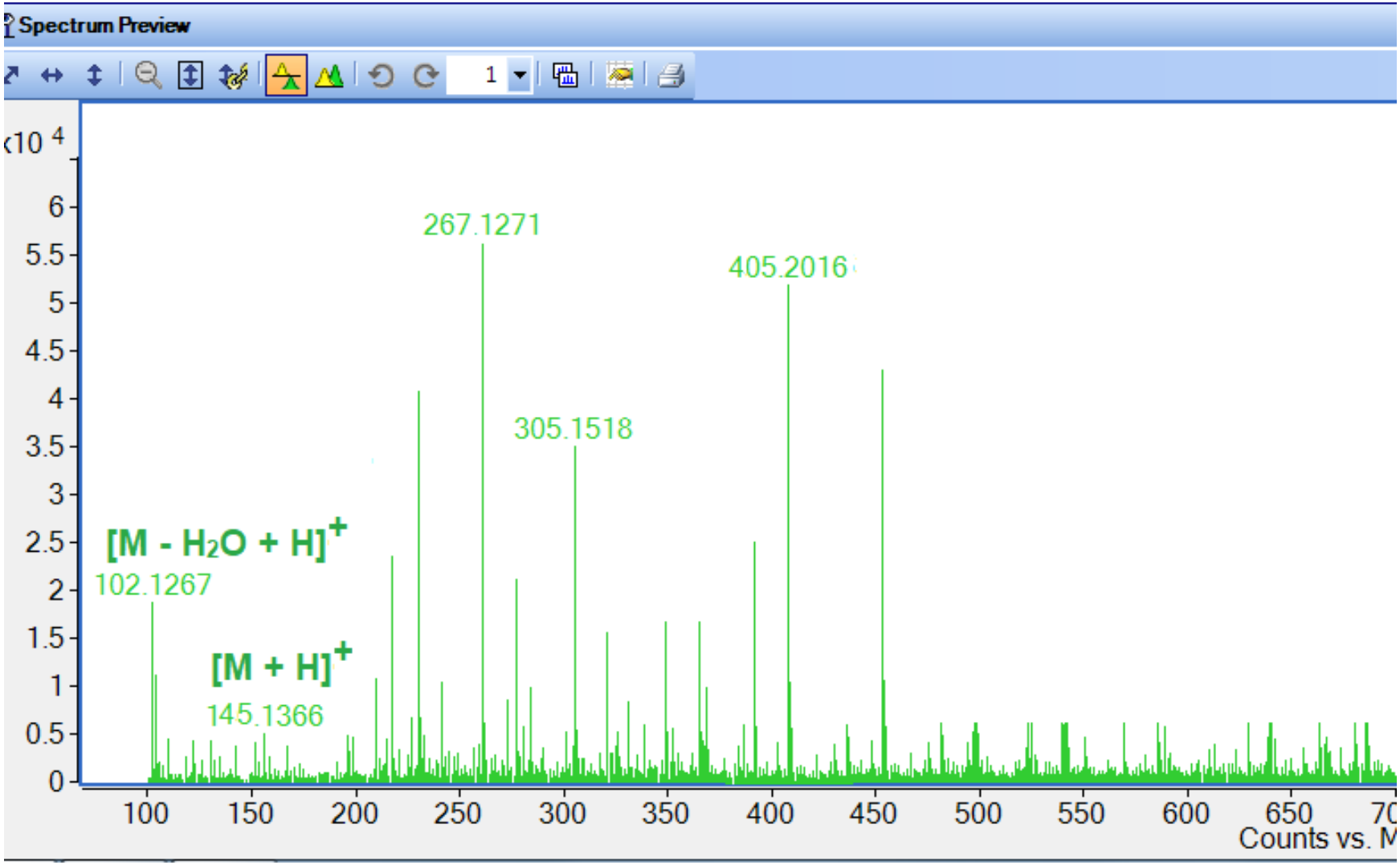
Supplementary Figure 8. ESI/MS (+) spectrum of ascosalipyrone (**4**).



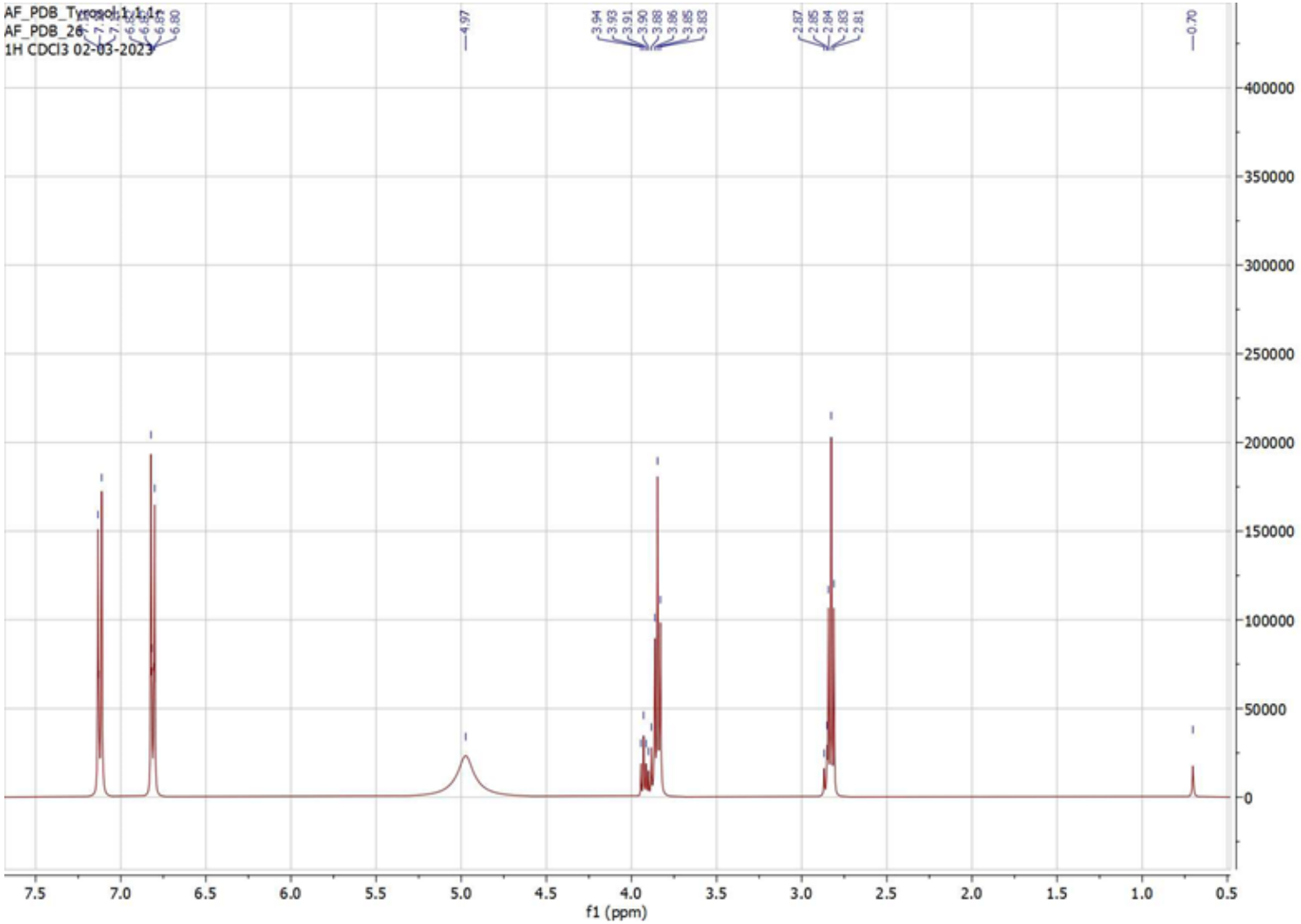
Supplementary Figure 9. ^{1}H NMR spectrum of benzoic acid (**5**) ($CDCl_3$, 400 MHz).



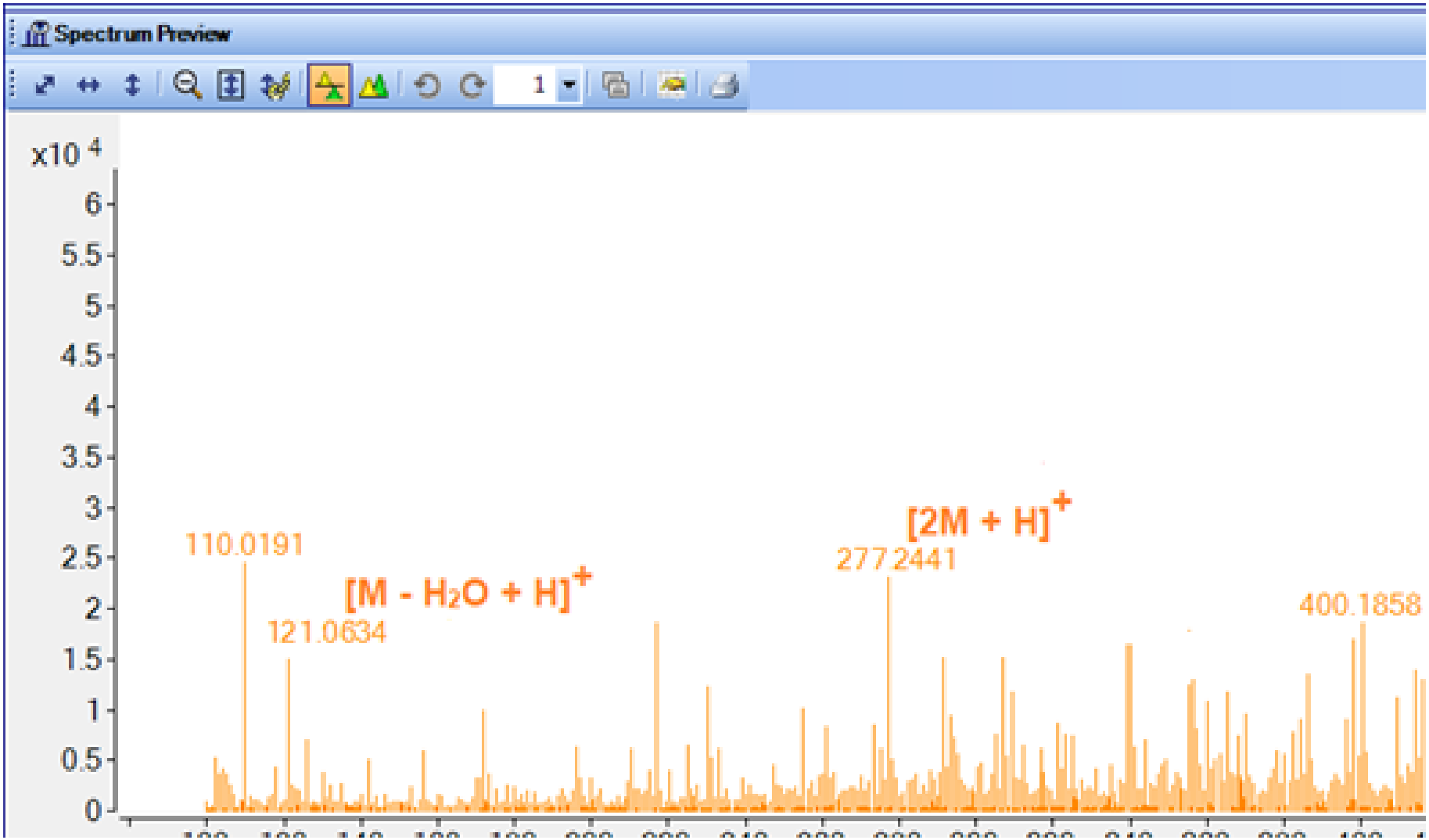
Supplementary Figure 10. ESI/MS (+) spectrum of benzoic acid (**5**).



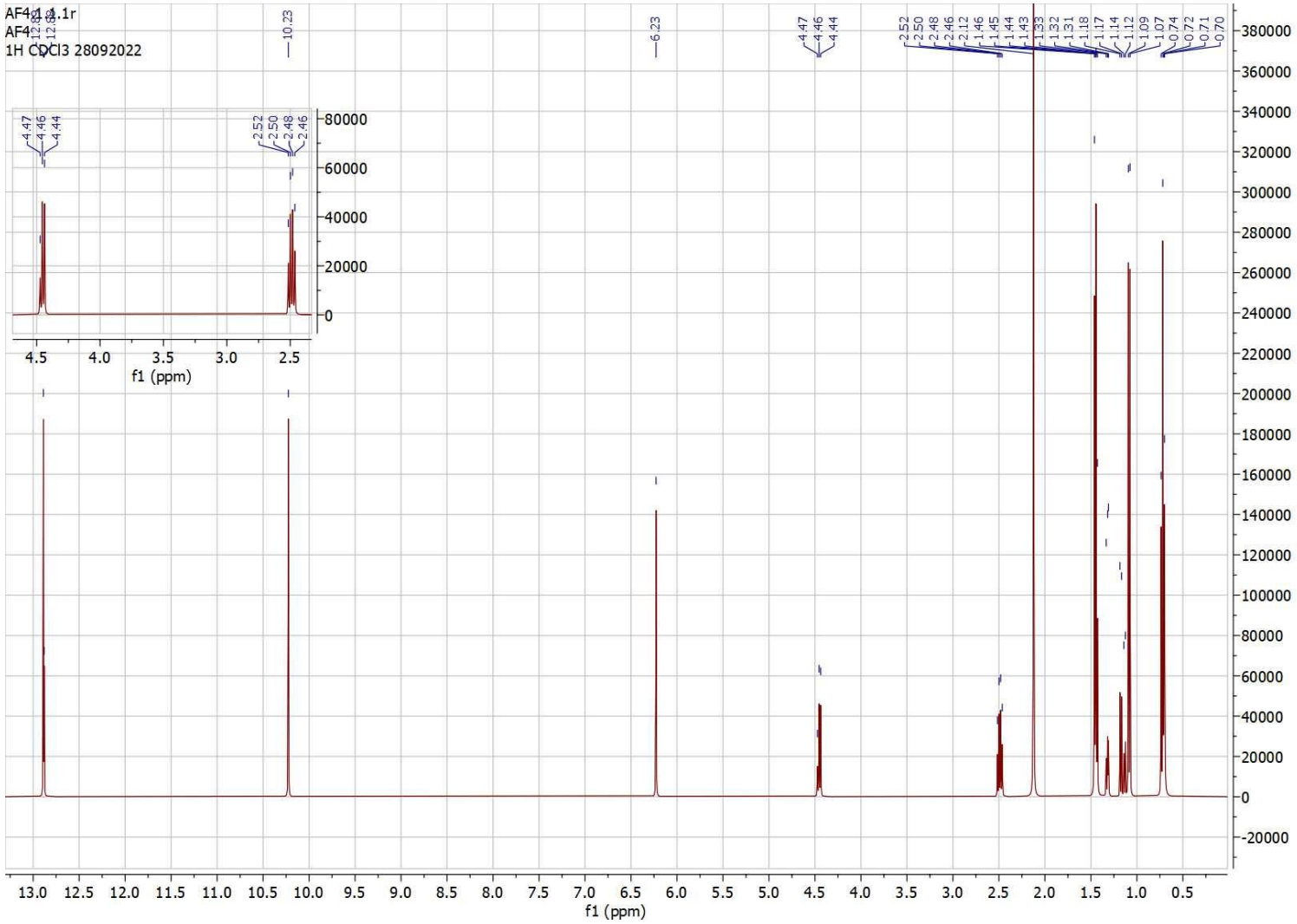
Supplementary Figure 11. ^{1}H NMR spectrum of tyrosol (**6**) ($CDCl_3$, 400 MHz).



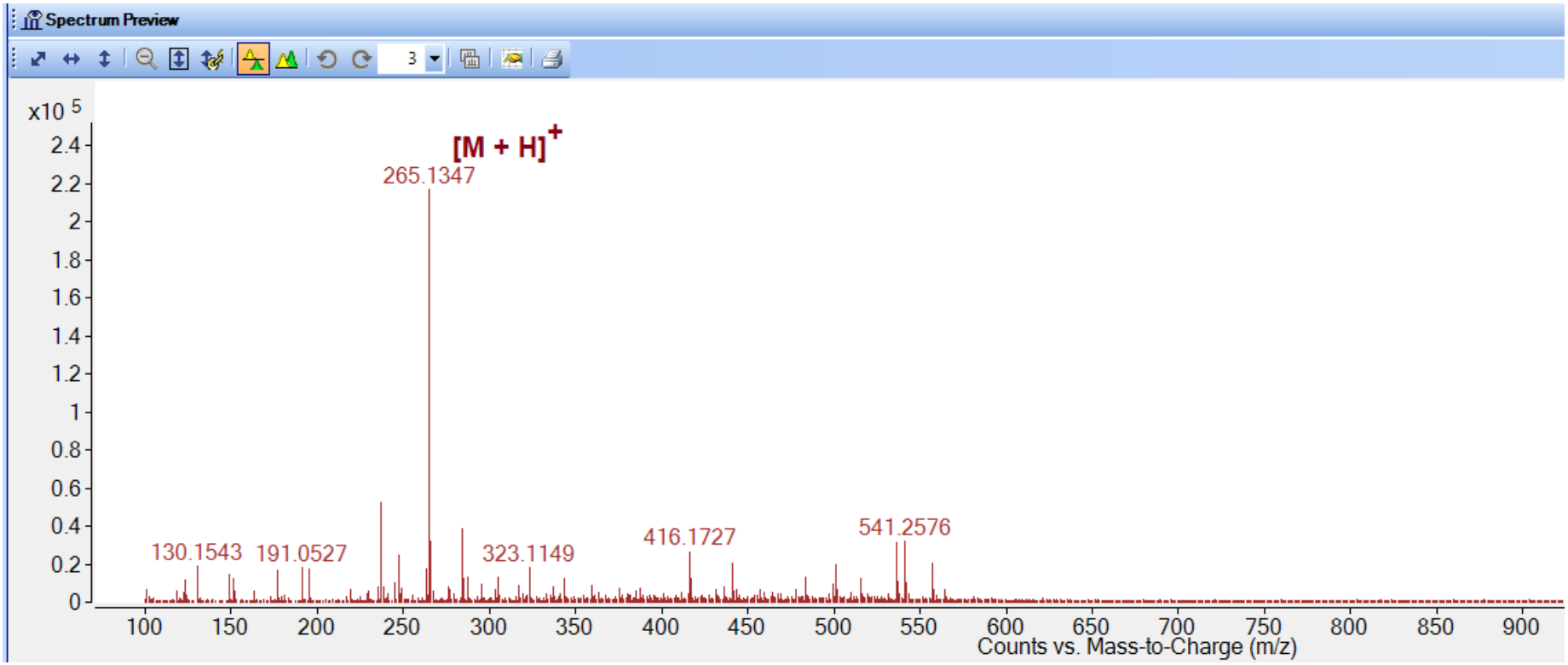
Supplementary Figure 12. ESI/MS (+) spectrum of tyrosol (**6**).



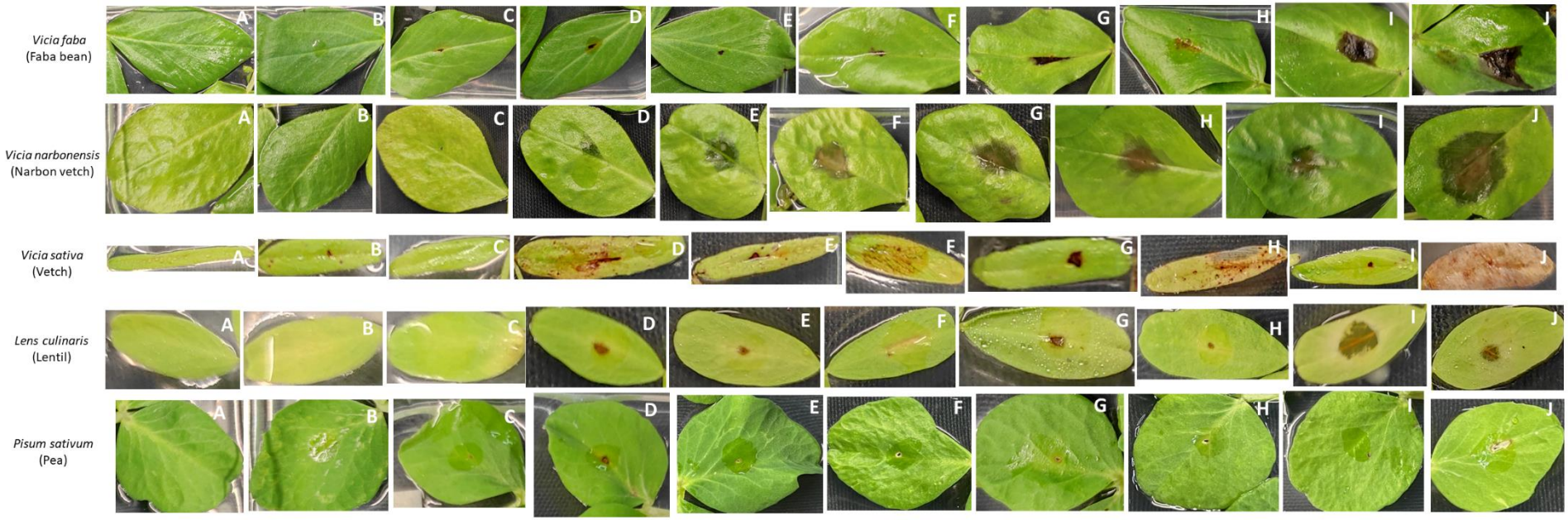
Supplementary Figure 13. ^{1}H NMR spectrum of ascosalitoxin (**7**) ($CDCl_3$, 400 MHz).



Supplementary Figure 14. ESI/MS (+) spectrum of ascosalitoxin (**7**).



Supplementary Figure 15. Symptoms developed on detached leaves of several legume hosts treated as follow: (a) uninoculated, (b) water, (c) methanol (MeOH 5%), (d) ascoclorin, (e) ascofuranol, (f) (R)-mevalonolactone, (g) ascosalipyrone, (h) benzoic acid, (i) tyrosol and (j) ascosalitoxin.



Supplementary Table 1. Diseased area (mm^2) measured on detached leaves of several legume crops with exudates from the fungus *Ascochyta fabae* growth in vitro on 3 different culture media (Czapek-Dox = CD, Potato Dextrose Broth = PDB and Rice) at concentrations of 0.5, 1 and 2 mg/mL. Negative (blank untreated, water and MeOH 5%) controls were also included. The experiment was repeated four times.

	Host plant									
Treatment	**Faba bean**		**Narbon vetch**		**Common vetch**		**Lentil**		**Pea**	
Concentration 0.5 mg/mL										
Blank	0.0±0.0	b	0.0±0.0	d	0.0±0.0	b	0.0±0.0	b	0.0±0.0	c
Water	0.0±0.0	b	2.9±1.6	d	0.2±0.1	b	0.0±0.0	b	0.3±0.2	c
MeOH	3.4±1.5	b	17.3±3.3	c	0.2±0.2	b	0.5±0.5	b	0.4±0.2	c
CD	45.2±11.1	a	65.3±17.1	a	25.6±4.7	a	16.1±8.5	a	16.1±3.6	a
PDB	63.8±29.1	a	53.5±8.9	a	16.8±9.2	a	0.0±0.0	b	9.9±6.5	b
Rice	61.7±16.3	a	36.9±4.7	b	3.9±3.4	b	0.0±0.0	b	7.8±2.6	b
Concentration 1 mg/mL										
Blank	0.0±0.0	c	0.0±0.0	d	0.0±0.0	b	0.0±0.0	b	0.0±0.0	c
Water	0.0±0.0	c	2.9±1.6	d	0.2±0.1	b	0.0±0.0	b	0.3±0.2	c
MeOH	3.4±1.5	c	17.3±3.3	c	0.2± 0.1	b	0.6± 0.5	b	0.4±0.2	c
CD	58.7±10.2	a	65.9±6.4	a	27.4±14.6	a	21.5±9.5	a	18.1±2.6	a
PDB	43.3±8.3	b	36.5±5.5	a	19.0±7.4	a	4.2±3.9	ab	8.1±4.4	b
Rice	82.3±24.5	a	47.6±19.3	b	30.1±10.4	a	16.9±7.5	a	12.3±4.4	b
Concentration 2 mg/mL										
Blank	0.0±0.0	b	0.0±0.0	b	0.0±0.0	c	0.0±0.0	c	0.0±0.0	b
Water	0.0±0.0	b	2.9±1.6	d	0.2±0.1	b	0.0±0.0	b	0.3±0.2	c
MeOH	3.4±1.5	c	17.3±3.3	c	0.2±0.2	b	0.5±0.4	b	0.4±0.3	c
CD	215.0±65.4	a	144.4±1.8	a	37.3±7.8	ab	20.6±7.8	a	80.7±57.4	a
PDB	157.0±25.33	a	43.9±7.9	b	44.2±12.9	a	1.6±0.8	bc	4.0±1.9	b
Rice	94.4±22.8	b	31.9±2.8	b	26.0±11.7	b	11.5±4.8	ab	9.2±3.4	b

Values, per column and treatment, followed by different letters differ significantly at $P < 0.01$.

Suplementary table 2. Diseased area (mm^2) measured in leaves detached from various legume crops with compounds produced by the exudate of the *Ascochyta fabae* fungus from the three-growth media at concentrations of 1, 10 and 100µM. Negative controls (untreated blank, water and MeOH 5%) were also included. *P*-value compared with value from MeOH 5% control.

Damage Area (mm^2)		***Vicia faba* (cv. Baraca)**		***Vicia narbonensis* (cv. Saoret)**		***Vicia sativa* (cv. Buzza)**		***Lens culinaris* (cv. Pardina)**		***Pisum sativum* (cv. Messire)**	
		Mean	p value	Mean	p value	Mean	p value	Mean	p value	Mean	p value
Blank		1.0±0.1	-	0.7±0.01	-	2.9±0.7	-	0.1±0.1	-	0.3±0.1	-
Water		5.0±0.9	-	3.6±0.8	-	2.6±0.5	-	0.9±0.2	-	1.3±0.2	-
MeOH		6.0±1.1	-	3.8±0.9	-	3.3±0.7	-	1.3±0.3	-	1.2±0.2	-
Ascochlorin (**1**)	1µM	12.1±2.9	0.011*	17.8±3.5	<0.001*	5.6±2.5	0.154	1.7±0.2	0.394	1.7±0.4	0.157
	10µM	12.7±4.2	0.004*	16.0±4.8	<0.001*	1.4±0.3	0.183	1.0±0.2	0.409	1.7±0.4	0.169
	100µM	14.0±1.9	<0.001*	22.9±4.0	<0.001*	2.0±0.8	0.35	1.3±0.1	0.871	1.9±0.3	0.067
Ascofuranol (**2**)	1µM	4.7±1.4	0.508	16.0±5.9	<0.001*	0.9±0.1	0.253	1.6±0.3	0.554	1.4±0.2	0.598
	10µM	8.5±2.5	0.179	18.2±2.1	<0.001*	5.8±3.4	0.213	1.8±0.4	0.295	1.5±0.4	0.456
	100µM	10.8±2.7	0.011*	41.9±7.4	<0.001*†	8.3±4.5	0.019*	2.3±0.7	0.055	3.0±1.2	<0.001*†
(*R*)-mevalonolactone (**3**)	1µM	11.7±3.6	0.25	8.0±2.5	0.221	0.8±0.1	0.066	1.8±0.4	0.387	1.6±0.2	0.176
	10µM	12.9±2.5	0.148	10.1±4.3	0.067	1.2±0.2	0.112	1.6±0.5	0.552	1.6±0.3	0.255
	100µM	20.6±11.5	0.003*	30.5±9.7	<0.001*†	2.4±0.9	0.491	1.7±0.5	0.457	1.5±0.4	0.245
Ascosalipyrone (**4**)	1µM	9.9±4.1	0.229	5.3±2.6	0.696	1.8±1.0	0.237	2.3±0.6	0.073	1.8±0.4	0.272
	10µM	12.7±4.6	0.063	7.4±2.2	0.328	2.4±1.0	0.48	2.2±0.4	0.104	2.0±0.5	0.175
	100µM	18.4±6.5	<0.001*	46.1±11.9	<0.001*†	2.4±1.1	0.498	2.5±0.8	0.067	4.2±1.5	<0.001*†
Benzoic acid (**5**)	1µM	7.2±2.2	0.712	25.5±9.8	<0.001*	1.7±0.8	0.658	2.3±0.7	0.193	1.4±0.2	0.608
	10µM	11.8±3.6	0.002*	24.4±9.2	<0.001*	5.6±3.0	0.498	2.0±0.7	0.370	1.5±0.3	0.501
	100µM	16.6±6.3	0.003*	27.3±6.6	<0.001*	16.5±9.6	<0.001*†	4.0±1.8	<0.001*†	3.1±1.3	<0.001*†
Tyrosol (**6**)	1µM	18.5± 4.1	0.008*	27.2±5.3	<0.001*	2.2±0.8	0.415	1.3±0.4	0.945	1.1±0.2	0.653
	10µM	17.7±6.3	0.016*	24.2±6.4	<0.001*	1.7±0.5	0.209	1.6±0.3	0.786	0.8±0.1	0.177
	100µM	20.5±8.8	<0.001*	31.9±5.8	<0.001*	3.3±1.6	0.977	7.3±3.2	<0.001*†	0.9±0.1	0.413
Ascosalitoxin (**7**)	1µM	9.9±2.3	0.187	39.1±4.8	<0.001*	5.0±3.2	0.597	0.9±0.4	0.375	1.6±0.3	0.293
	10µM	29.8±6.4	<0.001*	40.6±5.1	<0.001*	3.9±1.4	0.829	1.3±0.5	0.882	1.2±0.1	0.856
	100µM	26.2±5.7	<0.001*†	36.5±15.9	<0.001*	20.0±7.8	<0.001*†	1.7±0.3	0.438	3.7±0.7	<0.001*†

CHAPTER III

Uncovering Phytotoxic Compounds Produced by *Colletotrichum* spp. Involved in Legume Diseases Using an OSMAC–Metabolomics Approach

Uncovering Phytotoxic Compounds Produced by *Colletotrichum* spp. Involved in Legume Diseases Using an OSMAC–Metabolomics Approach

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This chapter is published in

Journal of Fungi 2023, 9(6), 610

DOI: https://doi.org/10.3390/jof9060610

Abstract: Different fungal species belonging to the *Colletotrichum* genus cause anthracnose disease in a range of major crops, resulting in huge economic losses worldwide. Typical symptoms include dark, sunken lesions on leaves, stems, or fruits. *Colletotrichum* spp. have synthesized, in vitro, a number of biologically active and structurally unusual metabolites that are involved in their host's infection process. In this study, we applied a one strain many compounds (OSMAC) approach, integrated with targeted and non-targeted metabolomics profiling, to shed light on the secondary phytotoxic metabolite panels produced by pathogenic isolates of *Colletotrichum truncatum* and *Colletotrichum trifolii*. The phytotoxicity of the fungal crude extracts was also assessed on their primary hosts and related legumes, and the results correlated with the metabolite profile that arose from the different cultural conditions. To the best of our knowledge, this is the first time that the OSMAC strategy integrated with metabolomics approaches has been applied to Colletotrichum species involved in legume diseases.

Keywords: *Colletotrichum* spp.; fungal metabolites; anthracnose; legumes; phytotoxins; metabolomics; chemotaxonomy.

1.Introduction

Legumes are crops that belong to the family Fabaceae, characterized by their high protein content, being important for human food and animal feed, and by their ability to fix nitrogen from the atmosphere in symbiosis with nitrogen-fixing soil bacteria, which is keyto the sustainability of cropping systems [1]. As with any crop, legumes are vulnerable to various biotic stresses, including fungal pathogens. These pathogens can cause devastating losses in crop yields, resulting in significant economic and environmental impacts [2]. Anthracnose, caused by pathogen *Colletotrichum* spp., is one of the most economically significant diseases affecting legumes. *Colletotrichum* spp. can survive for several years on plant debris that remains in the field after harvest [3]. Environmental high humidity ratesand temperatures up to 20 °C are required by these pathogens to infect the host plant [4].The initial symptoms on leaves are small yellow spots that enlarge into brown-colored lesions with a distinct dark margin. This might result in premature leaf drop. In the stem,the first lesions appear in its base from where they progress upwards [3]. Large stem lesions can cause wilting with subsequent plant death. In susceptible genotypes, more than 20% of the harvested seeds could show necrotic lesions, affecting their quality and market sale [5,6]. Among others, *Colletotrichum truncatum* and *Colletotrichum trifolii* are some of the most common fungal pathogens causing anthracnose in legumes. *C. truncatum* is a widespread pathogen that affects a range of legumes, including soybean, pea, and lentil [7–9], while *C. trifolii* primarily affects fodder legumes such as clover and barrel medic [10,11]. It has been observed that the host specificity of *Colletotrichum* isolates varies with the plant species from which they are obtained and isolates from a single host also exhibit pathogenic variation [12].

Fungal pathogens are known to produce a range of phytotoxic metabolites that could be involved in the development of disease symptoms in plants, or they could be viru- lence factors contributing to fungal pathogenicity [13–15]. These metabolites belong to different classes, including cyclohexanones, macrolides, polyketides, terpenes alkaloids, and peptides. Understanding the molecular mechanisms behind the production of these metabolites can lead to developing new and more effective control strategies for fungal diseases [16–18]. The lifestyles of the various *Colletotrichum* species vary from necrotrophic to hemibiotrophic [19], producing a significant number of secondary metabolites, some directly contributing to their pathogenicity [4]. Over

the decades, 189 secondary metabolites isolated from *Colletotrichum* spp. have been chemically and biologically characterized [20]. Nevertheless, only a few data are available on phytotoxic metabolites produced by *C. truncatum* [21,22]. While it is reported that *C. trifolii* can produce phytotoxic exopolysaccharides, no data are available on the isolation and characterization of phytotoxic low-molecular-weight metabolites.

The production of phytotoxic secondary metabolites is affected by environmental factors such as nutrient availability, temperature, and pH [23]. When altering these factors, it is fundamental to fully explore the structural diversity of secondary metabolites of fungal pathogens in order to induce the production of specific biomarkers that can also be useful for chemotaxonomy application [24,25]. In recent years, the one strain many compounds (OSMAC) approach has emerged as a powerful tool for exploring the chemical diversity of fungal secondary metabolites. This approach involves manipulating growth conditions, such as pH, temperature, and media composition, to elicit diverse secondary metabolites from a single fungal isolate, expanding its chemical repertoire [26]. OSMAC has also been applied in integration with metabolomics [27]. Indeed, untargeted and targeted metabolomics have emerged as essential tools for studying the chemical diversity of fungal metabolites [28]. Both approaches have successfully identified phytotoxic metabolites produced by fungal pathogens, including *Fusarium*, *Alternaria*, *Penicillium*, and *Aspergillus* species [28,29].

Considering this background, the primary aim of this work was to investigate the secondary phytotoxic metabolites produced by three *Colletotrichum* pathogens of economic importance isolated from infected legume plants: (i) *C. truncatum* C428 isolated from lentil (*Lens culinaris*); (ii) *C. truncatum* C431 isolated from soybean (*Glycine max*); (iii) *C. trifolii* C436 isolated from red clover (*Trifolium pratense*). The OSMAC strategy was integrated with targeted and non-targeted metabolomics profiling. Moreover, to assess if and how the cultural media could affect the phytotoxicity of *Colletotrichum* spp. extracts, their activity was assessed on their primary host and related legumes. The secondary objective of this research was to assess the feasibility of host-specialized fungal strains from *C. truncatum* and *C. trifolii* that could be differentiated according to their metabolic profiles, paving the way to chemotaxonomy. To our knowledge, this is the first time the OSMAC strategy integrated with metabolomics approaches has been applied to *Colletotrichum* species

involved in legume diseases. Moreover, it is the first time that phytotoxic metabolites have been dereplicated from an isolate of *C. trifolii.*

2. Materials and Methods

2.1. Fungal Strains, Plant Hosts, and Crossing Inoculations

Three previously well-characterized strains of Colletotrichum spp. kindly provided by Saskatoon Research and Development Centre (Canada) and maintained in the fungal collection belonging to the Institute for Sustainable Agriculture (IAS-CSIC, Córdoba, Spain) were used for the study (listed in Table 1). For the experiment, isolates were grown in Petri dishes containing potato dextrose agar (PDA) (Sigma Aldrich, Saint-Quentin Fallavier, France) under controlled conditions at 20 ± 2 °C under a 12 h photoperiod at 150 µmol m^{-2} s^{-1} photon flux density for 10 days, until sporulating mycelium was clearly visible. Then, a spore suspension for each fungal isolate was prepared by flooding the surface of 10-day-old cultures with sterile distilled water, gently scraping the colony with a glass rod and filtering the suspension through two layers of sterile cheesecloth.

Table 1. Fungal strains from *Colletotrichum* spp. Growth in different culture media and used for the plant bioassays.

Species	Host Plant	Fungal Code
C. truncatum	Lentil (*Lens culinaris*)	C428
C. truncatum	Soybean (*Glycine max*)	C431
C. trifolii	Red clover (*Trifolium pretense*)	C436

Disease responses were studied by performing cross-inoculation studies on a panel of legume species (listed in Table 2) that were grown under controlled conditions as follows: seeds were sown in pots (6 x 6 x 10 cm), filled with a potting mixture (sand/peat, 1:3 vol/vol), and kept in a growth chamber at 20 ± 2 °C and 65% relative humidity under a photoperiod at 14 h light/10 h dark with a light intensity of 200 µmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes, during a two-week period until the plants reached the 4–5-leaf stage. There were three independent replicates per fungal isolate and crop species, arranged in a completely randomized design. Each replicate consisted of 3 pots with 5 plants per pot. The experiments were repeated three times.

Table 2. Legume species and genotypes growth under controlled conditions and used in detached leaf assays with *Colletotrichum* spp. extracts.

Legume	Plant Specie	Genotype
Soybean	*Glycine max*	Creator
Faba bean	*Vicia faba*	Baraca
Lentil	*Lens culinaris*	Pardina
Pea	*Pisum sativum*	Messire
Barrel medic	*Medicago truncatula*	Paraggio
Red clover	*Trifolium pratense*	B1401
Subterranean clover	*Trifolium subterraneum*	E08
White clover	*Trifolium repens*	Anteria

The concentration of conidia from each spore suspension was determined with a hemocytometer and adjusted to 10^6 spores/mL. Tween 20 (VWR) was added as a wetting agent (two drops per 500 mL suspension). The conidial suspensions were sprayed at the 4–5-leaf stage using a handheld sprayer at a rate of 1 mL per plant. A pot of each plant species was sprayed with sterile deionized water as the non-inoculated control. After inoculation, plants were covered with a polyethylene sheet during the first 24 h in darkness, and high humidity was ensured by ultrasonic humidifiers operating for 15 min. every 2 h. Later on, the polyethylene cover was removed, and plants were maintained for 9 days in a growth chamber (under the conditions described above). Every 2 days, water was added to the trays to maintain high relative humidity (95–100%).

Anthracnose symptoms were rated for each fungal isolate on legume hosts ten days after inoculation. Disease response was assessed following a scale developed by Gossen [12] where lesions on the main stem of each plant were counted and grouped in categories as 1–10, 11–15, 16–20, 21–25, 26–30, and >30 lesions. Depth of penetration, position on the stem, and resulting degree of plant wilting were also integrated. As a result, numerical values were assigned to the levels within each descriptive category to permit statistical analysis. A final disease score was obtained by summing the values from each category [12].

2.2. Culture Medium and Growth Conditions for Fungal Organic Extract Production

For fungal organic extract production, each isolate was grown in four different culture media, as follows: (i) 6 Roux bottles (200 mL) containing each 100 mL of Richard's medium [30]. Each bottle was inoculated with 3 mycelial plugs (of 5 mm^2 each) of a 10-day-old mycelial plate of each isolate on PDA. The cultures were incubated at 24 °C, in constant stirring at 150 rpm, under a 12 h photoperiod at 150 µmol m^{-2} s^{-1} photon

flux density for 21 days after which the mycelial mats were removed by filtration through four layers of filter paper, centrifuged and kept at -20 °C until further processing; (ii) 6 Roux bottles (200 mL) containing each 100 mL of potato dextrose broth (PDB) (BD Difco®, Crystal Lake, NJ, USA) medium. Each bottle was inoculated and kept as described in point (i); (iii) 6 flasks containing each 100 gr of normal rice substrate. Water was added to the flask (45%, vol/vol) and left for 24 h to be absorbed. Then, the material was sterilized at 121 °C for 30 min. The flask was then inoculated with 3 mycelial plugs (of 5 mm^2 each) of a 10-day-old mycelial plate of each isolate on PDA. The cultures were incubated at 24 °C under a 12 h photoperiod at 150 µmol m^{-2} s^{-1} photon flux density for 30 days. Every 4 days, flasks were manually stirred to ensure fungal oxygenation. Then, the cultures were dried in a stove at 40 °C for 3 days and ground; (iv) 6 PDA Petri dishes were inoculated and incubated, as mentioned in point (iii), in static conditions for 10 days.

2.3. Reagents and Materials

Solvents such as EtOAc, n-hexane MeOH, i-PrOH, CHCl3, and CH_2Cl_2 were purchased from Panreac AppliChem (Barcelona, Spain). Colletochlorin A, orcinol, and tyrosol were isolated from in vitro cultures of *Colletotrichum gloeosporioides* [31]; colletochlorins E and F, colletopyrone, higginsianin A, and higginsianin B were isolated from *in vitro* cultures of *C. higginsianum* [32,33]; (*R*)-mellein was isolated from in vitro cultures of *Neofusicoccum parvum* [34]; 6-hydroxymellein were isolated from in vitro cultures of *Phoma chenopodiicola* [35]; resorcinol was isolated from in vitro cultures of *Dothiorella vidmadera* [36]; and 4-hydroxybenzaldehyde and 2-(4-hydroxyphenyl) acetic acid were isolated from in vitro cultures of *Spencermartinsia viticola* [37]. The identity and purity of all standard metabolites were checked using NMR analysis at the Department of Chemical Sciences of the University of Naples Federico II. As an internal standard (IS) for the UHPLC-QTOF-HRMS analysis, (±)-naringenin analytical standard, purchased from Sigma-Aldrich, Milan, Italy, was used.

2.4. Extraction of Secondary Metabolites from Different Culture Media

The culture filtrates (180 mL) of the PDB and Richard's media obtained from the three *Colletotrichum* isolates were exhaustively extracted with EtOAc (3x300 mL). The

organic extracts of each strain were combined, dried (Na_2SO_4), and evaporated under reduced pressure yielding dark-red and dark-brown solid residues in the case of PDB and Richard's medium, respectively. The solid rice cultures obtained from the three strains of Colletotrichum were air-dried as described in Section 2.2. The dried material (100 g) was minced using a laboratory mill and extracted with 100 mL of MeOH-H_2O (1% NaCl) (1:1). The mixture was centrifuged for 1 h at 10,000 rpm. The pellet was extracted again with the same solvent mixture in the same conditions, and the two supernatants were then pooled, defatted by n-hexane (2 × 250 mL), and extracted with CH_2Cl_2 (3 × 250 mL). The CH_2Cl_2 organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure yielding dark-brown solid residues. The solid PDA cultures obtained from the three strains of *Colletotrichum* were lyophilized (Epsioln 2-10D, Christ, Osterod, Germany), then extracted with EtOAc (3 × 50 mL), and finally the organic extracts were filtered and evaporated under reduced pressure yielding dark-red solid residues. The media PDA, PDB, Richard, and rice substrates were extracted in the same specific conditions reported above, and used as blanks in the UHPLC-QTOF-HRMS.

2.5. UHPLC-QTOF-HRMS Apparatus and Conditions

Before analysis, the 76 organic extracts were reconstituted in MeOH and filtered through a Millex syringe filter of 0.2 µm (Merck, Darmstadt, Germany), and the final concentration was 1 mg/mL. The internal standard (IS) was spiked in every standard solution at a 10 µg/mL concentration. Finally, 30 µL of each extract was taken to create 3 pooled QC samples.

The UHPLC-QTOF-HRMS analysis was carried out by the Metabolomics Platform of Agricultural Sciences, Food Science and Technology and Natural Resources (CEBAS)-CSIC, Murcia, Spain. Prior to mass spectrometric sample analysis, the chromatographic separation was realized using Acquity UPLC-I-class system (Waters Corporations, Milford, MA, USA). In total, 7 µL of the organic extract solutions was injected using a Sample Manager Fixed- Loop (SM-FL) (Waters Corporations, Milford, MA, USA). Chromatographic separation was performed using a Poroshell 120 EC-C18 Agilent column (100 x 3 mm, 2.7 µm, (Agilent Technologies, Waldbronn, Germany), operating at 30 °C and with a flow rate of 0.4 mL/min in an Acquity I-Class column oven system (Waters Corporations, USA). Compounds were separated using the following

gradient conditions, using H_2O + 0.1% formic acid (FA) (A) and MeCN + 0.1% FA (B): 0–10 min, 1–18% phase-B; 10–16 min, 18–38% phase-B; 16–22 min, 38–95% phase-B. Finally, the phase-B content was returned to the initial conditions (1%) for 1 min and the column was re-equilibrated for 5 min more. Software Compass HyStar (version 3.2 Bruker Daltonics, Bremen, Germany) was used for the operation of the UHPLC systems. The pooled quality controls (QCs) were used for metabolomic analysis quality. QCs, blanks, and (±)-naringenin solution (1 µg/mL) were injected three times during the batch test: beginning, middle, and end. A MaXis Impact QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was utilized for QTOF-HRMS experiments. Ionization in the mass spectrometers was performed using an ESI source (Bruker Daltonics, Bremen, Germany), which operated under optimized conditions. The parameters for ESI source were set as follows: Ionization was performed in the negative mode at -4.0 kV. Dry gas temperature was set to 200 °C at a flow rate of 9.0 L/min. Nebulizer gas pressure was 2 bar. The ESI ion source was operated at -4.0 kV with a probe gas temperature of 450 °C at a flow rate of 4 L/min. The dry gas temperature was set to 300 °C at a flow rate of 9.0 L/min. The nebulizer gas pressure was 2 bar. A mass range of 50-1200 m/z was covered, and the full scan and MS2 data were recorded at a spectra rate of 2 Hz. Data-independent acquisition in the broadband collision-induced dissociation (bbCID) mode was chosen for MS/MS experiments. Fragmentation took place in a collision-induced dissociation cell using nitrogen. Spectral acquisition was performed at a collision energy (CE) of 20 eV. To calibrate the mass axis, a 10 mM sodium formate cluster solution in 1:1 isopropanol–water was introduced into the ESI source at the beginning of each UHPLC run using a divert valve to calibrate instrument mass calibration and re-calibrate individual raw data files. The content of identified Level A metabolites was quantified by the selected IS, and the relative peak areas (analyte area/IS area) were used for quantification. Software Compass Control (software version 3.4, Bruker Daltonics, Bremen, Germany) was used for the operation of the mass spectrometer, and for data acquisition and conversion of raw spectra files to centroid mzML files.

2.6. Metabolite Annotation and Identification

In order to fully exploit the differences in the metabolite profiles obtained in different media cultures of *Colletotrichum* spp., three bioinformatic tools were integrated:

(1) MetaboAnalyst 5.0 [38], the web-based tool providing LC-HRMS spectra processing automated workflow integrated with comprehensive statistical data analysis and interpretation mod- ules; (2) MS-DIAL (Version 5.10)/MS-FINDER (Version 3.5), the computational approach which helps to characterize the structure of the metabolites rapidly [39]; (3) MetFreg [40], a freely available web software to assist the annotation of the high-precision tandem mass spectra of metabolites by in silico fragmentation.

This approach includes three main steps: Step 1: spectra processing and peak annotation with MetaboAnalyst 5.0. All the parameters are reported in Supplementary Table S1. Step 2: multivariate analysis of the global metabolites profile with MetaboAnalyst 5.0. Features with more than 50% of missing values in the samples were removed while missing values were estimated by a sample-wise k-nearest neighbors (KNN) algorithm. Datasets were normalized by the internal standard (±)-naringenin, and specific transformation and scaling conditions for the data sets are reported in Supplementary Table S2. The datasets were analyzed with PCA to explore potential patterns and heatmaps that could show clustering of the features and visualize the differences between groups. The top 20 (ANOVA $p < 0.05$) most significant features were selected for structural annotation. Moreover, PLS-DA was carried out to reveal the global profile changes and potential application for fungal classification according to metabolite composition. The VIP score cutoff value was 1.6. Cross-validation was carried out by LOOCV; R^2 and Q^2 values are reported in Supplementary Table S3. Step 3: structural annotation of the metabolites assisted by MS-DIAL/MS-FINDER and MetFrag. The metabolite ions were converted into structural information with MS-DIAL/MS-FINDER linked to MS/MS databases. The MS-DIAL parameters were MS1 tolerance of 0.01 Da; MS2 tolerance of 0.05 Da; and minimum peak height of 1000 (amplitude); for alignment, a QC sample was used as a reference file, and the retention time tolerance was set at 0.05 min. The MS/MS public databases used for peak identification were MSMS-Neg-MassBankEU, MSMS-Neg-GNPS, MSMS-Neg-MassBank, MSMS_Public_EXP_NEG _VS17, MSMS_Public_ExpBioInsilico_NEG_VS17, and MSMS-Neg-Vaniya-Fiehn_ Natural_Products_Library, and the identification score cutoff value was selected as 8. Significant metabolites with monoisotopic mass error within 5 mDa with no proper match in the selected databases were manually screened for mass spectral peak matching. The molecular formulas were searched on NpAtlas [41], Coconut [42], and Lotus [43] database to confirm whether they were previously produced by *Colletotrichum* spp. or by other microorganisms. SDF files were generated from the above-cited database and

uploaded to MetFrag to assist in identifying the metabolites with in silico fragmentation. The complete list of identified/annotated metabolites and confidence levels is reported in Supplementary Table S3. The careful manual curation of all assigned peaks was carried out, and the metabolites were annotated according to confidence levels [44,45], also considering the available fungal taxonomical information.

2.7. Bioassays

A macroscopical evaluation of all the *Colletotrichum* spp. extracts was performed using a detached leaf method [46]. Legume species (listed in Table 2) were grown under controlled conditions, as described in Section 2.1. Four-stage leaves from each legume specie were excised and placed, adaxial side up, on 4% technical agar in Petri dishes. For each legume species, fungal extract, and concentration, cut leaves were arranged in a randomized design with three replicates per treatment, with each replicate having four leaves. Due to different solubilities, the four organic fungal extracts were dissolved in MeOH (5%) and then brought to the final concentration with distilled water (1 and 2 mg/mL). Two droplets of the test solutions (25 µL) were applied on the adaxial leaf side. Untreated leaves, droplets (25 µL) of distilled water, and droplets (25 µL) of MeOH (5%) were applied as negative controls. The solvent was evaporated in a laminar flow cabinet until dry. The presence of symptoms, consisting of leaf alteration by discoloration or necrosis, was periodically observed (from 1 to 14 days after the extract's application). Total leaf area and leaf damaged area were measured in cm2 using the ImageJ (1.46 r) program (free license), and then the percentage of damage severity (%DS) was calculated. Additionally, the observed damage was classified as necrotic leaf area (N), or irregular discolored areas (DA) surrounded (or not) by a necrotic ring (NR).

2.8. Bioassay Data Analysis

All experiments followed a completely randomized design. The significance of the differences in leaf damage between plant species, treatments, and concentrations was estimated by one- or two-way analysis of variance (ANOVA). Before performing ANOVA tests, the normality and equality of variances were checked using Shapiro–Wilk and Levene's tests, respectively. Whenever the ANOVA test was statistically significant

($p \leq 0.05$), a Fisher's LSD multiple range test assessing the difference of the means between each treatment was performed. Null hypotheses were rejected when $p \leq 0.05$. All statistical analyses were performed using the Statistix 9.0 package (Analytical Software, Tallahase, FL, USA).

3. Results

3.1. Cross Inoculations

Cross inoculations performed with different *Colletotrichum* spp. showed that each isolate was more infectious to the legume species from which it was isolated, but that it also infected other legume species, although at a lower intensity. Isolate C428 of *C. truncatum*, collected from infected lentils, caused different severity indexes in the tested legume species ($p < 0.01$; Figure 1A), being most infectious to lentil (SI = 9.7), followed by white clover, pea, and faba bean (value ranging between 3.8 and 4.8), being even less infectious to subterranean clover, red clover, and soybean (SI < 2), and causing no symptoms in barrel medic (SI = 0) (Figure 1A). Isolate C431 of *C. truncatum*, collected from infected soybeans ($p < 0.01$; Figure 1B), was most infectious to soybean (SI = 9.3), followed by lentil, barrel medic, and white clovers (SI 5–4.2), and then by subterranean clover, red clover, pea, and faba bean (SI 3–1). Isolate C436 of *C. trifolii*, collected on red clover, was most infectious to red clover (SI = 9) (Figure 1C), followed by barrel medic and subterranean clover (SI 6.4–5.4), and then followed by faba bean, lentil, pea, and soybean (SI 3.5–3), and it was least infectious to white clover (SI = 1.6).

3.2. Targeted Analysis of Selected Phytotoxic Metabolites

Isolates C428 and C431 of *C. truncatum*, as well as isolate C436 of *C. trifolii* were grown in four different media: two solid substrates (PDA and rice) and two liquid media (PDB and Richard's medium), as reported in detail in the Materials and Methods section. The production of 13 pure phytotoxic metabolites commonly produced by *Colletotrichum* species was investigated by LC-HRMS-ESI-QTOF in negative mode. The selected metabolites included the following: colletochlorins A, E, and F, colletopyrone, higginsianins A and B (*R*)-mellein, 6-hydroxymellein, orcinol, resorcinol, tyrosol, 4-

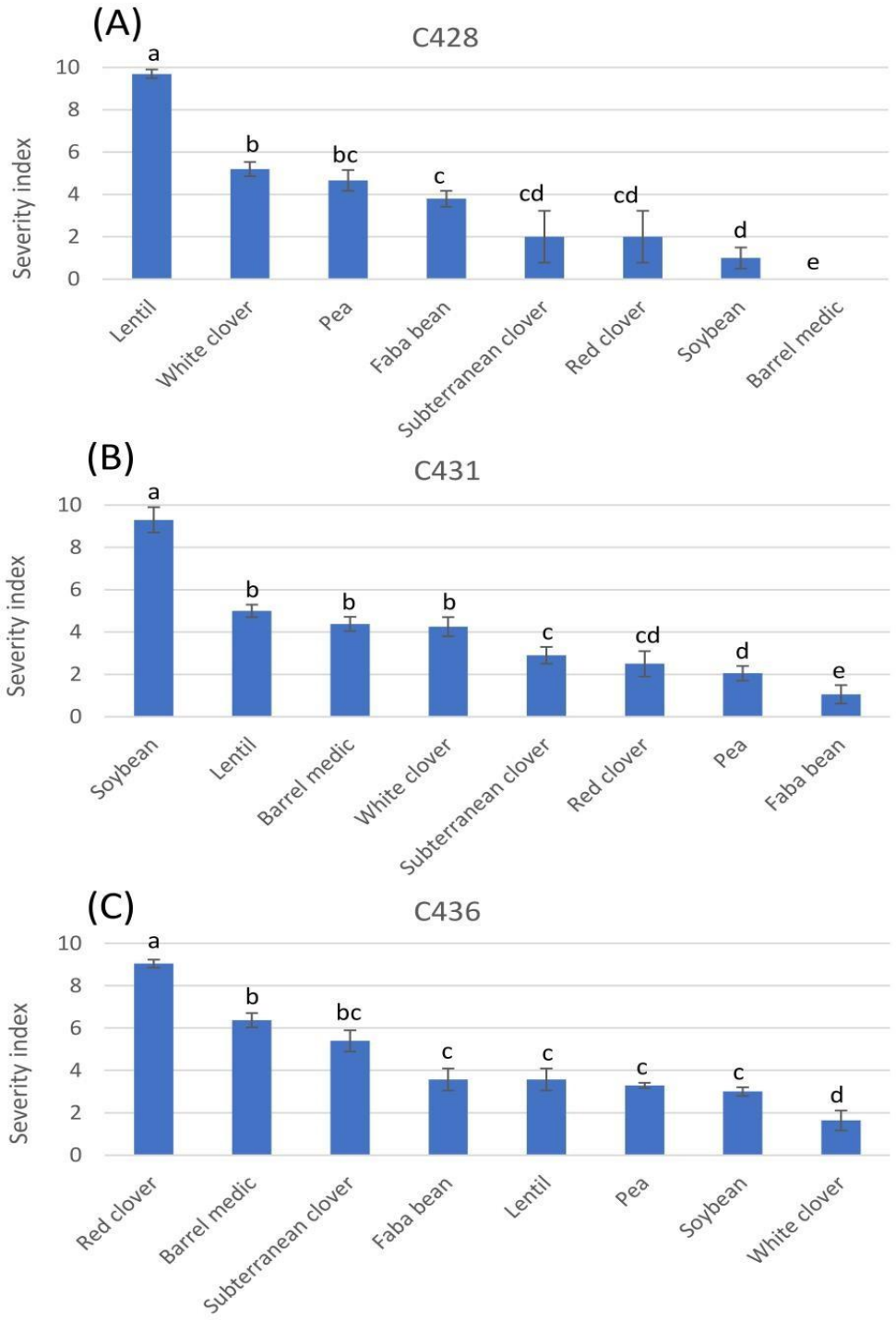

Figure 1. Severity index of anthracnose symptoms on legume species inoculated with (A) *Colletotrichum truncatum* isolate C428, (B) *C. truncatum* isolate C431, and (C) *C. trifolii* isolate C436, performed under controlled conditions, calculated following Gossen [12]. For each fungal isolate, data with the same letter, per column, are not significantly different (LSD test, $P < 0.01$).

hydroxybenzaldehyde, and 2-(4-hydroxyphenyl) acetic acid. Seventy-two extracts were analyzed in triplicate, and Compass software analyzed the quantitative data. The concentrations of the detected metabolites are reported in Table 3.

Table 3. Phytotoxic metabolites identified and quantified in the extracts of the selected *Colletotrichum*

Compound	*C.truncatum* C428				*C.truncatum* C431				*C.trifolii* C436			
	PDA	**PDB**	**RICE**	**RICHARD**	**PDA**	**PDB**	**RICE**	**RICHARD**	**PDA**	**PDB**	**RICE**	**RICHARD**
Colletochlorin E	n.d.	n.d.	n.d.	0.01 ± 0.004	n.d.	n.d.	n.d.	++	n.d.	n.d.	n.d.	n.d.
Colletopyrone	0.01 ± 0.001	8.13 ± 2.78	0.94 ± 0.11	+	0.04 ± 0.003	14.62 ± 1.32	0.04 ± 0.01	+	+	n.d.	0.18 ± 0.02	+
4-Hydroxybenzaldehyde	0.81 ± 0.66	0.05 ± 0.01	0.09 ± 0.02	0.59 ± 0.04	0.19 ± 0.05	0.06 ± 0.02	0.11 ± 0.02	0.74 ± 0.29	0.15 ± 0.03	0.09 ± 0.006	0.19 ± 0.03	0.49 ± 0.06
Resorcine	0.04 ± 0.02	n.d.	++	n.d.	n.d.	n.d.	n.d.	n.d.	++	n.d.	n.d.	++
Orcinol	n.d.	++	n.d.	n.d.	0.02 ± 0.002	+	n.d.	n.d.	n.d.	+	++	n.d.
Higginsianin A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	n.d.	n.d.	n.d.
Higginsianin B	0.01 ± 0.002	++	++	++	++	+	++	++	++	++	++	+
4-Hydroxyphenyl acetic acid	0.68 ± 0.14	+	0.06 ± 0.02	0.18 ± 0.03	0.09 ± 0.05	+	0.21 ± 0.02	0.06 ± 0.01	0.79 ± 0.60	n.d.	0.03±0.002	0.29 ± 0.02
6-Hydroxymellein	+	0.06 ± 0.004	0.30 ± 0.01	++	++	0.06 ± 0.004	0.41 ± 0.02	0.03 ± 0.003	+	+	++	++

n.d. = not detected; + = <LOD; ++ = <LOQ; a = concentration reported as µg/mL ± SD.

Colletochlorins A and F, (*R*)-mellein, and tyrosol were not detected in any organic extract. Colletopyrone, higginsianin B, 6-hydroxymellein, and 4-hydroxybenzaldehyde were detected in all the organic extracts of all three *Colletotrichum* species; however, the amount varied according to cultural conditions. In detail, colletopyrone was detected in the highest concentration in the PDB cultures of C428 and C431 (8.13 µg/mL and 14.62 µg/mL, respectively), while, in C436, a quantifiable amount (0.18 µg/mL) was detected in rice extract. A quantifiable amount of higginsianin B (0.01 µg/mL) was detected only in the PDA culture of *C. truncatum* C428. 6-Hydroxymellein was quantified in the highest concentration in the rice cultures of both *C. truncatum* isolates, while it was below the LOQ in C436, regardless of the cultural conditions. 4-Hydroxybenzaldehyde was detected and quantified in all the organic extracts of the three pathogens. In particular, the highest amount was detected in Richard's medium extracts.

All three *Colletotrichum* species produced orcinol, but its yield depended on cultural conditions. Indeed, it was detected only in the PDB extract of *C. truncatum* C428. On the other hand, it was quantified (0.02 µg/mL) in the PDA

extract of *C. truncatum* C431 and detected in the PDB extract. Moreover, it was detected in the organic extracts of the PDB and rice cultures of *C. trifolii* C436.

Resorcine was detected only in PDA (0.04 µg/mL) and in rice extracts of C428 and PDA and Richard's medium extracts of C436, respectively. Colletochlorin E was detected only in Richard's medium extracts of both *C. truncatum* isolates. In the extract of C428, colletochlorin E was also quantified at a rate of 0.01 µg/mL. Finally, higginsianin A was detected only in the PDA extract of *C. trifolii* C436.

3.3. Impact of Cultural Conditions on Secondary Metabolite Production by Colletotrichum *Species*

An untargeted metabolomics profiling was conducted to investigate the changes in secondary metabolite production due to in vitro cultural conditions. LC-HRMS-ESI-QTOF was used to compare the chemical profiles of the *Colletotrichum* species cultured. The LC-HRMS base peak chromatograms from the fungal cultures are shown in Supplementary Figures S1–S3.

The LC-HRMS data pretreatment, followed by multivariate analysis to identify the up- and downregulated metabolites in each culture, was performed using MetaboAnalyst 5.0. Unsupervised data analysis by PCA was performed to visualize the data, look for trends and groupings, and to identify possible outliers. Heatmaps, which provide an intuitive data table visualization, were used to identify unusually high/low compounds in different cultural conditions. The analysis was carried out for each *Colletotrichum* species independently.

The results were then cross-confirmed by MS-DIAL, while MS-FINDER and MetFrag were used to identify putative fungal metabolites according to MS/MS data starting from those commonly produced by *Colletrotrichum* species, as fully described in Section 2.5 of the Materials and Methods. The complete list of identified/annotated metabolites, compound classes, and confidence levels is reported in Supplementary Table S3. The structures of validated/putatively annotated compounds Levels A, B(i), and B(ii) are reported in Supplementary Figure S4. The results by pathogens are detailed and described in the sections below.

3.3.1. *Colletotrichum truncatum* C428 (from Lentil)

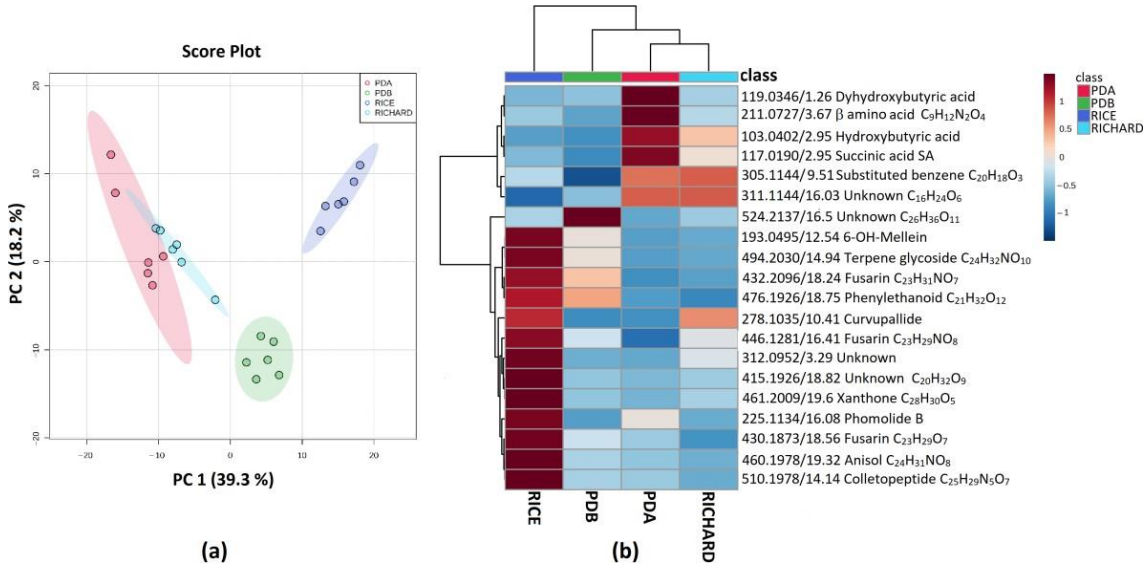

Figure 2. (a) PCA Scores plot between PC1 and PC2. The explained variances are shown in brackets; (b) heatmap of top 20 (ANOVA < 0.05) most different metabolites in the organic extracts of *C. truncatum* C428. The color key is based on the average peak intensity of each feature by class: red color for higher peak intensity (upregulated) and blue color for lower peak intensity (downregulated).

The obtained peak intensity table containing the complete features detected from the raw spectra processing of isolate C428 was submitted to the statistical data analysis module of Metabonalyst. Data were normalized by the internal standard (I.S., naringenin), square root transformed, and scaled using Pareto scaling to reduce systematic bias and improve overall data consistency.

The PCA analysis highlighted the presence of four different groups mainly separated along the PC1 direction (Figure 2a). The extracts clustered according to cultural conditions with PDA and Richard's medium extracts on the left quadrant of the score plot, while PDB and rice extracts are on the right quadrant (Figure 2a).

Among the compounds contributing to group discrimination, simple organic acids such as hydroxybutyric and succinic acids were produced in higher amounts in PDA and Richard's medium, while dihydroxybutyric acid was upregulated in PDA (Figure 2b). Interestingly, the production of a compound with *m/z* 278.1035 and formula $C_{14}H_{17}NO_5$ (putatively annotated as curvupallide, previously isolated from *Curvularia pallescens* [47]) was induced in both Richard's medium and rice culture. The high-resolution mass and MS/MS spectra agreed with both curvupallides A and B. However, they differed only in the absolute configuration of OH at the C3; thus, it was not possible to discriminate between the two structures (Supplementary Figure S4).

When growing on rice, several secondary metabolites belonging to a different class of natural compounds were produced in higher amounts by *C. truncatum* isolate C428. Among them, phomolide B, a 10-membered macrolide previously isolated from *Phomopsis* sp., was putatively annotated [48]. Moreover, a compound with molecular formula $C_{25}H_{29}N_5O_7$ was classified as hybrid peptide-polyketide cyclic tridepsipeptides, related to the class of the colletopeptides [49]. Finally, three compounds with molecular formulas $C_{23}H_{31}NO_7$, $C_{23}H_{29}NO_7$, and $C_{23}H_{29}NO_8$ were classified as fusarins, mycotoxins produced by the *Fusarium* species [50]. The $C_{23}H_{31}NO_7$ fusarin was also produced in high amounts in the PDB culture.

3.3.2. *Colletotrichum truncatum* C431 (from Soybean)

The obtained peak intensity table containing the complete features detected from the raw spectra processing of *C. truncatum* C431 was submitted to the statistical data analysis module of Metabonalyst. To reduce any systematic bias and to improve overall data consistency, data were normalized by the internal standard (I.S., naringenin), cube root transformed, and scaled using Pareto scaling.

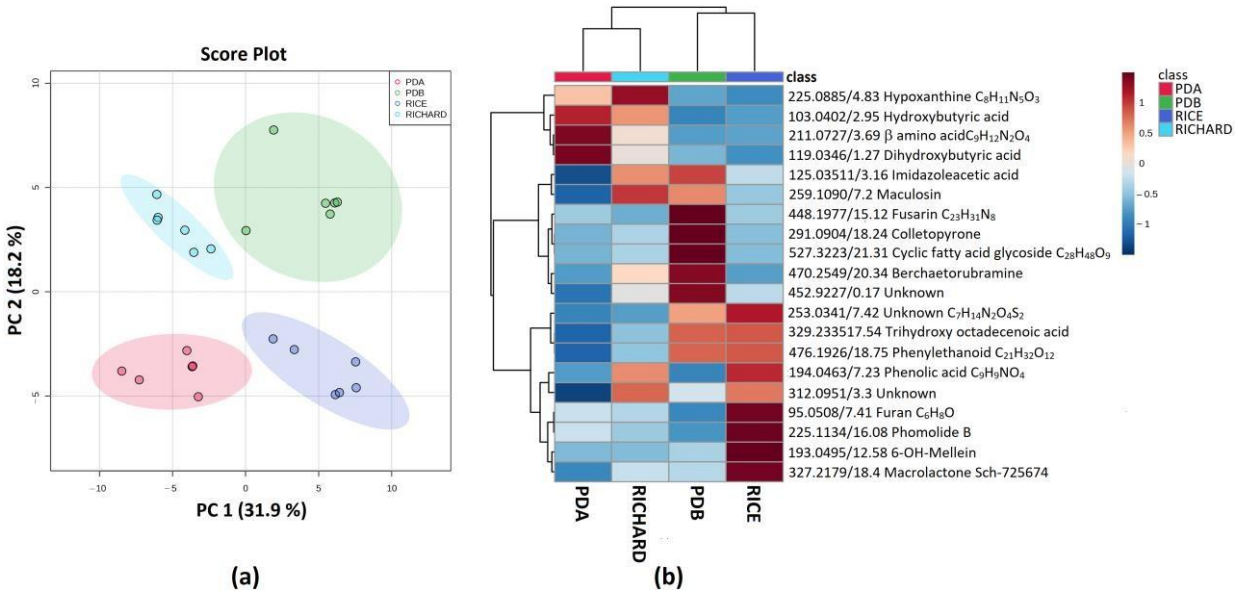

Figure 3. (a) PCA Scores plot between PC1 and PC2. The explained variances are shown in brack- ets; (b) heatmap of top 20 (ANOVA < 0.05) most different metabolites in the organic extracts of *C. truncatum* C431. The color key is based on the average peak intensity of each feature by class: red color for higher peak intensity (upregulated) and blue color for lower peak intensity (downregulated).

In addition, for isolate C431, the PCA analysis highlighted the presence of four different groups. Nevertheless, differently from *C. truncatum* C428, PDA and Richard's

medium extracts were separated from the PDB and rice extracts along the PC1 direction, while PDA and Richard's extracts could be discriminated along PC2. PDB and rice extracts could also be discriminated along PC2 (Figure 3a). Among the compounds contributing to group discrimination, hydroxybutyric and dihydroxybutyric acids were detected in higher amounts in PDA and Richard's extracts (Figure 3b). Imidazoleacetic acid was upregulated in Richard's medium and PDB (Figure 3b) Imidazoleacetic acid is an alkaloid, which was previously isolated from the mushroom *Coprinus atramantarius* [51]. Maculosin, a phytotoxic diketopiperazine that was previously isolated from pathogenic fungi and bacteria [52,53], was also upregulated in Richard's and PDB medium. Berkchaetorubramine, a red pigment belonging to the family of azaphilones, was previously isolated from *Pleurostomophora* spp. [54], and detected in a higher amount in PDB extract. Moreover, another fusarin with molecular mass $C_{23}H_{31}NO_8$ was detected in a high concentration in the PDB extract. Among the compounds upregulated in the rice culture, it was fascinating to denote the high concentration of phomolide B, together with another bioactive macrolide named Sch-725674, which was previously isolated from *Colletotrichum* spp. GDMU-1 isolated from the leaves of *Santalum album* [55] (Figure 3b).

3.3.3. *Colletotrichum trifolii* C436 (from Red Clover)

The obtained peak intensity table containing the complete features detected from the raw spectra processing of *C. trifolii* isolate C436 was submitted to the statistical data analysis module of Metabonalyst, normalized by the internal standard (I.S., naringenin), logarithmic transformed, and autoscaled.

The PCA analysis of *C. trifolii* C436 highlighted that Richard's medium extracts were separated along the PC1 from PDB and rice extracts, while it was possible to discriminate the PDA extracts along the PC2 (Figure 4a). PDA extracts were separated along PC1 from PDB and rice extracts; nevertheless, the latter extracts mostly overlapped in the score plot (Figure 4a). As shown in the heatmap (Figure 4b), among other compounds, PDB and rice substrate media shared the upregulation of two classes of secondary metabolites: colletopeptides and fusarins. Indeed, a colletopeptide with molecular formula $C_{26}H_{29}N_3O_6$ and fusarins with molecular formulas $C_{23}H_{29}NO_7$, $C_{23}H_{29}NO_8$, $C_{23}H_{30}NO_7$, and $C_{23}H_{31}NO_8$ were detected in higher concentrations.

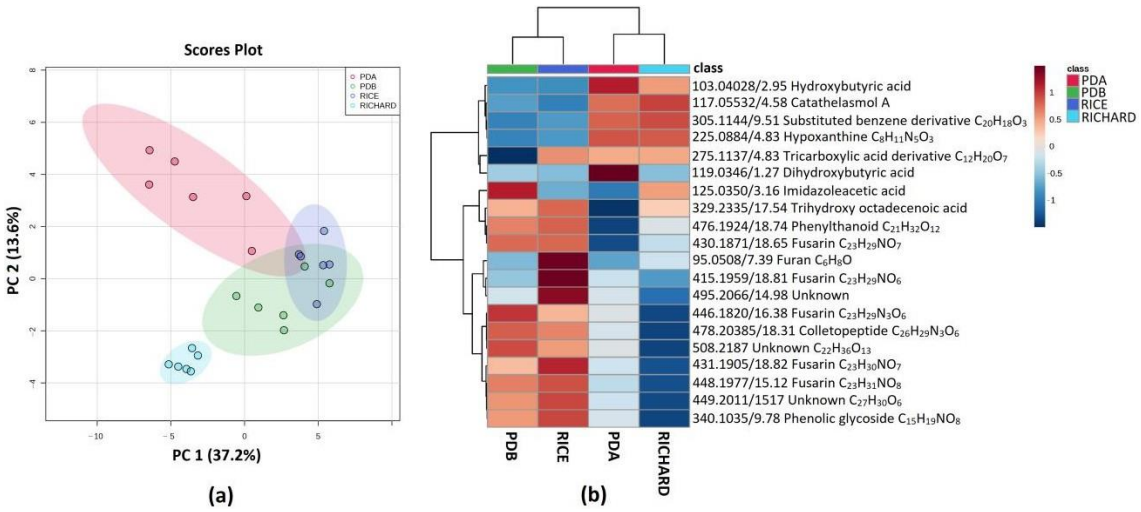

Figure 4. (a) PCA Scores plot between PC1 and PC2. The explained variances are shown in brackets; (b) heatmap of top 20 (ANOVA < 0.05) most different metabolites in the organic extracts of *C. trifolii* C436. The color key is based on the average peak intensity of each feature by class: red color for higher peak intensity (upregulated) and blue color for lower peak intensity (downregulated).

Moreover, another fusarin with molecular formula $C_{23}H_{29}NO_7$ was upregulated only in the PDB medium (Figure 4b).

Hydroxylated organic acids also contribute to group discrimination. A trihydroxy octadecanoic acid was detected in high amounts in PDA, Richard's medium, and rice extracts. In addition, hydroxybutyric acid was upregulated in PDA and Richard's medium, while dihydroxybutyric acid was upregulated only in PDA. Finally, a tricarboxylic acid derivative with molecular formula $C_{12}H_{20}O_7$ (Figure 4b) was detected in higher amounts in PDA and Richard's medium and rice extracts.

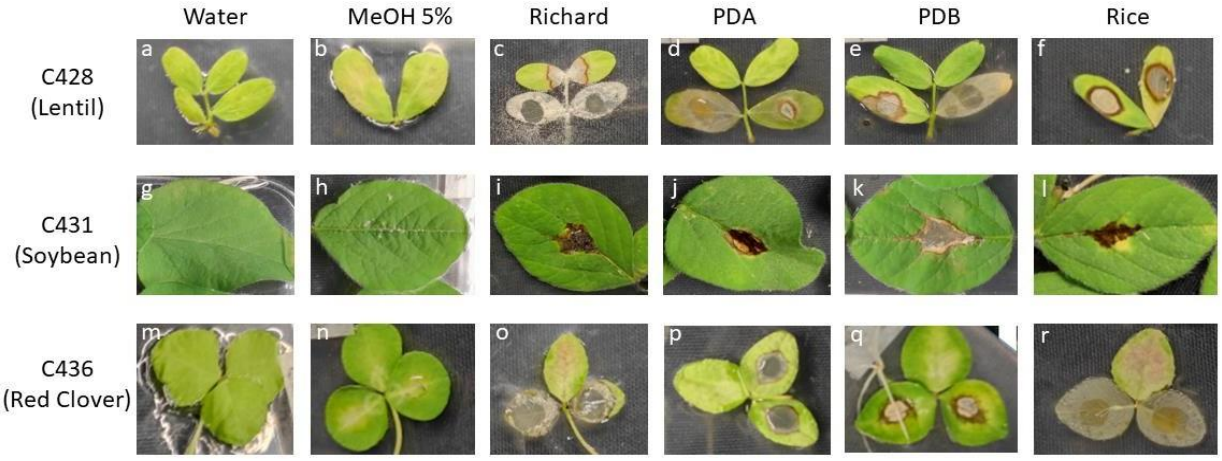

Figure 5. Symptoms observed on host legume leaves by *Colletotrichum* spp. culture media extracts after 13 days. (**a**,**g**,**m**) Negative control water; (**b**,**h**,**n**) negative control MeOH 5%; (**c**) effect of Richard's medium extract of C428; (**d**) effect PDA extract of C428; (**e**) effect of PDB extract of C428; (**f**) effect of rice culture extract of C428; (**i**) effect of Richard's medium extract of C431; (**j**) effect PDA extract of C431; (**k**) effect of PDB extract of C431; (**l**) effect of rice culture extract of C431; (**o**) effect of Richard's medium extract of C436; (**p**) effect PDA extract of C436; (**q**) effect of PDB extract of C436; (**r**) effect of rice culture extract of C428.

3.4. Effects of Culture Media on the Phytotoxicity of Organic Extracts

The liquid culture filtrates of *Colletotrichum* spp. were exhaustively extracted. The symptoms occurring on treated leaves started to appear on the sixth day after inoculation, and the severity increased during the following days, being maximal at 12-13 days after inoculation. The organic extracts showed differential phytotoxic activities depending on the fungal isolate, the culture media, the tested concentration, and the host plant, as shown in Supplementary Table S4.

3.4.1. *Colletotrichum truncatum* C428 (from Lentil)

In general, all the four tested fungal extracts from C428 showed significantly increased phytotoxicity (measured as DS%) compared with the controls (Figure 5a, b).

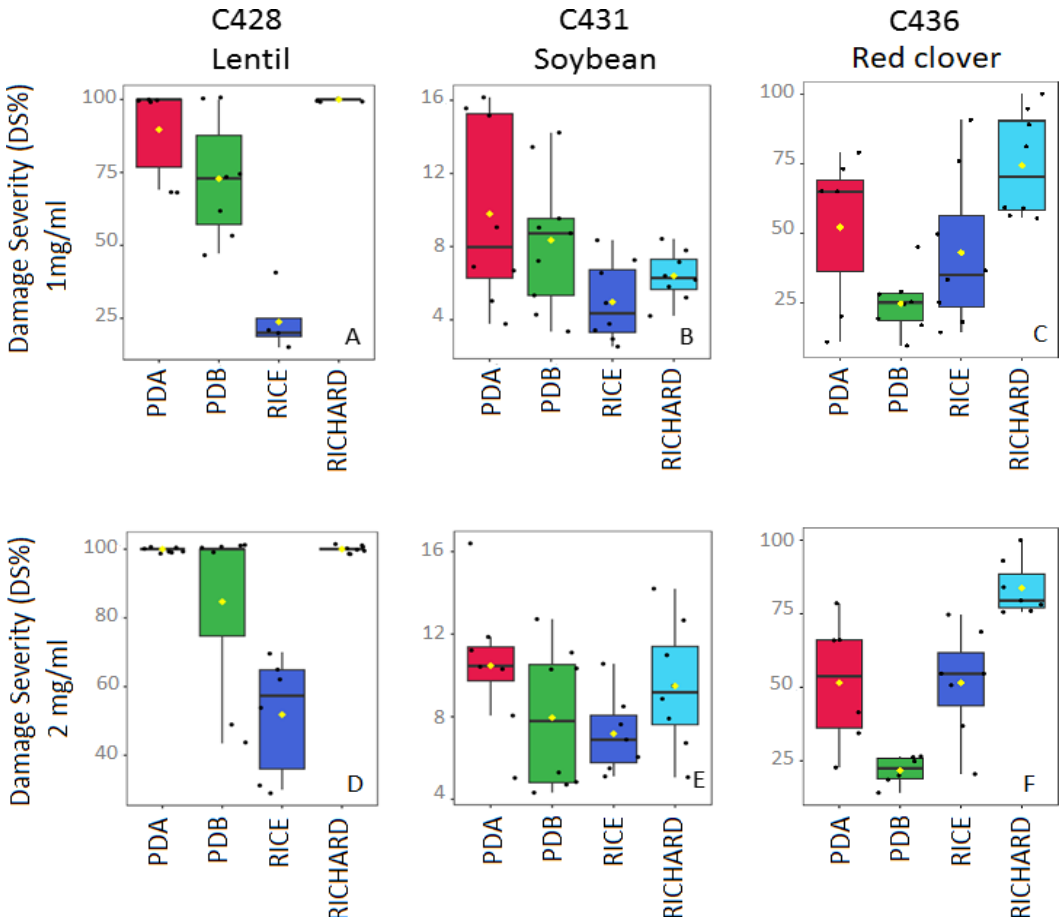

Figure 6. Box plots reporting the DS% of organic extracts of C428, C431, and C436 on the corresponding host, tested at 1 mg/mL and 2 mg/mL. (A) DS% of C428 extracts tested at 1 mg/mL on lentil;(B) DS% of C431 extracts tested at 1 mg/mL on soybean; (C) DS% of C436 extracts tested at 1 mg/mL on white clover; (D) DS% of C428 extracts tested at 2 mg/mL on lentil; (E) DS% of C431 extracts tested at 2 mg/mL on soybean; (F) DS% of C436 extracts tested at 2 mg/mL on white clover.

In terms of legume species, lentil was a crop with significantly higher DS% values, regardless of the culture media employed for its growth or the concentration applied. Necrotic area as well as discolored tissues surrounded by a necrotic ring reached together nearly the 100% of the host leaves in response to fungal extracts from Richard, PDA, and PDB media (Figure 5c-e). Lower values were achieved with rice substrate medium and were dose- dependent (Figures 5f and 6). Similarly, barrel medic also showed high DS% values, but only discolored areas surrounded by necrotic rings were observed. Soybean, faba bean, and pea were poorly affected by all of the culture filtrates, while all the clovers showed a discolored leaf area surrounded by a necrotic ring, with moderate to high DS% values ($17 < DS\% < 89.5$) (Figure 6; Supplementary Table S4). Clovers were especially sensitive to Richard's extracts, as well as to rice, with a dose-dependent effect. In general, C428 fungal exudate from Richard's medium was the most phytotoxic.

3.4.2. *Colletotrichum truncatum* C431 (from Soybean)

Regardless of the culture media, the four tested fungal extracts from C431 showed phytotoxicity on soybean with necrosis and a discolored area surrounded by a necrotic ring, in spite of reduced DS values (<11%) (Figures 5i-l and 6), and with effects that were not dose-dependent except for in Richard's medium. Similar symptoms but higher DS% values were achieved by Richard's medium extract in red clover, white clovers, and lentil (Supplementary Table S4), as well as by rice medium in red clover and barrel medic. In clovers and barrel medic, extended discolored areas are incited by PDA, with associated necrotic rings (in PDB-treated leaves), and with moderate to high DS% values. No symptoms, or only few necrotic spots, were developed with any of the C431 extracts on pea and faba bean. An overall difference in phytotoxic activity between culture media was not found.

3.4.3. *Colletotrichum trifolii* C436 (from Red Clover)

In clovers, all the fungal extracts from C436 caused impaired leaf tissues (discolored areas surrounded by a necrotic ring) compared with the controls (Figure 5). The presence of additional necrotic areas, as well as increasing fungal severity, depends on the cultural media and the concentration applied, with Richard's and rice media being

the most phytotoxic (DS% > 53 and 18 and DS% > 83 and 42, for Richard's and rice medium at 1 and 2 mg/mL, respectively) (Figure 6). In terms of legume species, subterranean clover and barrel medic were the crops with significantly higher sensitivity in almost all media employed for their growth (Supplementary Table S4). In Richard's medium, the diseased area reached up to 80% of the host leaves. Similarly, Richard's medium was also phytotoxic to pea, faba bean, and soybean leaves, but with low to moderate DS% values (11 < DS% < 57). The symptoms in lentil were strongly influenced by the culture media, with the crop being highly susceptible to Richard's medium at concentrations of 2 mg/mL, moderately susceptible to PDB and rice at 2 mg/mL, or poorly diseased following the application of PDA extracts at any concentration.

3.5. Secondary Metabolite Profiles as a Tool for Chemotaxonomy of Colletotrichum *Species*

To explore the possibility of using the untargeted metabolomics profiles for chemo- taxonomical purposes, partial least squares (PLS)-discriminant analysis (DA) was used to differentiate groups and identify the intrinsic variations in the data sets. The peak lists from each cultural media were reorganized, and the analysis was carried out using the *Colletotrichum* isolates/species as classes. As a result, the secondary metabolites specifically produced by *C. truncatum* C428 and C431 and *C. trifolii* C436 could discriminate between fungal species and isolates, regardless of the cultural media (Figure 7). A threshold of 1.6 was used for the VIP score. The normalization process of each data set and the Q2 and R2 values of each PLS-DA model, developed according to the cultural media, are reported in Supplementary Table S2.

Figure 7a shows the score plot of the PLS-DA for the PDA extracts; only eight metabolites had a VIP score higher than 1.6, and half of them capable of discriminating between fungal species and isolates were organic acids and organic acid derivatives, including succinic acid, succinic anhydride, 3-nitropropionic acid, and an organic acid with an *m*/*z* of 115.0033 for which MS/MS data could agree with both fumaric acid and maleic acid. The detection of 3-nitropropionic acid is particularly interesting because it is a potent antimicrobial agent produced by plants and fungi [56,57]. All of these acids were detected in higher amounts in *C. truncatum* C428. Colletopyrone also contributed to discriminating the fungal pathogens, being highly produced by *C. truncatum* C431 in this medium (Figure 7b).

When the PLS-DA analysis was carried out on PDB extracts, 15 metabolites having a VIP score > 1.6 contributed to the class discrimination (Figure 7c,d). Among them, two fusarins with formulas $C_{23}H_{29}NO_6$ and $C_{23}H_{29}NO_9$ and one colletopeptide with the formula $C_{31}H_{33}N_3O_7S$ were upregulated in *C. trifolii* C436. Meanwhile, 3,4-dehydro-6- hydroxymellein, an isocoumarine previously isolated from *Ceratocystis minor* [58] and *Torrubiella tenuis* [59], together with a jasmonic acid derivative with a molecular formula of $C_{12}H_{20}O_3$ were produced in a higher amount by *C. truncatum* C428. Several fungi have been reported to produce jasmonic acids to manipulate and/or hijack plant hormone defense signaling cascades for their own benefit [60,61]. Another isocoumarine, dereplicated as fusarentin 6,7-dimethyl ether and previously isolated from *Colletotrichum* spp. [62], together with methylsidowate, a sesquiterpenoid previously isolated from *Aspergillus* spp. [63], and berkchaetorubramine contributed to the discrimination being produced in higher amounts in this medium by *C. truncatum* C431.

The score plot of PLS-DA analysis of the metabolic profiles of the rice extracts, reported in Figure 5e, shows three clear groups according to the *Colletotrichum* species and isolates. Eighteen compounds had a VIP score value higher than 1.6. Most of the discriminant metabolites were detected in higher concentrations in the extracts of *C. trifolii* C436. Interestingly, different from the PDB, the production of fusarentin 6,7-dimethyl ether is upregulated in *C. trifolii* C436 (Figure 7f). Moreover, three colletopeptides with formulas $C_{25}H_{29}N_5O_7$, $C_{28}H_{42}N_4O_5$, and $C_{29}H_{45}N_5O_5$, a fusarin with formula $C_{23}H_{31}NO_8$, and colletol and colletodiol, two macrolides previously isolated from *Colletotrichum capsici* [64], were also detected in higher amounts by C436. Among the metabolites upregulated in *C. truncatum* C431 that also contributed to the class discrimination, two isocoumarines, alternariol, a mycotoxin previously isolated from *Alternaria* species [65] but also from *Colletotrichum* species [66], and 6-hydroxymellein were dereplicated (Figure 7f).

Finally, the secondary metabolite profile of Richard's medium extracts allows for categorizing the three *Colletotrichum* according to isolates and species (Figure 7g). Fifteen metabolites showed a VIP score > 1.6 and contributed to the class discrimination (Figure 7h).

Most discriminant metabolites were detected in higher concentrations in *C. truncatum* C431. Among them, berkchaetorubramine, TMC-205, an indole alkaloid previously isolated from unidentified fungal strain TC 1630 [67], pantothenic acid, also known as vitamin B5 and important for homeostasis and virulence of *Aspergillus*

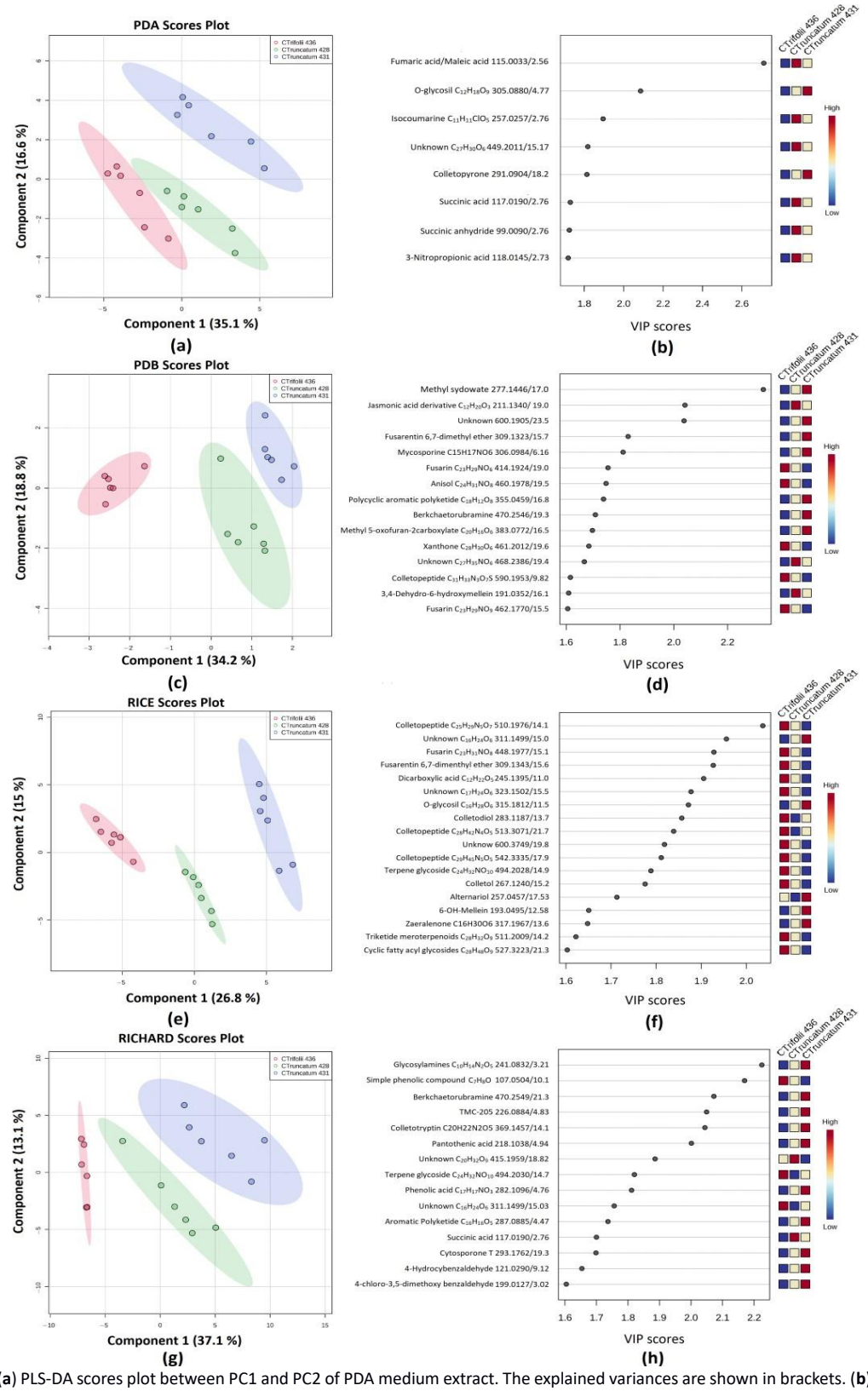

Figure 7. (**a**) PLS-DA scores plot between PC1 and PC2 of PDA medium extract. The explained variances are shown in brackets. (**b**) Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in *C. trifolii* C436, *C. truncatum* C428 and C431 extracts, respectively. (**c**) PLS-DA scores plot between PC1 and PC2 of PDB medium extract. The explained variances are shown in brackets. (**d**) Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in *C. trifolii* C436*, C. truncatum* C428 and C431 extracts, respectively. (**e**) PLS- DA scores plot between PC1 and PC2 of RICE medium extract. The explained variances are shown in brackets. (**f**) Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in *C. trifolii* C436, *C. truncatum* C428, and *C. truncatum* C431 extracts, respectively. (**g**) PLS-DA scores plot between PC1 and PC2 of Richard's medium extract. The explained variances are shown in brackets. (**h**) Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in C. trifolii C436, C. truncatum C428, and C. truncatum C431 extracts, respectively.

fumigatus [68], cytosporone T, an octaketides previously isolated from *Phomopsis* sp. IFB-ZS1-S4 [69], 4-hydroxybenzaldehyde and 4-chloro-3,5-dimethoxybenzaldehyde, a chlorinated orcinol derivative with antimicrobial activity and previously isolated from *Hericium erinaceum* [70], were also putatively annotated and upregulated in *C. truncatum* C431 (Figure 5h).

4. Discussion

Among the diseases affecting legumes, anthracnose, caused by fungal pathogens from the genus *Colletotrichum*, is one of the most economically significant [2]. Fungal isolates such as *C. truncatum* and *C. trifolii* affect different agronomically important grain and forage legumes [3], and their host specificity varies with the plant species from which they are obtained [71]. In the present work, we were studying the differential disease severity caused by three *Colletotrichum* isolates on a panel of different legumes, with a special focus on the phytotoxic metabolites produced by each pathogen, how these metabolites could be responsible for the exhibited host specificity, and how the in vitro growth conditions could affect their production. To the best of our knowledge, this is the first time that the OSMAC strategy, integrated with targeted and untargeted metabolomics approaches, has been applied to *Colletotrichum* species involved in legume diseases. Nevertheless, this strategy was previously applied to other fungal species [27]. Moreover, this is the first time that low-molecular-weight phytotoxins have been dereplicated from an isolate of *C. trifolii*.

It has been described that isolates of *C. truncatum* from lentil and from soybean produced distinct symptoms in their own hosts, as well as in different legume crops under controlled conditions. Our results on host specificity support the case for taxonomic separation of the pathogen *C. truncatum*, which agrees with previous studies [12,72]. Under controlled conditions, in the current study, the isolate from lentil was pathogenic to lentil, white clover, faba bean, and pea, while few or no symptoms were found in soybean and barrel medic, confirming previous findings [12]. In contrast, inoculation with the isolate from soybean produced visible lesions in soybean, and, to a lesser extent, also in lentils, barrel medic, and clovers. However, pea and faba bean were the hosts that developed the fewest symptoms of infection. This is also in line with previously developed studies on several hosts under both field and controlled conditions, where

important differences in latent period and infection strategies between isolates from lentil and soybean were found [12,72]. Several reports indicate that anthracnose symptoms appear to be triggered by a change in host physiology, especially when plant tissues are under stress [73,74]. Indeed, many chemical and physical factors directly or indirectly contribute to metabolic pathway activation, including phytotoxic secondary metabolites produced by the fungus [26]. In this study, the metabolomics analysis performed on the fungal extracts, together with the chemometrics analysis, has pinpointed a total of 84 discriminant metabolites. In detail, 9 compounds were validated with pure standards (level A), 20 were putatively annotated (levels B(i) and B(ii)), and for 43 compounds it was possible to assign only the natural product class (level C(i) and C(ii)), while 12 remained unknown. As a general result, these secondary metabolites produced by *Colletotrichum* species belong to numerous natural products classes, including alkaloids, terpenoids, coumarins, chromones, xanthones, polyketides, quinones, peptides, phenols, and macrolides. These results agree with the capability of this fungal genus to produce a wide range of interesting bioactive compounds [4,20]. Furthermore, our data show that their production is heavily dependent on the selected cultural media.

Among specific *Colletotrichum* spp., all the fungal pathogens studied in this investigation produced colletopyrone, and higginsianin B, the identity of which was validated with pure standards. Moreover, their concentrations depend on the cultural conditions. Colletopyrone is a bioactive polyketide-derived compound that contains a fused six-membered and eight-membered ring system with a lactone and a ketone group. It is produced by various species of *Colletotrichum* and exhibits a range of biological activities, including phytotoxicity, cytotoxicity, and antifungal properties [20]. Higginsianin B, a diterpenoid α-pyrones, was previously isolated from *Colletotrichum higginsianum* and showed cytostatic activity against cancer cells [32]. Furthermore, 6-hydroxymellein was also detected in all the organic extracts, and its concentration depended on the cultural conditions. This compound belongs to the class of isocoumarins and is reported to have weak antimicrobial, cytotoxic, and phytotoxic activity [75]. Even though it was previously produced by several fungal species, this is the first time that has been reported in *Colletotrichum* species. Another interesting result that arose from the targeted analysis was that the production of colletochlorin E and higginsianin A seems to be species-specific and cultural medium-specific. Indeed, colletochlorin E was detected only in Richard's medium extracts of *C. truncatum* isolates. This metabolite is tetrasubstituted pyran-2-one, previously isolated from *C. higginsianum*, and it showed

only modest phytotoxic activity in *Sonchus arvensis* and tomato leaves [33]. On the other hand, higginsianin A was detected only in the PDA extracts of *C. trifolii* C436. This metabolite was isolated together with higginsianin B from *C. higginsianum* and showed cytostatic activity.

The chemometric analysis of the untargeted metabolomics profiles of the organic extracts showed that the selected cultural media (PDA, PDB, Richard's medium, and rice) influence the production of specific secondary metabolites, and even though the up-regulated or downregulated compounds depend on the *Colletotrichum* species, as reported in the results sections, some general patterns can be highlighted. Various small and/or hydroxylated organic acids, including succinic acid, fumaric acid/maleic acid, trihydroxy octadecanoic acid, hydroxybutyric acid, and dihydroxybutyric acid were upregulated in PDA and Richard's media. It is important to underline that these compounds are primary metabolites, but they are usually released in the culture medium by the fungus. The optimization of their production and extraction process from fungi is an important challenge in the biotechnology field [76]. Nevertheless, pathogenic fungi can produce various small organic acids, including succinic, oxalic acid, or fumaric acid, which play important roles in fungal pathogenesis [77,78]. Moreover, the hydroxylated derivatives of fatty acids might have a role in fungal quorum sensing [79]. The production of colletopeptides and fusarins was upregulated when the *Colletotrichum* species were grown in PDB and rice media. This result was more evident for *C. trifolii* C436. Five colletopeptides were annotated at the level of confidence of C(ii). Several cyclic depsipeptides have been isolated from microorganisms, especially fungi in the genera *Aspergillus*, *Beauveria*, *Fusarium*, *Penicillium*, and *Colletotrichum* [80,81]. They show different biological activities, and they can play a role in the fungal defensive response. Seven fusarins were annotated at the level of confidence of C(ii). Fusarins are mycotoxins produced by *Fusarium* and other fungal species [82,83]. They are important virulence factors for *Fusarium* species, as they can suppress the host's immune response and promote fungal colonization [82]. Moreover, their production could be influenced by various genetic and environmental factors [83,84]. Finally, the production of macrolides colletodiol, colletol in *C. trifolii* C436, and Sch-725674, and phomolide B in *C. truncatum* isolates was upregulated in rice culture. These results further support the capability of *C. truncatum* isolates to produce macrolides, as recently reported [22]. Fungi, including the *Colleotrichum* genus, are an important source of macrolides [20,85]. Various enzymes, including polyketide synthases and non-ribosomal peptide synthetases, mediate their

biosynthesis. These compounds have diverse biological activities, including antimicrobial, antifungal, anticancer, and insecticidal properties, making them attractive targets for drug discovery and agricultural applications [86,87]. Regarding the latter, fungal diseases of crops of agronomic importance are usually chemically controlled by commercial fungicides from multiple chemical compound groups when no genetic resistance is available. However, pathogens have the ability to develop resistance to several chemical classes of compounds within a few years. This situation has decreased the efficacy of the major fungicides that are employed against crop pathogens, leading to the application of general integrated disease management strategies such as dose limitation, mixtures, and the continuous search for novel antimicrobial compounds [23]. Many plant pathogens, especially necrotrophic fungi such as *Colletotrichum* species, as we have seen here, are capable of producing a broad panel of natural substances representing an unexploited source of potential bio-fungicides with new molecular structures and modes of action against several crop diseases that should be tested in the near future.

Overall, these results could be a useful starting point for more in-depth studies, while also integrating them with other omics techniques to shed light on the effects of cultural conditions on bioactive metabolite pathway activation in *Colletotrichum* species. The im- provement in the culture conditions and a better knowledge of the regulation of secondary metabolism in *Colletotrichum* species are also essential in order to evaluate the impact on the phytotoxicity of extracts. Our results showed that their phytotoxicity varies depend- ing on legume species, fungal isolates, cultural conditions, and the tested concentration. Furthermore, the high phytotoxicity of Richard's medium extracts of *C. truncatum* C428 and *C. trifolii* C436 agrees with data previously reported [88–90]. These pathogens in this medium can produce some non-host-specific phytotoxins capable of inducing necrosis on all the plant species tested in our studies, even with different intensities. This variation in phytotoxicity of organic extracts on different plant species might be attributed to several factors, including differences in plant physiology and the chemical composition of the extract. Indeed, as highlighted by the metabolomics analysis, the chemical composition of organic extracts varied according to the cultural conditions, which affected their interaction with different legumes. This caused varied responses ranging from low effects to severe damage. Our results could help to select a specific cultural condition to investigate target phytotoxic metabolites involved in each pathosystem. For example, the severity of symptoms induced in lentil by *C. truncatum*

C428 was higher for both PDA and Richard's broth extracts, followed by PDB. Thus, the former extracts could be studied to investigate the role of small and/or hydroxylated organic acids in pathogenicity, while PDB extract could be investigated to shed light on the roles of colletopeptides and fusarins in disease development. On the contrary, for *C. truncatum* isolate C431, despite inducing high disease symptoms in a cross-inoculation assay, a low phytotoxicity of the organic extract was observed, regardless of the cultural conditions. These differences in symptom development, also observed for other legumes in a cross-inoculation assay, may be attributed to the pathogen's ability to produce high-molecular-weight phytotoxins that remain unextracted under the used condition. These unextracted compounds could have a synergistic effect, exacerbating the observed symptoms [91]. Indeed, toxic exopolysaccharides and extracellular peptides have been isolated from other fungal species [92,93]. Hence, future investigations could explore the production of high-molecular-weight phytotoxins by *Colletotrichum* species to gain a better understanding of their role in pathogenicity. It is noteworthy that the highest phytotoxicity on all studied clover species for *C. trifolii* isolate C436 was observed with Richard's medium extract. However, since the isolate was collected from red clover, which exhibited the highest severity index in the cross-inoculation assay, the PDA's extract was the most specific, causing greater phytotoxicity in *Trifolium pratense* compared to other *Trifolium* species. Studying specific compounds could help us to fully understand the mechanisms behind host-pathogen interactions. Indeed, follow-up studies are underway to correlate the phytotoxic activity with specific metabolites. Nevertheless, the in vitro bioassay should be considered only as a starting point in investigating the role of secondary metabolites in symptom development. Only in planta studies can confirm their involvement in the pathogenicity or virulence, or if they have another role in the ecology of these fungi.

Information on the chemotaxonomy of *Colletotrichum* spp. is minimal. Our results showed that secondary metabolites could help to classify the three *Colletotrichum* at both species and isolate levels. These results could assist species identification, enabling precise classification and enhancing our understanding of fungal diversity. Furthermore, they could support ecological studies by unraveling interactions between fungi and their host plants. In our research, C428 was isolated from lentils, while C431 was isolated from soybeans, and the observed metabolic differences may reflect the level of specificity in host selection. However, further in-depth studies are required to identify appropriate biomarkers for *Colletotrichum* spp. chemotaxonomy. This outcome is also essential for

the selection of a proper phytotoxic metabolites pool for further in planta studies with the aims of (i) understanding the role of phytotoxins in the pathogenicity ad virulence of *C. truncatum* and *C. trifolii*, and (ii) selecting potential specific biomarkers for the early detection of anthracnose-assisted molecular methods.

At this stage, it is essential to highlight the importance of chemometric tools in metabolomics studies to fully explore the biological meaning behind the feature tables [94]. Indeed, applying a combination of univariate and multivariate analysis such as ANOVA, heatmap, PCA, and PLS-DA allows us to visualize hidden patterns in the data. Nevertheless, the PLS-DA algorithm should be regarded as just one building block in the steps used to develop a classification model, mainly when the study involves small sample sizes and large numbers of variables [95], such as the secondary metabolites produced by fungi. Furthermore, fungal metabolites' vast chemical diversity and complexity contribute to the main bottleneck of untargeted metabolomics using LC-MS/MS: the unbiased structure assignment to metabolites of interest. In fact, despite outstanding progress made in the last decade in growing the number of metabolites in databases, many of the signals detected in metabolomics experiments cannot be directly assigned to specific metabolites because of the absence of their spectra in metabolomics databases [28,96]. This is particularly true for fungal metabolites due to the absence of wide and specific database spectra libraries. Modern dereplication strategies have been developed to tackle this problem, including machine learning, in silico fragmentation, or molecular networking [97–100]. Moreover, adding taxonomical information, or, in general, metadata from previous biological knowledge, and using a combination of bioinformatic tools, as was carried out in this investigation, could assist in obtaining a correct dereplication of the fungal metabolites [101,102]. Another significant limitation of this approach is that standard spectral library matching or in silico fragmentation cannot distinguish between potential stereo- and regioisomers, resulting in a level C annotation, or the fragmentation profile could agree with several classes, resulting in an unknown classification. This was also clear in our study, where more than 65% of the discriminant metabolites were level C or unknown. Dereplication strategies are fundamental to screen the crude extracts for the presence of known compounds and to identify compounds produced in a very low amount that could be undetected or lost during tedious purification steps. However, using orthogonal analytical methods, such as NMR, to validate metabolites, reach level A identification, and dereplicate unknown or characterize novel metabolites is unavoidable. Only by elucidating unknown metabolites, including

assigning absolute or no relative configuration to chiral carbons, can we decipher complex biological systems.

5. Conclusions

Studying secondary metabolites, and in particular phytotoxic metabolites, is essential in order to gain information on the pathogenicity or virulence of a specific pathogen, and also in order to develop more sustainable control methods. Indeed, this research could be beneficial for investigations dealing with fungal chemotaxonomy [24], host–pathogen interactions and searching for potential fungal biomarkers [103–105], or, more generally, investigations dealing with genomics, transcriptomics, and proteomics in order to provide a more comprehensive understanding of fungal metabolism and physiology [106,107]. Finally, considering that *Colletotrichum* species are an important source of bioactive metabolites, as well as the continuous search for novel antimicrobial compounds and the increasing rate of fungal diseases worldwide, these data could also be used for multidisciplinary studies in life science and drug discovery.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/jof9060610/s1, Figure S1. *m*/*z* distribution diagrams and Base Peak Chromatograms of *C. truncatum* C428 (from lentil) extracts. (a) PDA extract; (b) PDB extract; Rice extract; (d) Richard extract; Figure S2. *m*/*z* distribution diagrams and Base Peak Chro- matograms of *C. truncatum* C431 (from soybean) extracts. (a) PDA extract; (b) PDB extract; (c) Rice extract; (d) Richard extract; Figure S3. *m*/*z* distribution diagrams and Base Peak Chromatograms of *C. trifolii* C436 (from clover) extracts. (a) PDA extract; (b) PDB extract; (c) Rice extract; (d) Richard extract; Figure S4. Structure of validate and putatively identified metabolites: validated metabolites with pure standards (level A; red); putatively identified and produced by *Colletotrichum* spp. (level B(i), blue); putatively identified and produced by other fungal species (level B(ii), black); Table S1. Parameters Metaboanalyst 5.0 for LC-MS spectra processing; Table S2. Features lists transformation and scaling for PLS-DA analysis. Q2 and R2 values of PLS-DA models in cross validation; Table S3. Metabolites dereplicated by targeted and untargeted metabolomics analysis, organized according to identification

level; Table S4. Phytotoxic bioassay carried out on various legumes with organic extracts from *Colletotrichum* spp. Symptoms observed after 13 days.

Author Contributions: Conceptualization, P.R., D.R. and E.B.; methodology, P.R. and E.B.; validation, D.R., A.E. and M.M.; investigation, P.R., F.J.A.-J. and E.B.; data curation, P.R., E.B. and F.J.A.-J.; writing—original draft preparation, P.R. and E.B.; writing—review and editing, F.J.A.-J., A.E., M.M. and D.R.; visualization, P.R. and F.J.A.-J.; supervision, D.R.; funding acquisition, D.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Agencia Estatal de Investigación (AEI), grant PID2020-114668RB-I00.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data that arose from this research are contained in the manuscript and Supplementary Materials. Data sets and raw mass spectrometry data are available on request from the authors.

Acknowledgments: We acknowledge the Metabolomics Platform of Agricultural Sciences, Food Science and Technology and Natural Resources (CEBAS)-CSIC, Murcia, Spain, for carrying out the UHPLC-QTOF-HRMS metabolomics analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Stagnari, F.; Maggio, A.; Galieni, A.; Pisante, M. Multiple benefits of legumes for agriculture sustainability: An overview. *Chem. Biol. Technol. Agric.* **2017**, *4*, 2. [CrossRef]
2. Rubiales, D.; Fondevilla, S.; Chen, W.; Gentzbittel, L.; Higgins, T.J.; Castillejo, M.A.; Singh, K.B.; Rispail, N. Achievements and challenges in legume breeding for pest and disease resistance. *Crit. Rev. Plant Sci.* **2015**, *34*, 195–236. [CrossRef]
3. Cannn, P.; Damm, U.; Johnston, P.; Weir, B. *Colletotrichum*: Current status and future directions. *Stud. Mycol.* **2012**, *73*, 181–213.[CrossRef]
4. García-Pajón, C.; Collado, I.G. Secondary metabolites isolated from *Colletotrichum* species. *Nat. Prod. Rep.* **2003**, *20*, 426–431. [CrossRef]

5. O'Connell, R.J.; Thon, M.R.; Hacquard, S.; Amyotte, S.G.; Kleemann, J.; Torres, M.F.; Damm, U.; Buiate, E.A.; Epstein, L.; Alkan, N. Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. *Nat. Genet.* **2012**, *44*, 1060–1065. [CrossRef]
6. Boufleur, T.R.; Ciampi-Guillardi, M.; Tikami, Í.; Rogério, F.; Thon, M.R.; Sukno, S.A.; Massola Junior, N.S.; Baroncelli, R. Soybean anthracnose caused by *Colletotrichum* species: Current status and future prospects. *Mol. Plant Pathol.* **2021**, *22*, 393–409.
7. Begum, M.; Sariah, M.; Puteh, A.; Abidin, M.Z. Pathogenicity of *Colletotrichum truncatum* and its influence on soybean seed quality. *Int. J. Agric. Biol.* **2008**, *10*, 393–398.
8. Tullu, A.; Buchwaldt, L.; Lulsdorf, M.; Banniza, S.; Barlow, B.; Slinkard, A.; Sarker, A.; Tar'an, B.; Warkentin, T.; Vandenberg, A. Sources of resistance to anthracnose (*Colletotrichum truncatum*) in wild *Lens* species. *Genet. Resour. Crop Evol.* **2006**, *53*, 111–119. [CrossRef]
9. O'Connell, R.; Uronu, A.; Waksman, G.; Nash, C.; Keon, J.; Bailey, J. Hemibiotrophic infection of *Pisum sativum* by *Colletotrichum truncatum*. *Plant Pathol.* **1993**, *42*, 774–783. [CrossRef]
10. Schubiger, F.; Streckeisen, P.; Boller, B. Resistance to southern anthracnose (*Colletotrichum trifolii*) in cultivars of red clover (*Trifolium pratense*). *Czech J. Genet. Plant Breed.* **2003**, *39*, 309.
11. Mould, M.J.; Boland, G.; Robb, J. Ultrastructure of the *Colletotrichum trifolii-Medicago sativa* pathosystem. I. Pre-penetration events.*Physiol. Mol. Plant Pathol.* **1991**, *38*, 179–194. [CrossRef]
12. Gossen, B.; Anderson, K.; Buchwaldt, L. Host specificity of *Colletotrichum truncatum* from lentil. *Can. J. Plant Pathol.* **2009**, *31*, 65–73. [CrossRef]
13. Pradhan, A.; Ghosh, S.; Sahoo, D.; Jha, G. Fungal effectors, the double edge sword of phytopathogens. *Curr. Genet.* **2021**, *67*, 27–40.
14. Evidente, A.; Cimmino, A.; Masi, M. Phytotoxins produced by pathogenic fungi of agrarian plants. *Phytochem. Rev.* **2019**, *18*, 843–870. [CrossRef]
15. Möbius, N.; Hertweck, C. Fungal phytotoxins as mediators of virulence. *Curr. Opin. Plant Biol.* **2009**, *12*, 390–398. [CrossRef]
16. Cox, R.J. Polyketides, proteins and genes in fungi: Programmed nano-machines begin to reveal their secrets. *Org. Biomol. Chem.* **2007**, *5*, 2010–2026. [CrossRef]

17. Scharf, D.H.; Heinekamp, T.; Brakhage, A.A. Human and plant fungal pathogens: The role of secondary metabolites. *PLoS Pathog.* **2014**, *10*, e1003859. [CrossRef]
18. Pusztahelyi, T.; Holb, I.J.; Pócsi, I. Secondary metabolites in fungus-plant interactions. *Front. Plant Sci.* **2015**, *6*, 573. [CrossRef]
19. De Silva, D.D.; Crous, P.W.; Ades, P.K.; Hyde, K.D.; Taylor, P.W. Life styles of *Colletotrichum* species and implications for plant biosecurity. *Fungal Biol. Rev.* **2017**, *31*, 155–168. [CrossRef]
20. Moraga, J.; Gomes, W.; Pinedo, C.; Cantoral, J.M.; Hanson, J.R.; Carbú, M.; Garrido, C.; Durán-Patrón, R.; Collado, I.G. The current status on secondary metabolites produced by plant pathogenic *Colletotrichum* species. *Phytochem. Rev.* **2019**, *18*, 215–239. [CrossRef]
21. Stoessl, A.; Stothers, J. Colletruncoic acid methyl ester, a unique meroterpenoid from *Colletotrichum truncatum*. *Z. Für Nat. C* **1986**, *41*, 677–680. [CrossRef]
22. Masi, M.; Castaldi, S.; Sautua, F.; Pescitelli, G.; Carmona, M.A.; Evidente, A. Truncatenolide, a bioactive disubstituted nonenolide produced by *Colletotrichum truncatum*, the causal agent of Aanthracnose of soybean in Argentina: Fungal antagonism and SAR Sstudies. *J. Agr. Food Chem.* **2022**, *70*, 9834–9844. [CrossRef]
23. Agudo-Jurado, F.J.; Reveglia, P.; Rubiales, D.; Evidente, A.; Barilli, E. Status of phytotoxins isolated from necrotrophic fungi causing diseases on grain legumes. *Int. J. Mol. Sci.* **2023**, *24*, 5116. [CrossRef]
24. Frisvad, J.C.; Andersen, B.; Thrane, U. The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. *Mycol. Res.* **2008**, *112*, 231–240. [CrossRef]
25. Kim, W.; Peever, T.L.; Park, J.-J.; Park, C.-M.; Gang, D.R.; Xian, M.; Davidson, J.A.; Infantino, A.; Kaiser, W.J.; Chen, W. Use of metabolomics for the chemotaxonomy of legume-associated *Ascochyta* and allied genera. *Sci. Rep.* **2016**, *6*, 20192. [CrossRef]
26. Pan, R.; Bai, X.; Chen, J.; Zhang, H.; Wang, H. Exploring structural diversity of microbe secondary metabolites using OSMAC strategy: A literature review. *Front. Microbiol.* **2019**, *10*, 294. [CrossRef]
27. Farinella, V.F.; Kawafune, E.S.; Tangerina, M.M.; Domingos, H.V.; Costa-Lotufo, L.V.; Ferreira, M.J. OSMAC strategy inte- grated with molecular networking for

accessing griseofulvin derivatives from endophytic fungi of *Moquiniastrum polymorphum* (Asteraceae). *Molecules* **2021**, *26*, 7316. [CrossRef]

28. Li, G.; Jian, T.; Liu, X.; Lv, Q.; Zhang, G.; Ling, J. Application of metabolomics in fungal research. *Molecules* **2022**, *27*, 7365. [CrossRef]
29. Ball, B.; Langille, M.; Geddes-McAlister, J. Fun (gi) omics: Advanced and diverse technologies to explore emerging fungal pathogens and define mechanisms of antifungal resistance. *Mbio* **2020**, *11*, e01020. [CrossRef]
30. Khan, A.I.; Bhandari, R.R.; Pokhrel, A.; Yadav, R.N. A study on root exudation pattern and effect of plant growth promoting fungi during biotic and abiotic stress in pigeon pea. *World J. Agric. Res.* **2018**, *6*, 122–131.
31. Masi, M.; Zonno, M.C.; Cimmino, A.; Reveglia, P.; Berestetskiy, A.; Boari, A.; Vurro, M.; Evidente, A. On the metabolites produced by *Colletotrichum gloeosporioides* a fungus proposed for the *Ambrosia artemisiifolia* biocontrol; spectroscopic data and absolute configuration assignment of colletochlorin A. *Nat. Prod. Res.* **2017**, *32*, 1537–1547. [CrossRef]
32. Cimmino, A.; Mathieu, V.; Masi, M.; Baroncelli, R.; Boari, A.; Pescitelli, G.; Ferderin, M.N.; Lisy, R.; Evidente, M.; Tuzi, A.; et al. Higginsianins A and B, two diterpenoid α-pyrones produced by *Colletotrichum higginsianum*, with in vitro cytostatic activity. *J. Nat. Prod.* **2016**, *79*, 116–125. [CrossRef]
33. Masi, M.; Cimmino, A.; Boari, A.; Tuzi, A.; Zonno, M.C.; Baroncelli, R.; Vurro, M.; Evidente, A. Colletochlorins E and F, new phytotoxic tetrasubstituted pyran-2-one and dihydrobenzofuran, isolated from *Colletotrichum higginsianum* with potential herbicidal activity. *J. Agr. Food Chem.* **2017**, *65*, 1124–1130. [CrossRef]
34. Masi, M.; Reveglia, P.; Baaijens-Billones, R.; Górecki, M.; Pescitelli, G.; Savocchia, S.; Evidente, A. Phytotoxic metabolites from three *Neofusicoccum* species causal agents of Botryosphaeria dieback in Australia, luteopyroxin, neoanthraquinone, and Lluteoxepinone, a disubstituted furo-α-pyrone, a hexasubstituted anthraquinone, and a trisubstituted oxepi-2-one from *Neofusicoccum luteum*. *J. Nat. Prod.* **2020**, *83*, 453–460.
35. Cimmino, A.; Andolfi, A.; Zonno, M.C.; Avolio, F.; Berestetskiy, A.; Vurro, M.; Evidente, A. Chenopodolans A–C: Phytotoxic furopyrans produced by *Phoma chenopodiicola*, a fungal pathogen of *Chenopodium album*. *Phytochemistry* **2013**, *96*, 208–213. [CrossRef]

36. Reveglia, P.; Savocchia, S.; Billones-Baaijens, R.; Cimmino, A.; Evidente, A. Isolation of phytotoxic phenols and characterization of a new 5-hydroxymethyl-2-isopropoxyphenol from *Dothiorella vidmadera*, a causal agent of grapevine trunk disease. *J. Agr. Food Chem.* **2018**, *66*, 1760–1764. [CrossRef]
37. Reveglia, P.; Savocchia, S.; Billones-Baaijens, R.; Masi, M.; Evidente, A. Spencertoxin and spencer acid, new phytotoxic derivatives of diacrylic acid and dipyridinbutan-1,4-diol produced by *Spencermartinsia viticola*, a causal agent of grapevine Botryosphaeria dieback in Australia. *Arab. J. Chem.* **2018**, *13*, 1803–1808. [CrossRef]
38. Pang, Z.; Chong, J.; Zhou, G.; de Lima Morais, D.A.; Chang, L.; Barrette, M.; Gauthier, C.; Jacques, P.-É.; Li, S.; Xia, J. MetaboAnalyst 5.0: Narrowing the gap between raw spectra and functional insights. *Nucleic Acids Res.* **2021**, *49*, W388–W396. [CrossRef]
39. Tsugawa, H.; Cajka, T.; Kind, T.; Ma, Y.; Higgins, B.; Ikeda, K.; Kanazawa, M.; VanderGheynst, J.; Fiehn, O.; Arita, M. MS-DIAL: Data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nat. Met.* **2015**, *12*, 523–526. [CrossRef]
40. Ruttkies, C.; Neumann, S.; Posch, S. Improving MetFrag with statistical learning of fragment annotations. *BMC Bioinform.* **2019**, *20*, 376. [CrossRef]
41. van Santen, J.A.; Poynton, E.F.; Iskakova, D.; McMann, E.; Alsup, T.A.; Clark, T.N.; Fergusson, C.H.; Fewer, D.P.; Hughes, A.H.; McCadden, C.A. The Natural Products Atlas 2.0: A database of microbially-derived natural products. *Nucleic Acids Res.* **2022**, *50*, D1317–D1323. [CrossRef]
42. Sorokina, M.; Merseburger, P.; Rajan, K.; Yirik, M.A.; Steinbeck, C. COCONUT online: Collection of open natural products database. *J. Cheminform.* **2021**, *13*, 2. [CrossRef]
43. Rutz, A.; Sorokina, M.; Galgonek, J.; Mietchen, D.; Willighagen, E.; Gaudry, A.; Graham, J.G.; Stephan, R.; Page, R.; Vondrášek, J. The LOTUS initiative for open knowledge management in natural products research. *Elife* **2022**, *11*, e70780. [CrossRef]
44. Alseekh, S.; Aharoni, A.; Brotman, Y.; Contrepois, K.; D'Auria, J.; Ewald, J.; Ewald, J.C.; Fraser, P.D.; Giavalisco, P.; Hall, R.D. Mass spectrometry-based metabolomics: A guide for annotation, quantification and best reporting practices. *Nat. Met.* **2021**, *18*, 747–756. [CrossRef]

45. Sumner, L.W.; Amberg, A.; Barrett, D.; Beale, M.H.; Beger, R.; Daykin, C.A.; Fan, T.W.-M.; Fiehn, O.; Goodacre, R.; Griffin, J.L. Proposed minimum reporting standards for chemical analysis. *Metabolomics* **2007**, *3*, 211–221. [CrossRef]
46. Andolfi, A.; Cimmino, A.; Villegas-Fernández, A.; Tuzi, A.; Santini, A.; Melck, D.; Rubiales, D.; Evidente, A. Lentisone, a new phytotoxic anthraquinone produced by *Ascochyta lentis*, the causal agent of Ascochyta blight in *Lens culinaris*. *J. Agric. Food Chem.* **2013**, *61*, 7301–7308. [CrossRef]
47. Abraham, W.-R.; Meyer, H.; Abate, D. Curvupallides, a new class of alkaloids from the fungus *Curvularia pallescens*. *Tetrahedron* **1995**, *51*, 4947–4952. [CrossRef]
48. Du, X.; Lu, C.; Li, Y.; Zheng, Z.; Su, W.; Shen, Y. Three new antimicrobial metabolites of *Phomopsis* sp. *J. Antibiot.* **2008**, *61*, 250–253. [CrossRef]
49. Feng, L.; Wang, J.; Liu, S.; Zhang, X.-J.; Bi, Q.-R.; Hu, Y.-Y.; Wang, Z.; Tan, N.-H. Colletopeptides A-D, anti-inflammatory cyclic tridepsipeptides from the plant endophytic fungus *Colletotrichum* sp. S8. *J. Nat. Prod.* **2019**, *82*, 1434–1441.
50. Kleigrewe, K.; Aydin, F.; Hogrefe, K.; Piecuch, P.; Bergander, K.; Würthwein, E.-U.; Humpf, H.-U. Structure elucidation of new fusarins revealing insights in the rearrangement mechanisms of the *Fusarium* mycotoxin fusarin C. *J. Agric. Food Chem.* **2012**, *60*, 5497–5505. [CrossRef]
51. List, P.; Reith, H. Basische Pilzinhaltsstoffe, X. Imidazolderivate im Faltentintling, *Coprinus atramentarius* Bull. no. *Jahresband* **1960**, *319*, 17–21.
52. Lee, H.-B.; Choi, Y.-C.; Kim, S.-U. Isolation and identification of maculosins from *Streptomyces rochei* 87051-3. *Appl. Biol. Chem.* **1994**, *37*, 339–342.
53. Park, S.H.; Strobel, G.A. Cellular protein receptors of maculosin, a host specific phytotoxin of spotted knapweed (*Centaurea maculosa* L.). *Biochim. Biophys. Acta Gen. Subj.* **1994**, *1199*, 13–19. [CrossRef]
54. Stierle, A.A.; Stierle, D.B.; Girtsman, T.; Mou, T.; Antczak, C.; Djaballah, H. Azaphilones from an acid mine extremophile strain of a *Pleurostomophora* sp. *J. Nat. Prod.* **2015**, *78*, 2917–2923. [CrossRef]
55. Liu, W.; Chen, S.; Li, J.; Yang, X.; Yan, C.; Liu, H. A new β-tetralonyl glucoside from the *Santalum album* derived endophytic fungus *Colletotrichum* sp. GDMU-1. *Nat. Prod. Res.* **2019**, *33*, 354–359. [CrossRef]

56. Evidente, A.; Capretti, P.; Giordano, F.; Surico, G. Identification and phytotoxicity of 3-nitropropanoic acid produced in vitro by *Melanconis thelebola*. *Experientia* **1992**, *48*, 1169–1172. [CrossRef]
57. Francis, K.; Smitherman, C.; Nishino, S.F.; Spain, J.C.; Gadda, G. The biochemistry of the metabolic poison propionate 3-nitronate and its conjugate acid, 3-nitropropionate. *IUBMB Life* **2013**, *65*, 759–768. [CrossRef]
58. Ayer, W.A.; Attah-Poku, S.K.; Browne, L.M.; Orszanska, H. The chemistry of the blue stain fungi. Part 3. Some metabolites of *Ceratocystis minor* (Hedgcock) Hunt. *Can. J. Chem.* **1987**, *65*, 765–769. [CrossRef]
59. Kornsakulkarn, J.; Thongpanchang, C.; Lapanun, S.; Srichomthong, K. Isocoumarin glucosides from the scale insect fungus *Torrubiella* tenuis BCC 12732. *J. Nat. Prod.* **2009**, *72*, 1341–1343. [CrossRef]
60. Chini, A.; Cimmino, A.; Masi, M.; Reveglia, P.; Nocera, P.; Solano, R.; Evidente, A. The fungal phytotoxin lasiojasmonate A activates the plant jasmonic acid pathway. *J. Exp. Bot.* **2018**, *69*, 3095–3102. [CrossRef]
61. Fonseca, S.; Radhakrishnan, D.; Prasad, K.; Chini, A. Fungal production and manipulation of plant hormones. *Curr. Med. Chem.* **2018**, *25*, 253–267. [CrossRef]
62. Tianpanich, K.; Prachya, S.; Wiyakrutta, S.; Mahidol, C.; Ruchirawat, S.; Kittakoop, P. Radical scavenging and antioxidant activities of isocoumarins and a phthalide from the endophytic fungus *Colletotrichum* sp. *J. Nat. Prod.* **2011**, *74*, 79–81. [CrossRef]
63. Wei, M.-Y.; Wang, C.-Y.; Liu, Q.-A.; Shao, C.-L.; She, Z.-G.; Lin, Y.-C. Five sesquiterpenoids from a marine-derived fungus *Aspergillus* sp. isolated from a gorgonian *Dichotella gemmacea*. *Mar. Drugs* **2010**, *8*, 941–949. [CrossRef]
64. MacMillan, J.; Simpson, T.J. Fungal products. Part V. The absolute stereochemistry of colletodiol and the structures of related metabolites of *Colletotrichum capsici*. *J. Chem. Soc. Perkin Trans.* **1973**, 1487–1493. [CrossRef]
65. Ostry, V. Alternaria mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J.* **2008**, *1*, 175–188. [CrossRef]
66. Zhi-jun, Y.; Yu, Y.; Zhi-qiang, W.; Tian, Y.; Daijie, C. Cytotoxic metabolites of endophytic fungus *Colletotrichum* sp. From *Aristolochia* spp. *Nat. Prod.*

Res. Devel. **2012**, *24*, 329–332.

67. Sakurai, M.; Kohno, J.; Nishio, M.; Yamamoto, K.; Okuda, T.; Kawano, K.; Nakanishi, N. TMC-205 a new transcriptional up- regulator of SV40 promoter produced by an unidentified fungus fermentation, isolation, physico-chemical properties, structure determination and biological activities. *J. Antibiot.* **2001**, *54*, 628–634. [CrossRef]
68. Dietl, A.-M.; Meir, Z.; Shadkchan, Y.; Osherov, N.; Haas, H. Riboflavin and pantothenic acid biosynthesis are crucial for iron homeostasis and virulence in the pathogenic mold *Aspergillus fumigatus*. *Virulence* **2018**, *9*, 1036–1049. [CrossRef]
69. Wu, Q.; Guo, Y.; Guo, Z.; Chu, Y.; Wang, T.; Tan, R. Two new cytosporones from the culture of endophytic *Phomopsis* sp. *Chem. Nat. Comp.* **2013**, *48*, 938–941. [CrossRef]
70. Okamoto, K.; Sakai, T.; Shimada, A.; Shirai, R.; Sakamoto, H.; Yoshida, S.; Ojima, F.; Ishiguro, Y.; Kawagishi, H. Antimicrobial chlorinated orcinol derivatives from mycelia of *Hericium erinaceum*. *Phytochemistry* **1993**, *34*, 1445–1446. [CrossRef]
71. Buchwaldt, L.; Anderson, K.; Morrall, R.; Gossen, B.; Bernier, C. Identification of lentil germ plasm resistant to *Colletotrichum truncatum* and characterization of two pathogen races. *Phytopathology* **2004**, *94*, 236–243. [CrossRef] [PubMed]
72. Chongo, G.; Gossen, B.; Bernier, C. Infection by *Colletotrichum truncatum* in resistant and susceptible lentil genotypes. *Can. J. PlantPathol.* **2002**, *24*, 81–85. [CrossRef]
73. Latunde-Dada, A.O. *Colletotrichum*: Tales of forcible entry, stealth, transient confinement and breakout. *Mol. Plant Pathol.* **2001**, *2*, 187–198. [CrossRef] [PubMed]
74. Cerkauskas, R.; Sinclair, J. Use of paraquat to aid detection of fungi in soybean tissues. *Phytopathology* **1980**, *70*, 1036–1038. [CrossRef]
75. Reveglia, P.; Masi, M.; Evidente, A. Melleins—Intriguing natural compounds. *Biomolecules* **2020**, *10*, 772. [CrossRef]
76. Ruijter, G.; Kubicek, C.; Visser, J. Production of organic acids by fungi. In *Industrial Applications*; Springer: Berlin/Heidelberg, Germany, 2002; pp. 213–230.

77. Andolfi, A.; Boari, A.; Evidente, M.; Cimmino, A.; Vurro, M.; Ash, G.; Evidente, A. Gulypyrones A and B and Phomentrioloxins B and C produced by *diaporthe gulyae*, a potential mycoherbicide for saffron thistle (*Carthamus lanatus*). *J. Nat. Prod.* **2015**, *78*, 623–629. [CrossRef]
78. Wang, Y.; Wang, Y. Oxalic acid metabolism contributes to full virulence and pycnidial development in the poplar canker fungus *Cytospora chrysosperma*. *Phytopathology* **2020**, *110*, 1319–1325. [CrossRef]
79. Tian, X.; Ding, H.; Ke, W.; Wang, L. Quorum sensing in fungal species. *Ann. Rev. Microbiol.* **2021**, *75*, 449–469. [CrossRef]
80. Wang, X.; Gong, X.; Li, P.; Lai, D.; Zhou, L. Structural diversity and biological activities of cyclic depsipeptides from fungi. *Molecules* **2018**, *23*, 169. [CrossRef]
81. Evidente, A. Bioactive Lipodepsipeptides Produced by Bacteria and Fungi. *Int. J. Mol. Sci.* **2022**, *23*, 12342. [CrossRef]
82. Desjardins, A.; Proctor, R. Molecular biology of *Fusarium* mycotoxins. *Int. J. Food Microbiol.* **2007**, *119*, 47–50. [CrossRef] [PubMed]
83. Awuchi, C.G.; Ondari, E.N.; Ogbonna, C.U.; Upadhyay, A.K.; Baran, K.; Okpala, C.O.R.; Korzeniowska, M.; Guiné, R.P. Mycotoxins affecting animals, foods, humans, and plants: Types, occurrence, toxicities, action mechanisms, prevention, and detoxification strategies—A revisit. *Foods* **2021**, *10*, 1279. [CrossRef] [PubMed]
84. Janevska, S.; Tudzynski, B. Secondary metabolism in *Fusarium fujikuroi*: Strategies to unravel the function of biosynthetic pathways. *App. Microbiol. Biotechnol.* **2018**, *102*, 615–630. [CrossRef] [PubMed]
85. Evidente, A. Fungal bioactive macrolides. *Nat. Prod. Rep.* **2022**, *39*, 1591–1621. [CrossRef]
86. Omura, S. *Macrolide Antibiotics: Chemistry, Biology, and Practice*, 2nd ed.; Academic Press: Tokyo, Japan, 2002.
87. Lumaret, J.P.; Errouissi, F.; Floate, K.; Rombke, J.; Wardhaugh, K. A review on the toxicity and non-target effects of macrocyclic lactones in terrestrial and aquatic environments. *Curr. Pharm. Biotechnol.* **2012**, *13*, 1004–1060. [CrossRef]
88. Amusa, N. Production, partial purification and bioassay of toxic metabolites of three plant pathogenic species of *Colletotrichum* in Nigeria. *Mycopathologia*

1994, *128*, 161–166. [CrossRef]

89. Kulkarni, S. Studies on the cultural characteristics of *Colletotrichum truncatum* the causal organism of green gram anthracnose. *J. Pharm. Phytochem.* **2019**, *8*, 2366–2369.
90. Marak, T.; Umbrey, Y.; Mahapatra, S.; Das, S. Cultural, morphological and bio-chemical variability of different isolates of *Colletotrichum truncatum* causing anthracnose of greengram. *Arch. Phytopathol.* **2019**, *52*, 141–154. [CrossRef]
91. Caesar, L.K.; Cech, N.B. Synergy and antagonism in natural product extracts: When 1 + 1 does not equal 2. *Nat. Prod. Rep.* **2019**, *36*, 869–888. [CrossRef]
92. Cimmino, A.; Cinelli, T.; Evidente, M.; Masi, M.; Mugnai, L.; Silva, M.A.; Michereff, S.J.; Surico, G.; Evidente, A. Phytotoxic fungal exopolysaccharides produced by fungi involved in grapevine trunk diseases. *Nat. Prod. Commun.* **2016**, *11*, 1481–1484. [CrossRef]
93. Paper, J.M.; Scott-Craig, J.S.; Adhikari, N.D.; Cuomo, C.A.; Walton, J.D. Comparative proteomics of extracellular proteins in vitro and in planta from the pathogenic fungus *Fusarium graminearum*. *Proteomics* **2007**, *7*, 3171–3183. [CrossRef] [PubMed]
94. Paul, A.; de Boves Harrington, P. Chemometric applications in metabolomic studies using chromatography-mass spectrometry. *Trends Anal. Chem.* **2021**, *135*, 116165. [CrossRef]
95. Brereton, R.G.; Lloyd, G.R. Partial least squares discriminant analysis: Taking the magic away. *J. Chemom.* **2014**, *28*, 213–225. [CrossRef]
96. Bittremieux, W.; Wang, M.; Dorrestein, P.C. The critical role that spectral libraries play in capturing the metabolomics community knowledge. *Metabolomics* **2022**, *18*, 94. [CrossRef]
97. Guo, J.; Yu, H.; Xing, S.; Huan, T. Addressing big data challenges in mass spectrometry-based metabolomics. *Chem. Commun.* **2022**, *58*, 9979–9990. [CrossRef]
98. Allard, P.-M.; Péresse, T.; Bisson, J.; Gindro, K.; Marcourt, L.; Pham, V.C.; Roussi, F.; Litaudon, M.; Wolfender, J.-L. Integration of molecular networking and in-silico MS/MS fragmentation for natural products dereplication. *Anal. Chem.* **2016**, *88*, 3317–3323. [CrossRef] [PubMed]
99. Colby, S.M.; Nuñez, J.R.; Hodas, N.O.; Corley, C.D.; Renslow, R.R. Deep

learning to generate in silico chemical property libraries and candidate molecules for small molecule identification in complex samples. *Anal. Chem.* **2019**, *92*, 1720–1729. [CrossRef] [PubMed]

100. Dührkop, K.; Nothias, L.-F.; Fleischauer, M.; Reher, R.; Ludwig, M.; Hoffmann, M.A.; Petras, D.; Gerwick, W.H.; Rousu, J.; Dorrestein, P.C. Systematic classification of unknown metabolites using high-resolution fragmentation mass spectra. *Nat. Biotechnol.* **2021**, *39*, 462–471. [CrossRef]
101. Rutz, A.; Dounoue-Kubo, M.; Ollivier, S.; Bisson, J.; Bagheri, M.; Saesong, T.; Ebrahimi, S.N.; Ingkaninan, K.; Wolfender, J.-L.; Allard, P.-M. Taxonomically informed scoring enhances confidence in natural products annotation. *Front. Plant Sci.* **2019**, *10*, 1329. [CrossRef]
102. Gauglitz, J.M.; West, K.A.; Bittremieux, W.; Williams, C.L.; Weldon, K.C.; Panitchpakdi, M.; Di Ottavio, F.; Aceves, C.M.; Brown, E.; Sikora, N.C. Enhancing untargeted metabolomics using metadata-based source annotation. *Nat. Biotechnol.* **2022**, *40*, 1774–1779. [CrossRef]
103. Allwood, J.W.; Clarke, A.; Goodacre, R.; Mur, L.A. Dual metabolomics: A novel approach to understanding plant–pathogen interactions. *Phytochemistry* **2010**, *71*, 590–597. [CrossRef] [PubMed]
104. Chen, F.; Ma, R.; Chen, X.L. Advances of metabolomics in fungal pathogen–plant interactions. *Metabolites* **2019**, *9*, 169. [CrossRef] [PubMed]
105. Perez de Souza, L.; Alseekh, S.; Naake, T.; Fernie, A. Mass spectrometry-based untargeted plant metabolomics. *Curr. Protoc. Plant Biol.* **2019**, *4*, e20100. [CrossRef] [PubMed]
106. Crandall, S.G.; Gold, K.M.; Jiménez-Gasco, M.D.M.; Filgueiras, C.C.; Willett, D.S. A multi-omics approach to solving problems in plant disease ecology. *PLoS ONE* **2020**, *15*, e0237975. [CrossRef] [PubMed]
107. van Der Hooft, J.J.; Mohimani, H.; Bauermeister, A.; Dorrestein, P.C.; Duncan, K.R.; Medema, M.H. Linking genomics and metabolomics to chart specialized metabolic diversity. *Chem. Soc. Rev.* **2020**, *49*, 3297–3314. [CrossRef] [PubMed]

Supplementary Materials:

Uncovering Phytotoxic Compounds Produced by *Colletotrichum* spp. Involved in Legume Diseases Using an OSMAC–Metabolomics Approach

Supplementary material

Figure S1. m/z distribution diagrams and Base Peak Chromatograms of *C. truncatum* isolate C428 (from lentil) extracts. (a) PDA extract; (b) PDB extract; (c) Rice extract; (d) Richard extract.

Figure S2. m/z distribution diagrams and Base Peak Chromatograms of *C. trucatum* isolate C431 (from soybean) extracts. (a) PDA extract; (b) PDB extract; (c) Rice extract; (d) Richard extract.

Figure S3. m/z distribution diagrams and Base Peak Chromatograms of *C. trifolii* isolate C436 (from clover extracts. (a) PDA extract; (b) PDB extract; (c) Rice extract; (d) Richard extract.

Figure S4. Structure of: validated metabolites with pure standards (level A; red); putatively identified and produced by *Colletotrichum* spp. (level B(i), blue); putatively identified and produced by other fungal species (level B(ii), black); Structures of Curvupallide A and B (Level C(i), brown).

Table S1. Parameters Metaboanalyst 5.0 for LC-MS spectra processing.

Table S2. Features lists transformation and scaling for PLS – DA analysis. Q2 and R2 values of PLS – DA models in cross validation.

Table S3. Metabolites dereplicated by targeted and untargeted metabolomics analysis, organised according to identification level.

Table S4. Metabolites dereplicated by targeted and untargeted metabolomics analysis, organised according to identification level.

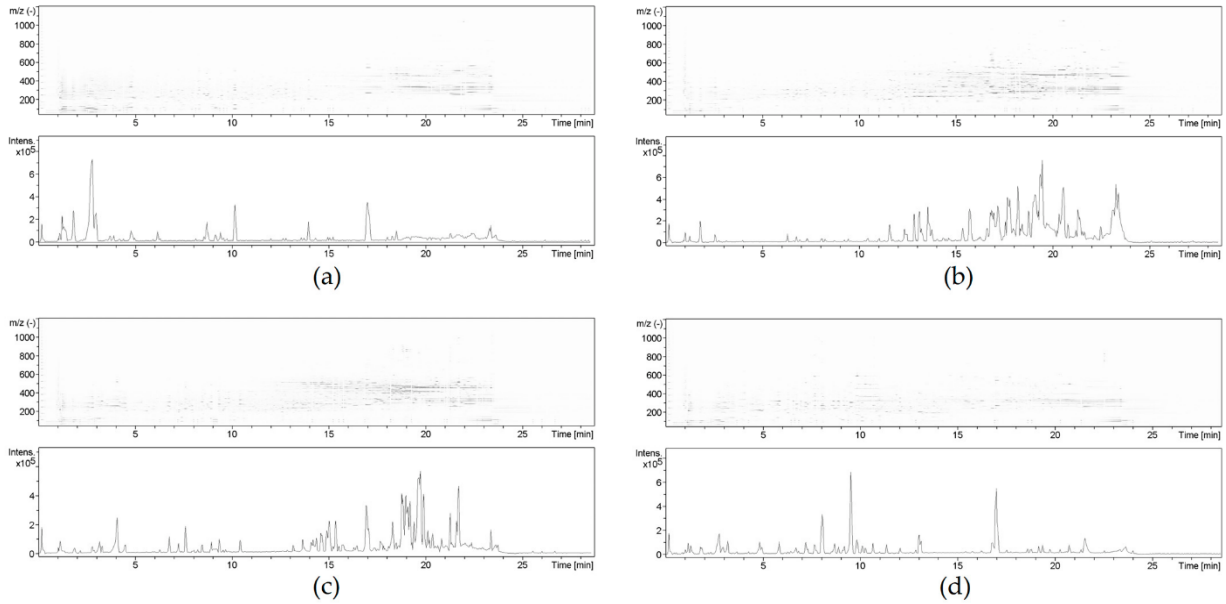

Figure S1. m/z distribution diagrams and Base Peak Chromatograms of *C. truncatum* isolate C428 (from lentil) extracts. (a) PDA extract; (b) PDB extract; (c) Rice extract; (d) Richard extract.

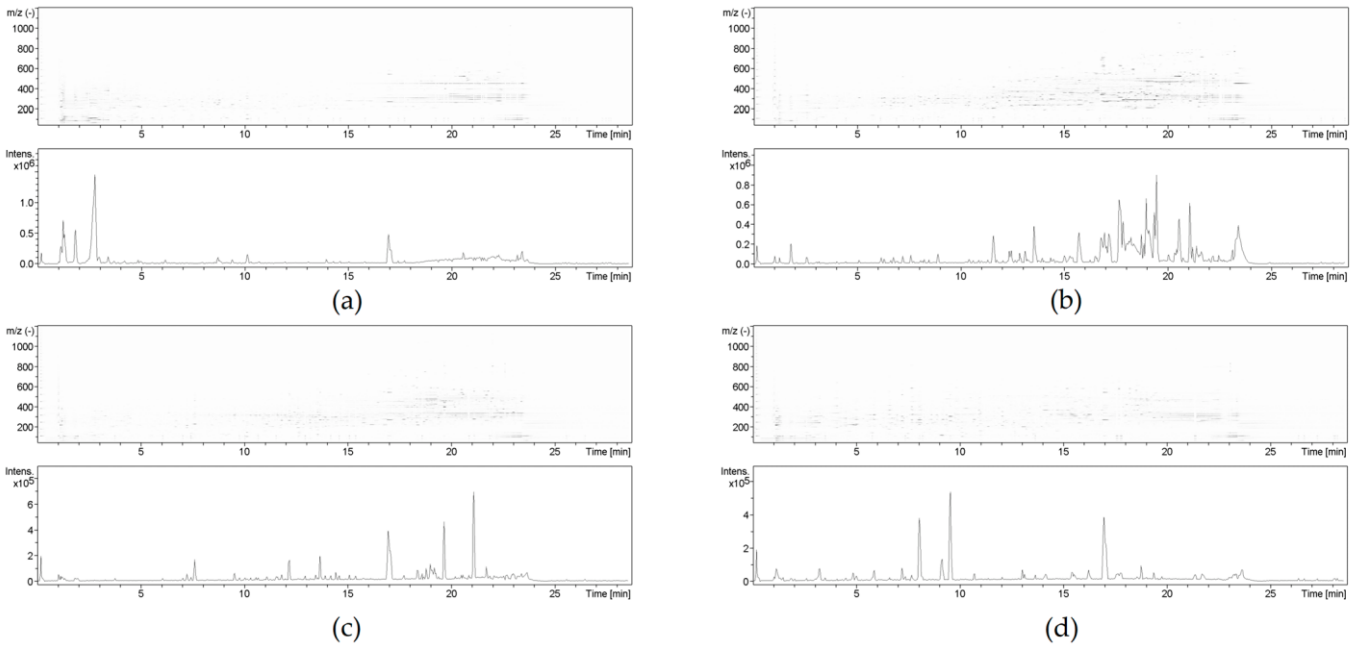

Figure S2. m/z distribution diagrams and Base Peak Chromatograms of *C. truncatum* isolate C431 (from soybean) extracts. (a) PDA extract; (b) PDB extract; (c) Rice extract; (d) Richard extract.

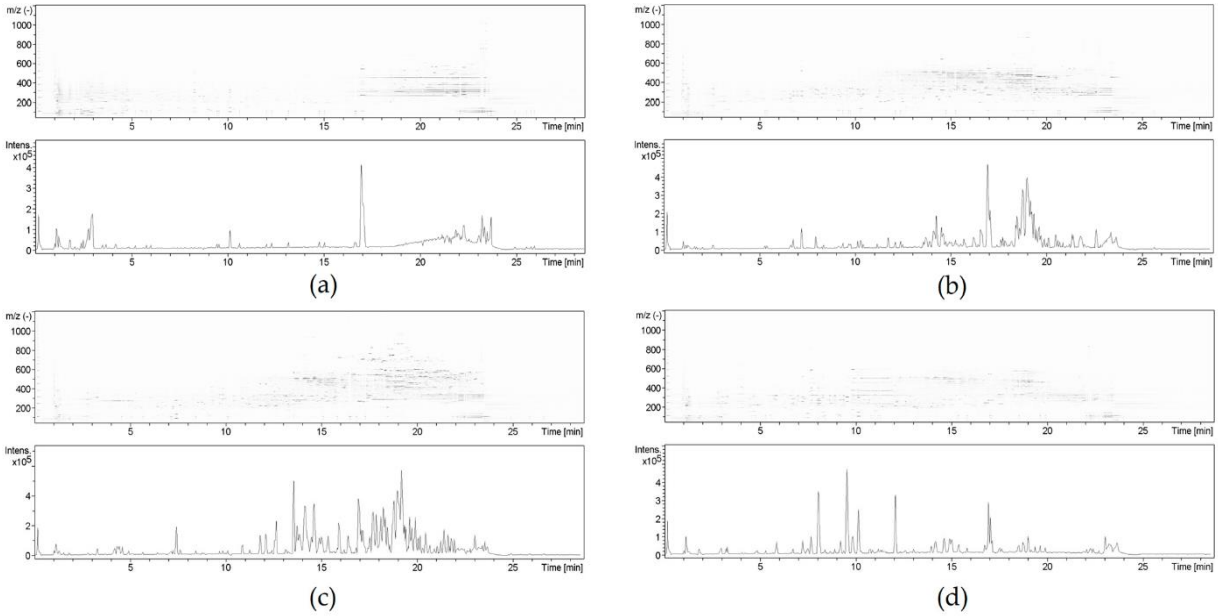

Figure S3. m/z distribution diagrams and Base Peak Chromatograms of *C. trifolii* isolate C436 (from clover) extracts. (a) PDA extract; (b) PDB extract; (c) Rice extract; (d) Richard extract.

Table S1. Parameters Metaboanalyst 5.0 for LC-MS spectra processing

Platform selection	**Parameters**
UPLC-Q/TOF	Auto-Optimized
Peak Picking	**Parameters**
Method	centWave min_peakwidth
max_peakwidth	20.0
ppm	15 ppm
mzdiff	0.01
snthresh	6.0
noise	1000.0
prefilter	3.0
value_of_prefilter	100.0
Peak Alignment	**Parameters**
Method	obiwarp
Bandwidth	30.0
minFraction	0.8
minSamples	1
maxFeatures	100
integrate	1
extra	1
span	0.25
profStep	1
Peak Alignment	**Parameters**
Polarity	Negative
Adducts	$[M]^-$;$[M-H]^-$; $[M-2H]^-$; $[M-3H]^-$; $[M- H+Cl]^{2-}$; $[M+Cl]^-$; $[M+2Cl]^{2-}$;$[2M-H]^-$
Perc_fwhm	0.6
Mz_abs_iso	0.005
Max_charge	2
Corr_eic_th	0.85
Mz_abs_add	0.001
Contaminant removal	Yes
Blank sustraction	Yes

Table S2. Features lists transformation and scaling for PLS – DA analysis. Q2 and R2 values of PLS – DA models in cross validation

Cultural media extracts	Transformation	Scaling	Q2 value	R2 value
PDA	Logarithmic	Pareto scaling	0.8331*	0.9900*
PDB	Logarithmic	Range scaling	0.9106**	0.9997**
Rice	Logarithmic	Pareto scaling	0.9501**	0.9998**
Richard	Cube root	Autoscaling	0.7625***	0.9967***

*values using the top 4 components; ** values using the top 5 components; ***values using the top 3 components.

Table S3. Metabolites dereplicated by targeted and untargeted metabolomics analysis, organised according to identification level.

Putative metabolites name	Biosynthetic origin and Class of compound	Molecular formula	Ret. Time	ES (-) Theor. m/z	ES (-) Found m/z	m/z error (mDa)	MS/MS	ID Levels (A – D)*	Databases Code**
6-hydroxymellein	Polyketide_Isocoumarine	$C_{10}H_{10}O_4$	12.58	193.0501 $[M-H]^-$	193.0495 $[M-H]^-$	0.6	149.0601; 131.0498; 105.0715	A	CNP0395266 NPA028477
Colletopyrone	Polyketides_Cyclic polyketides	$C_{15}H_{16}O_6$	18.24	291.0869 $[M-H]^-$	291.0904 $[M-H]^-$	-3.5	224.0162; 139.0394; 95.0505	A	CNP0267088 Q82653372
Resorcinol	Shikimates and Phenylpropanoids_Simple phenol	$C_6H_6O_2$	10.11	109.0289 $[M-H]^-$	109.0284 $[M-H]^-$	0.5	93.0330; 81.0331	A	CNP0414137 Q82653372
4-Hydroxybenzaldehyde	Shikimates and Phenylpropanoids_Simple phenolic acids	$C_7H_6O_2$	9.12	121.0289 $[M-H]^-$	121.0291 $[M-H]^-$	-0.2	108.0200; 92.0271; 65.0407	A	CNP0230702 NPA032546
Orcinol	Shikimates and Phenylpropanoids_Simple phenol	$C_7H_8O_2$	4.76	123.0446 $[M-H]^-$	123.0446 $[M-H]^-$	-	109.0292; 108.0190; 91.0180; 81.0350	A	CNP0300146 NPA032537
4-Hydroxyphenylacetic acid	Shikimates and Phenylpropanoids_Simple phenolic acids	$C_8H_8O_3$	8.97	151.0395 $[M-H]^-$	151.0390 $[M-H]^-$	0.5	118.8785; 109.0283; 107.049; 93.0333	A	CNP0212603 NPA032547
Colletochlorin E	Terpenoids & Polyketides_Tetraketide meroterpenoids	$C_{20}H_{23}ClO_6$	18.72	393.1104 $[M-H]^-$	393.1105 $[M-H]^-$	-0.1	241.0638; 151.0398; 95.0504	A	-
Higginsianin A	Terpenoids& Polyketides_Triketide meroterpenoids	$C_{27}H_{38}O_4$	22.50	425.2692 $[M-H]^-$	425.2686 $[M-H]^-$	0.6	325.1839; 265.1463; 265.1478; 223.0281	A	CNP0152211 NPA021815

Higginsianin B	Terpenoids& Polyketides_Triketide meroterpenoids	$C_{27}H_{40}O_4$	22.66	427.2848 $[M-H]^-$	427.2843 $[M-H]^-$	0.5	357.2645; 315.2527; 283.2276; 155.0351	A	CNP0073478 NPA021814
Imidazoleacetic acid	Alkaloids_Imidazole alkaloids	$C_5H_6N_2O_2$	3.15	125.0351 $[M-H]^-$	125.0350 $[M-H]^-$	0.1	81.04434; 71.6317; 97.0401	B(i)	CNP0253943
Maculosin	Amino acids and Peptides_Dipeptides	$C_{14}H_{16}N_2O_3$	7.22	259.1083 $[M-H]^-$	259.1090 $[M-H]^-$	-0.5	98.0251; 121.0293; 124.0398	B(i)	CNP0320469 NPA007345
Succinic Acid	Fatty Acids_ Dicarboxylic acids	$C_4H_6O_4$	2.74	117.0188 $[M-H]^-$	117.0190 $[M-H]^-$	-0.2	99.00871; 73.0295	B(i)	Q213050
Succinic Anhydride	Fatty Acids_ Anhydride of dicarboxylic acid	$C_4H_4O_3$	2.76	99.0082 $[M-H]^-$	99.0091 $[M-H]^-$	-0.9	73.0138; 59.0155	B(i)	CNP0079889 Q417847
Pantothenic acid	Fatty acids_ Beta amino acids and derivatives	$C_9H_{17}NO_5$	4.94	218.1028 $[M-H]^-$	218.1038 $[M-H]^-$	-1	88.0413; 99.0459; 146.0810, 71.01495	B(i)	CNP0153142
3,4-Dehydro-6-hydroxymellein	Polyketides_ Isocoumarine	$C_{10}H_8O_4$	16.10	191.0344 $[M-H]^-$	191.0352 $[M-H]^-$	-0.8	149.0234; 147.0441; 123.0439; 87.0458	B(i)	CNP0389619 Q27105447
Fusarentin 6,7-dimethyl ether	Polyketides_ Isocoumarine	$C_{16}H_{22}O_6$	15.70	309.1338 $[M-H]^-$	309.1323 $[M-H]^-$	1.5	265.1440; 253.1075; 233.1208; 125.0243	B(i)	CNP0219353 Q27138491
Colletol	Polyketides_Macrolides	$C_{14}H_{20}O_5$	15.2	267.1235 $[M-H]^-$	267.1240 $[M-H]^-$	-0.5	223.1343; 213.1496; 121.0296	B(i)	CNP0157857 NPA002061
Colletodiol	Polyketides_Macrolides	$C_{14}H_{20}O_6$	13.7	283.1182 $[M-H]^-$	283.1187 $[M-H]^-$	-0.5	111.0822; 253.1077; 207.1020; 239.1284	B(i)	Q27896955

Macrolactone Sch-725674	Polyketides_Macrolides	$C_{18}H_{32}O_5$	18.4	327.2172 [M-H]$^-$	327.2179 [M-H]$^-$	-0.7	209.1179; 227.1282; 293.1770	B(i)	CNP0221935 NPA008159
Alternariol	Polyketides_Isocoumarine	$C_{14}H_{10}O_5$	17.53	257.0450 [M-H]$^-$	257.0457 [M-H]$^-$	-0.7	111.0087; 213.0557; 68.9974	B(i)	CNP0184929 NPA020438
TMC-205	Alkaloids_Simple indole alkaloids	$C_{14}H_{13}NO_2$	4.83	226.0868 [M-H]$^-$	226.0.884 [M-H]$^-$	-1.6	121.0294; 135.0440 167.0367	B(ii)	CNP0402436 NPA017297
3-Nitropropionic Acid	Fatty Acids_Oxo fatty acids	$C_3H_5NO_4$	2.73	118.0140 [M-H]$^-$	118.0145 [M-H]$^-$	-0.5	99.0075; 73.0281; 61.9896	B(ii)	CNP0439550 NPA007876
Cytosporone T	Polyketides_Aromatic polyketides	$C_{17}H_{26}O_4$	19.30	293.1752 [M-H]$^-$	293.1762 [M-H]$^-$	-1	275.16412; 221.15503; 236.10591; 83.05134; 149.09648	B(ii)	CNP0264849 NPA018477
Berkchaetorubramine	Polyketides_Azaphilones	$C_{27}H_{37}NO_6$	20.34	470.2543 [M-H]$^-$	470.2549 [M-H]$^-$	-0.6	414.1930; 301.1450; 251.1654; 111.0095	B(ii)	CNP0357282 NPA004011
Catathelasmol A	Polyketides_furans	$C_5H_{10}O_3$	4.58	117.0552 [M-H]$^-$	117.0553 [M-H]$^-$	-0.1	101.0246; 85.0300; 73.0304; 68.0517	B(ii)	CNP0357577 NPA010903
Phomolide B	Polyketides_Macrolides	$C_{12}H_{18}O_4$	16.08	225.1127 [M-H]$^-$	225.1134 [M-H]$^-$	-0.7	137.0970; 89.0248; 151.0764; 121.0295; 127.0764; 195.0647; 68.9970	B(ii)	CNP0206816 NPA005349

Methyl 2-[(3-formyl-4-hydroxyphenyl)methyl]-4-hydroxy-3-(4-hydroxyphenyl)-5-oxo-2,5-dihydrofuran-2-carboxylate	Shikimates and Phenylpropanoids_Lignans	$C_{20}H_{16}O_8$	16.5	351.0869 $[M-H]^-$	383.0772 $[M-H]^-$	-0.5	339.0872; 157.0136; 95.0550; 89.0252	B(ii)	CNP0354744 NPA014159
4-chloro-3,5-dimethoxy benzaldehyde	Shikimates and Phenylpropanoids_Simple phenolic acids	$C_9H_9ClO_3$	3.02	199.0162 $[M-H]^-$	199.0127 $[M-H]^-$	3.5	135.04427; 121.0297; 93.03511	B(ii)	CNP0247714 NPA002948
(+)-Methyl Sydowate	Terpenoids_Sesquiterpenoids	$C_{16}H_{22}O_4$	17.00	277.1439 $[M-H]^-$	277.1446 $[M-H]^-$	-0.7	134.0379; 127.1134; 121.0300; 111.0092; 77.0408	B(ii)	CNP0134087 NPA010996
Curvupallide A or B	Polyketides_ Isoindolazinfuranones	$C_{14}H_{17}NO_5$	10.41	278.1028 $[M-H]^-$	278.1035 $[M-H]^-$	-0.7	205.0868; 248.0924; 163.0757; 178.0858; 124.0402; 135.0445	C(i)	NPA004814/ NPA015714
3-Hydroxy dodecanedioic acid; Dibutyl malate;	Fatty Acids_ Dicarboxylic acids	$C_{12}H_{22}O_5$	11.0	245.1389 $[M-H]^-$	245.1395 $[M-H]^-$	-0.6	173.0810; 111.0818; 141.0919; 103.0509	C(i)	-
Fumaric acid; Maleic acid;	Fatty Acids_ Dicarboxylic acids	$C_4H_4O_4$	2.56	115.0031 $[M-H]^-$	115.0033 $[M-H]^-$	-0.2	87.0091; 71.0139	C(i)	-
Dihydroxybutyric acid	Fatty Acids_Dihydroxy Fatty acid	$C_4H_8O_4$	1.26	119.0344 $[M-H]^-$	119.0346 $[M-H]^-$	-0.2	71.01505; 86.02505; 101.02489	C(i)	-
Hydroxybutyric acid	Fatty Acids_Hydroxy Fatty acid	$C_4H_8O_3$	2.95	103.0395 $[M-H]^-$	103.0402 $[M-H]^-$	-0.7	57.0366; 74.0233; 85.02810; 101.0232	C(i)	-

Trihydroxy octadecenoic acid	Fatty Acids_Long Chain Fatty Acids	$C_{18}H_{34}O_5$	17.54	329.2328 $[M-H]^-$	329.2335 $[M-H]^-$	0.7	229.14413; 211.1333; 171.1020; 162.02217	C(i)	-
p-Cresol; m-Cresol; o-Cresol;	Shikimates and Phenylpropanoids_Simple phenol	C_7H_8O	10.1	107.0497 $[M-H]^-$	107.0504 $[M-H]^-$	-0.7	85.0304; 79.0540; 65.0380	C(i)	-
Benzyl alcohol; Anisole; 2,5-Dimethylfuran; 2-Ethylfuran	Polyketides_Furans	C_6H_8O	7.41	95.0497 $[M-H]^-$	95.0508 $[M-H]^-$	-1.1	82.0305; 68.9968; 55.0207	C(i)	-
Putative jasmonic acid derivative	Fatty acids_Octadecanoids_Jasmonic acids	$C_{12}H_{20}O_3$	19.0	211.1334 $[M-H]^-$	211.1340 $[M-H]^-$	-0.6	111.0091; 151.0762; 149. 0458	C(ii)	-
Putative colletotryptin	Alkaloids_Tryptophan alkaloids	$C_{20}H_{22}N_2O_5$	14.1	369.1450 $[M-H]^-$	369.1457 $[M-H]^-$	-0.7	325.1548; 255.0776; 221.0931	C(ii)	-
Putative glycosylamine	Carbohydrates and carbohydrate conjugate	$C_{10}H_{14}N_2O_5$	3.21	241.0824 $[M-H]^-$	241.0832 $[M-H]^-$	-0.8	223.0721; 197.0935; 144.0658; 145.0501	C(ii)	-
Putative hypoxanthine	Alkaloids _Hypoxanthines	$C_8H_{11}N_5O_3$	4.83	225.0862 $[M]^-$	225.0885 $[M]^-$	-2.3	165.0653; 88.0413; 82.0306; 131.0347; 113.0606	C(ii)	-
Putative colletopeptide	Cyclic peptides _ Depsipeptides	$C_{25}H_{29}N_5O_7$	14.14	510.1989 $[M-H]^-$	510.1978 $[M-H]^-$	1.1	494.2041; 448.1982; 281.1398; 162.0547	C(ii)	-
Putative colletopeptide	Cyclic peptides _ Depsipeptides	$C_{26}H_{29}N_3O_6$	18.31	478.1978 $[M-H]^-$	478.2036 $[M-H]^-$	5.0	448.1977; 281.1394; 267.1588	C(ii)	-

Putative colletopeptide	Cyclic peptides _ Depsipeptides	C28H42N4O5	21.70	513.3077 [M-H]-	513.3071 [M-H]-	0.6	423.2756; 358.2028; 297.2437	C(ii)	-
Putative colletopeptide	Cyclic peptides _ Depsipeptides	$C_{29}H_{45}N_5O_5$	17.9	542.3342 [M-H]-	542.3335 [M-H]-	0.7	527.3226; 515.3228; 501.3428; 499.3272; 489.2980	C(ii)	-
Putative colletopeptide	Cyclic peptides _ Depsipeptides	$C_{31}H_{33}N_3O_7S$	9.82	590.1961 [M-H]-	590.1953 [M-H]-	0.8	502.1041; 444.0982; 275.1383; 178.0865	C(ii)	-
Putative benzene derivative	Benzenoids _Benzene and substituted derivatives	$C_{20}H_{18}O_3$	9.51	305.1178 [M-H]-	305.1144 [M-H]-	3.4	261.1863; 305.1752; 155.0141; 111.0094	C(ii)	-
Putative O-glycosil compound	Carbohydrates and carbohydrate conjugate_O-glycosil compounds	$C_{12}H_{18}O_9$	4.77	305.0873 [M-H]-	305.0880 [M-H]-	-0.7	306.8868; 225.0893; 181.1003; 131.0336 82.0305	C(ii)	-
Putative O-glycosil compound	Carbohydrates and carbohydrate conjugate_O-glycosil compounds	$C_{16}H_{28}O_6$	11.5	315.1808 [M-H]-	315.1812 [M-H]-	-0.4	283.1550; 211.0609; 68.9967	C(ii)	-
Putative phenolic glycoside	Carbohydrates and carbohydrate conjugate_Phenolic glycosides	$C_{15}H_{19}NO_8$	9.78	340.1032 [M-H]-	340.1035 [M-H]-	-0.3	294.0978; 221.0702; 192.0661; 138.0202; 108.0457	C(ii)	-
Putative mycosporine	Amino acids and Peptides_ Mycosporine derivatives	$C_{15}H_{17}NO_6$	6.16	306.0977 [M-H]-	306.0984 [M-H]-	-0.7	260.1046; 121.02924; 124.0404	C(ii)	-

Putative tricarboxylic acid derivative	Carboxylic acid and derivatives_tricarboxylic acid and derivatives	$C_{12}H_{20}O_7$	17.54	275.1131 $[M-H]^-$	275.1137 $[M-H]^-$	-0.6	238.0693; 214.0169; 129.0194; 112.9852	C(ii)	-
Putative cyclic fatty acyl glycosides	Fatty Acids_ Fatty acyl glycosides	$C_{28}H_{48}O_9$	21.31	527.3220 $[M-H]^-$	527.3223 $[M-H]^-$	-0.3	470.2545; 400.2484; 348.2541; 279.2345; 148.9299; 263.0919; 189.0532	C(ii)	-
Putative isocoumarine	Polyketides_ Isocoumarine	$C_{11}H_{11}ClO_5$	2.76	257.0217 $[M-H]^-$	257.0252 $[M-H]^-$	-3.5	103.0389; 89.02373; 73.03042	C(ii)	-
Putative aromatic polyketide	Polyketides_Aromatic Polyketides	$C_{16}H_{16}O_5$	4.47	287.0919 $[M-H]^-$	287.0885 $[M-H]^-$	3.4	107.05028; 96.95872; 79.95595	C(ii)	-
Putative polycyclic aromatic polyketides	Polyketides_Polycyclic aromatic polyketides	$C_{18}H_{12}O_8$	16.80	355.0454 $[M-H]^-$	355.0459 $[M-H]^-$	-0.5	341.0662; 271.1446; 244.1556	C(ii)	-
Putative zearalenones	Polyketides_Zearalenones	$C_{16}H_{30}O_6$	14.20	317.1964 $[M-H]^-$	317.1967 $[M-H]^-$	-0.3	148.92999; 263.09186; 189.0532; 116.0503	C(ii)	-
Putative β amino acid derivative	Beta amino acids and derivatives	$C_9H_{12}N_2O_4$	3.67	211.0718 $[M-H]^-$	211.0727 $[M-H]^-$	-0.9	167.0816; 114.0196; 102.9327; 70.0304	C(ii)	
Putative phenolic acid	Shikimates and Phenylpropanoids_ Phenolic acids (C6-C1)	$C_{17}H_{17}NO_3$	4.76	282.1130 $[M-H]^-$	282.1096 $[M-H]^-$	3.4	192.0671; 92.05122; 148.0767	C(ii)	-

Putative phenolic acid	Shikimates and Phenylpropanoids_ Simple phenolic acids	$C_9H_9NO_4$	7.23	194.0453 [M-H]$^-$	194.0463 [M-H]$^-$	-1	150.0561; 137.0240; 108.0460; 107.0377	C(ii)	-
Putative phenylethanoid	Shikimates and Phenylpropanoids_Phenylethanoids	$C_{21}H_{32}O_{12}$	18.75	476.1893 [M-H]$^-$	476.1926 [M-H]$^-$	-3.3	460.1980; 430.1876; 237.1130; 149.0462	C(ii)	-
Putative anisol	Benzenoids _Anisoles	$C_{24}H_{31}NO_8$	19.32	460.1971 [M-H]$^-$	460.1978 [M-H]$^-$	-0.7	414.1924; 384.1817; 366.1709; 309.1538; 88.0047	C(ii)	-
Putative xanthone	Shikimates and Phenylpropanoids_Xanthones	$C_{28}H_{30}O_6$	19.60	461.1964 [M-H]$^-$	461.2009 [M-H]$^-$	-4.5	415.1001; 253.0480; 275.1405	C(ii)	-
Putative triketide meroterpenoid	Terpenoids& Polyketides_Triketide meroterpenoids	$C_{28}H_{32}O_9$	14.20	511.1968 [M-H]$^-$	511.2009 [M-H]$^-$	-4.1	421.1874; 403.1775; 375.1814; 369.1350; 299.1290	C(ii)	-
Putative terpene glycoside	Terpene glycosides	$C_{24}H_{32}NO_{10}$	14.94	494.2026 [M]$^-$	494.2030 [M]$^-$	-0.4	416.175; 369.1549; 323.1500	C(ii)	-
Putative fusarin	Terpenoids_Sesquiterpenoids	$C_{23}H_{30}NO_7$	18.82	431.1944 [M-H]$^-$	431.1905 [M-H]$^-$	3.9	416.1710; 402.1710; 389.1598; 324.1452; 303.0860	C(ii)	-
Putative fusarin	Terpenoids_Sesquiterpenoids	$C_{23}H_{29}NO_6$	19.01	414.1917 [M-H]$^-$	414.1924 [M-H]$^-$	-0.7	387.1815; 301.1440; 352.21173; 213.12711	C(ii)	-

Putative fusarin	Terpenoids_Sesquiterpenoids	$C_{23}H_{29}NO_7$	18.56	430.1866 $[M-H]^-$	430.1873 $[M-H]^-$	0.7	400.1759; 357.1609; 320.1550; 151.0766; 139.0042; 138.0199	C(ii)	-
Putative fusarin	Terpenoids_Sesquiterpenoids	$C_{23}H_{29}NO_8$	16.41	446.1815 $[M-H]^-$	446.1821 $[M-H]^-$	-0.6	414.1925; 400.1767; 241.1453	C(ii)	-
Putative fusarin	Terpenoids_Sesquiterpenoids	$C_{23}H_{29}NO_9$	15.50	462.1764 $[M-H]^-$	462.1770 $[M-H]^-$	-0.6	448.1987; 416.1725; 323.1037; 307.1085; 196.0982	C(ii)	-
Putative fusarin	Terpenoids_Sesquiterpenoids	$C_{23}H_{31}NO_7$	18.24	432.2022 $[M-H]^-$	432.2029 $[M-H]^-$	-0.7	416.1710; 402.1710; 389.1598; 324.1452; 303.087	C(ii)	-
Putative fusarin	Terpenoids_Sesquiterpenoids	$C_{23}H_{31}NO_8$	15.12	448.1971 $[M-H]^-$	448.1977 $[M-H]^-$	-0.6	432.1654; 416.1712; 167.0717; 155.0461	C(ii)	-
Unknown	-	$C_{20}H_{32}O_9$	18.82	415.1968 $[M-H]^-$	415.1959 $[M-H]^-$	0.9	-	D	-
Unknown	-	$C_{17}H_{24}O_6$	15.50	323.1495 $[M-H]^-$	323.1502 $[M-H]^-$	-0.7	-	D	-
Unknown	-	$C_7H_{14}N_2O_4S_2$	7.42	253.0317 $[M-H]^-$	253.0341 $[M-H]^-$	-2.4	-	D	-
Unknown	-	$C_{16}H_{24}O_6$	15.03	311.1495 $[M-H]^-$	311.1499 $[M-H]^-$	-0.4	-	D	-

Unknown	-	$C_{22}H_{36}O_{13}$	16.63	508.2155 [M]$^-$	508.2187 [M]$^-$	-3.2	-	D	-
Unknown	-	$C_{26}H_{36}O_6$	16.50	524.2137 [M-H]$^-$	524.2137 [M-H]$^-$	-	-	D	-
Unknown	-	$C_{27}H_{30}O_6$	15.17	449.1964 [M-H]$^-$	449.2011 [M-H]$^-$	-4.7	-	D	-
Unknown	-	$C_{27}H_{35}NO_6$	19.4	468.2386 [M-H]-	468.2386 [M-H]-	-	-	D	-
Unknown	-	-	3.29	-	312.0872	-	-	D	-
Unknown	-	-	23.5	-	600.1905	-	-	D	-
Unknown	-	-	0.17	-	452.9227	-	-	D	-
Unknown	-	-	14.98	-	495.2018	-	-	D	-

*Confidence levels: Level A: validate metabolite. Comparison with pure standard analysed in the same experimental LC-MS/MS condition; Level B(i): Putative identification using taxonomical information. Confident match based on MS/MS data with reference standard of selected spectral libraries in MSDIAL/MSFINDER analysis (Match Score ≥ 8) and/or complete match of *in silico* fragmentation of MS/MS data of compounds restricting interrogation to *Colletrotrichum* species using MetFrag; Level B(ii): Putative identification. Complete match of *in silico* fragmentation of MS/MS, using MetFrag data restricting interrogation to fungal metabolites. Level C(i): Tentative structure. MS/MS data in agreement with different structural isomers of reference standards of selected database in MSDIAL/MSFINDER belonging to the same natural compound class; Level C(ii): Compound Class. Partial match of MS/MS data on the spectral libraries used in MSDIAL/MSFIDER (6 ≤ Match Score < 8) with specific metabolites class and/or MS/MS data in agreement with a specific class of fungal metabolites according to *in silico* fragmentation using MetFrag; Level D: Only molecular formula and/or High-resolution mass.

**Databases identifiers: ID starting with CNPXXX are Coconunt ID: https://coconut.naturalproducts.net/; ID starting with NPAXXX are Natural Product Atlas ID: https://www.npatlas.org/; ID starting with QXXX are Lotus ID: https://lotus.naturalproducts.net/.

Table S4. Metabolites dereplicated by targeted and untargeted metabolomics analysis, organised according to identification level.

		Richard					PDA					PDB					Rice				
Fungal species	**Legume specie tested**	**N[a]**	**DA[a]**	**NR[a]**	**%DS 1mg/mL**	**%DS 2mg/mL**	**N[a]**	**DA[a]**	**NR[a]**	**%DS 1mg/mL**	**%DS 2mg/mL**	**N[a]**	**DA[a]**	**NR[a]**	**%DS 1mg/mL**	**%DS 2mg/mL**	**N[a]**	**DA[a]**	**NR[a]**	**%DS 1mg/mL**	**%DS 2mg/mL**
Colletotrichum truncatum isolate C428	Lentil	+	++	+	100	100	+	++	+	89.7±14.5	100	+	++	+	72.8±19.5	72.8±19.5	+	+	-	23.7±9.6	51.8±16*
	Soybean	-	+	+	11.9±5	12.2±4.8	+	-	-	4.4±1.6	4.6±1.9	+	-	-	3.4±0.9	3.6±1.1	+	-	-	7.1±1.5	8.5±1.3
	Faba bean	+	-	-	4.3±2.5	9±2.1*	+	-	-	4.3±2.1	2.9±1	+	+	-	2.4±1.6	6.4±2.4*	+	-	-	11.6±2.3	19±4.8*
	Pea	-	+	+	20.2±8.3	26.5±4.8	+	+	-	4.4±2.3	5.9±2.7	-	+	-	4.2±2.3	35±14*	+	+	+	9.3±4.2	8.4±5.9
	Red clover	-	+	+	35.5±10.5	80.6±16*	-	+	-	31±8.9	34.4±11.4	-	+	+	27.1±6.5	28.7±2.8	-	+	+	42±13.4	72.1±18.5*
	White clover	-	+	+	35±5.7	43.5±7.3*	-	+	+	58.7±22	51.4±18	-	+	+	36.6±7.4	35.7±10.4	-	+	+	41.3±6.1	39.9±3.4
	Subterranean clover	-	+	+	86.4±12.6	89.5±9	-	+	+	16.7±2.1	44.8±8.5*	-	+	+	30.9±3.7	32.4±1.8	+	+	+	23.9±4.9	49.7±11.4*
	Barrel medic	-	+	+	94.3±6.4	100	-	+	-	89±12.5	93.5±8.4	-	+	+	92.8±12	100	-	+	+	100	96.5±4.1
Colletotrichum truncatum isolate C431	Lentil	+	++	+	66±24	84.4±8.8	+	++	-	100	97.6±2.4	-	+	-	49.9±11.9	47±21.7	-	+	+	4.5±2.6	7.8±4
	Soybean	+	+	+	6.40±1.3	9.48±2.8*	+	+	+	9.8±4.7	10.5±3	+	+	+	8.4±3.6	8±3.2	+	+	+	5±2.1	7.2±1.8
	Faba bean	+	-	-	5.6±2.3	5.9±1.5	+	-	-	3.7±0.9	4.2±0.1	+	-	-	3.5±2	3.4±0.2	+	-	-	7.1±1.3	13.3±5.5*
	Pea	+	-	-	2.1±1.1	1.9±0.6	-	+	-	0.98±0.4	0.9±0.4	-	+	-	6.2±1.1	5.4±1.6	-	+	-	1.2±0.9	3±1.1*
	Red clover	+	+	+	33.4±12.5	51.8±17*	-	+	-	56.6±17	61.9±14.7	-	+	+	41.5±14	85±15*	+	+	+	30.6±10.1	38.7±12.8
	White clover	+	+	+	27.6±3.9	33.2±5.8	-	+	-	43.9±7.5	91±8.3*	-	+	+	25±13	47±14.8*	-	+	+	18.1±8.6	29±7.8*
	Subterranean clover	-	+	+	51.3±8	54.1±18.2	-	+	-	90.8±10	98.1±3.3	-	+	+	57.4±16.3	92.4±11*	-	+	+	42.6±11.7	86.9±12.2*
	Barrel medic	-	+	+	93.1±8.1	92.7±5.4	-	+	-	45.9±14.2	44.1±2.3	-	+	-	47.8±11.9	43.6±0.9	+	+	+	92.1±7	90.2±5.7
Colletotrichum trifolii isolate C436	Lentil	-	++	+	35.4±14.4	95.6±4*	-	+	-	0.1±0.1	4.4±1.6	+	+	-	11.5±3.2	26.2±4.3*	-	+	-	4.7±0.9	15.2±2.8*
	Soybean	+	+	+	6.4±1.1	9.5±2.8*	+	+	+	5.4±1.8	5.8±2.3	-	+	+	7.2±3.3	6±2.5	+	-	-	13.2±3	10.2±0.7
	Faba bean	+	+	+	11.5±2.7	17.2±5.8	+	-	-	13.2±6.6	10.6±1.2	-	+	-	6.4±2.8	5.9±1.8	+	+	-	7.8±3.6	46.7±4.7*
	Pea	+	+	+	21±10.5	57±14.9*	+	-	-	2.4±0.9	2±0.4	+	-	-	1±0.5	2.5±0.9	+	-	-	3.2±1.4	9.6±3.9*
	Red clover	+	+	+	74.2±17.6	83.7±8.7	-	+	+	52.1±19	46.1±17	+	-	-	24.7±9.7	21.7±4.5	+	+	+	42.9±19.6	51.7±17
	White clover	+	+	+	53.3±5.6	85.3±16*	-	+	+	16.7±9.3	14±3.9	-	+	+	9±1.7	10.3±3	+	+	+	17.7±2.4	90±5.7*
	Subterranean clover	+	+	+	90.4±8.9	88±9.2	+	+	+	20±3.9	14±5.6	+	+	+	37.5±16	66±13*	+	+	+	51.8±12	90.6±9*
	Barrel medic	+	+	+	100	100	-	+	+	82.7±14.3	84.4±13.9	-	+	+	39.7±11.7	77.5±19*	+	+	+	45.7±8.1	42.1±2.6
CT	Untrated	-	-	-	0	0	-	-	-	0	0	-	-	-	0	0	-	-	-	0	0
	Water	-	-	-	0	0	-	-	-	0	0	-	-	-	0	0	-	-	-	0	0
	MeOH 5%	-	-	-	0	0	-	-	-	0	0	-	-	-	0	0	-	-	-	0	0

a:(N) necrotic leaf area, (DA) irregular discolored areas surrounded (or not) by a (NR) necrotic ring;

* %DS at 1 mg/ml and at 2 mg/ml is significantly different ($p < 0.05$)

CHAPTER IV

Anthraquinones and their analogues as potential biocontrol agents of rust and powdery mildew diseases of field crops

Anthraquinones and their analogues as potential biocontrol agents of rust and powdery mildew diseases of field crops

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This chapter is published in

Pest Management Science 2022, 78(8), 3489-3497

DOI: https://doi.org/10.1002/ps.6989

Background: Rusts and powdery mildews are severe fungal diseases of major crops worldwide, including cereals and legumes. They can be managed by chemical fungicide treatments, with negative consequences as environmental pollution and risk for human and animal health. Bioactive natural products could be the safest alternative for pest control. The family of anthraquinones, as well as analogue compounds containing an anthraquinone moiety or some modified anthraquinone rings, has been reported to exhibit certain antibiotic activity. Thus, the potential antifungal activity of some anthraquinones isolated from *Ascochyta lentis*, was assayed in this study for their effectiveness to reduce rust and powdery mildew diseases on pea and oat. Their effect on fungal development was macro- and microscopically assessed on inoculated leaves, and compared to the control achieved by the chemical fungicide (Tetraconazol 12.5% and Azoxystrobin 25%). In addition, the most promising com- pound was also tested at different concentrations in inoculated whole plants in order to evaluate its preventive and curative potential against fungal infection.

Results: All metabolites studied strongly reduced the development of rust and powdery

mildews in both pea and oat, being pachybasin and lentiquinone C the most effective ones in hampering fungal spore germination and appressoria formation. Some of them also affected post-penetration events reducing colony size and number of haustoria per colony. Results were confirmed for pachybasin in whole plants assays, showing an efficacy similar to the commercial fungicide to control fungal diseases, both in preventive and curative applications.

Conclusions: Some fungal anthraquinones and close metabolites, especially pachybasin, could be very promising molecules with effective potential as antifungal agents against both rust and powdery mildew of both pea and oat. Some structure-activity relationships feature have also been evaluated.

Keywords: Biocontrol; fungal metabolites; anthraquinones; pachybasin; powdery mildew; rust.

1.Introduction

Foliar diseases caused by biotrophic fungal pathogens, such as rusts and powdery mildews are major limiting factors for yield production in field crops worldwide, including legumes and cereals, and are responsible for losses ranging between 20-40% of global agricultural productivity [1]. There are many species of rusts and powdery mildew fungi, being *Puccinia* and *Uromyces*, and *Blumeria* and *Erysiphe* the most important genus of rusts and powdery mildews, respectively [2,3]. Among these, *Puccinia coronata* f.sp. *avenae* and *Uromyces pisi* cause rust disease, and *Blumeria graminis* f.sp. *avenae* and *Erysiphe pisi*, cause powdery mildew diseases on oat and pea, respectively [2,4].

Biotrophic pathogens have very efficient spreading mechanisms, hampering their management. Several efforts were made to develop resistant cultivars [1, 2, 4], but to date, the use of chemical is the most diffuse method for rust and powdery mildew management [5, 6], even though fungicides have low specificity, are not easily biodegradable and pathogens tend to develop resistance after a prolonged use [7]. These problems prompt the search for ecofriendly alternative methods also to satisfy the global pressing requests of policy makers and consumers.

Many efforts have been made to discover natural products with known and/or new structures and modes of actions. Among them bioactive natural products with potential and different practical applications in agriculture were recently isolated from different fungi [8-10], as well as from weeds and cultivated plants [11,12]. Cyclopaldic acid and

epi-epoformin [13,14] produced by *Seiridium cupressi* and *Diplodia quercivora*, pathogen of the forest plant cypress (*Cupressus sempervirens*) and oak (*Quercus canariensis*), respectively, have been reported to inhibit *Puccinia* and *Uromyces* spore germination and hypha infection [15,16]. Also, cavoxin [17], inuloxin C [12] and sphaeropsidin A [18] have been reported to inhibit pea powdery mildew (*E. pisi*) spore germination and haustoria formation, resulting in disease reduction [19].

Anthraquinones represent a well-known class of natural compounds produced by plants and fungi [20,21]. They include phytotoxins produced by pathogenic fungi of field [22] and forest crops [23]. Among many examples, some different anthraquinones such as chrysophanol, emodin, pachybasin and ω-hydroxypachybasin were isolated from *Trichoderma* spp [24]. Others, such as lentiquinones A, B, and C and lentisone, which are close fungal metabolites, together with pachybasin, ω-hydroxypachybasin and phomarin were recently isolated from *Aschochyta lentis*, the causal agent of lentil's Ascochyta blight. These anthraquinones have shown antibacterial, antiparasitic, insecticidal, fungicidal, and antiviral activities [25-27].

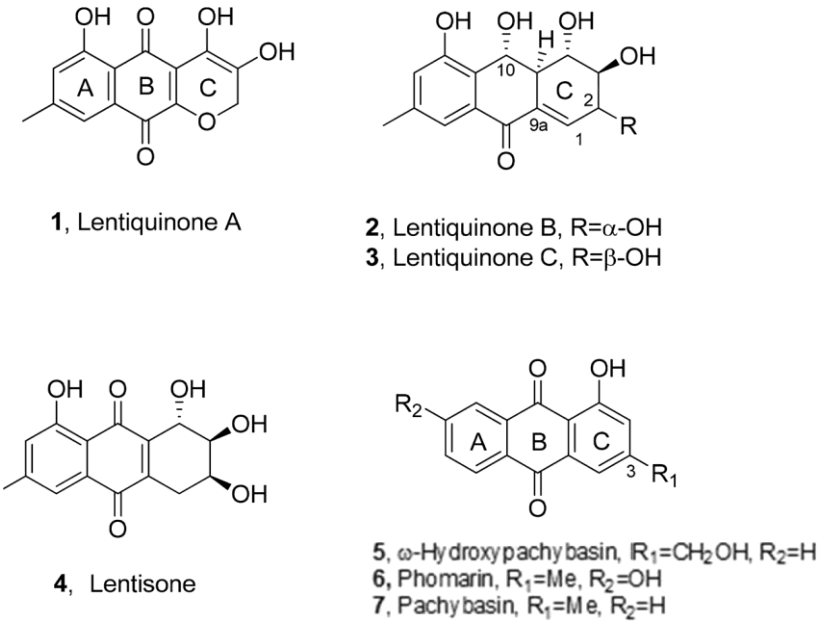

Figure 1. Structures of lentiquinones A–C, lentisone, ω-hydroxypachybasin, phomarin and pachybasin (**1**-**7**).

On viewing these results, seven metabolites isolated from *A. lentis* (specifically lentiquinones A, B and C, lentisone, pachybasin, ω-hydroxypachybasin and phomarin) belonging or closely related to the anthraquinone's family, were tested for their potential as natural fungicides against agronomically damaging pea and oat rusts (*Uromyces pisi* and *Puccinia coronata* f.sp. *avenae*) and pea and oat powdery mildews (*Erysiphe pisi* and

Blumeria graminis f. sp. *avenae*) under controlled conditions both in detached leaves and in whole plant assays.

2. Materials and Methods

2.1. Fungal bioactives metabolites

The anthraquinones pachybasin, ω-hydroxypachybasin and phomarin as well as the close lentiquinones A-C, and lentisone (Fig. 1), were obtained from the culture filtrates and mycelium of Ascochyta lentis isolated from diseased lentil (*Lens culinaris*) plants and purified as recently detailed by Masi *et al.* [27].

2.2. Plant growth and pathogen multiplication

Seedlings of pea (*P. sativum* subsp. *sativum*) cv. Messire and oat (*Avena sativa*) cv. Selma, highly susceptible to pea and oat rusts and powdery mildews, respectively, were used both for pathogen multiplication and assays. Plants were raised from seeds in pots (6 × 6 × 10 cm) filled with a potting mixture (sand/peat, 1:3 vol/vol), then were grown in a growth chamber at 20 ± 2 °C and 65% relative humidity under a photoperiod at 14 h light/10 h dark with light intensity of 200 μmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes.

Both rust and powdery mildew are obligate biotrophic fungi, needing living plants for their multiplication as they do not grow in artificial culture media. Rust spores can easily be long term stored frozen or even short term maintained at air temperature. However, powdery mildew spores cannot be stored, and the fungus must be maintained on living plants. Because of this, the pea powdery mildew (*E. pisi*) isolate CO-01 derived from mildew population collected at Córdoba (southern Spain) was maintained on Messire seedlings, and the oat powdery mildew (*Blumeria graminis* f.sp. *avenae*) race 5 was maintained on Selma seedlings. Both isolates are permanently maintained at IAS-CSIC premises for use in different experiments. Leaves with heavy sporulation were shaken 1 day before inoculation ensuring that only young, vigorous spores were used for inoculum the following day (Fig. 2).

Figure 2. Susceptible pea (*Pisum sativum*) cv. Messire infected with *Uromyces pisi* (a) or with *Erysiphe pisi* (b); susceptible oat (*Avena sativa*) cv. Selma infected with *Puccinia coronata* f.sp. *avenae* (c) or with *Blumeria graminis* f.sp. *avenae* (d).

For rust, 12-day-old seedling plants of each species were inoculated with their respective fungal isolates, preserved at −80 °C, for spore multiplication. Rust isolates used were *U. pisi* isolate UPC-04 and *P. coronata* f.sp. *avenae* isolate Co-04 belonging to the IAS-CSIC fungal collection. Plants were inoculated by dusting the leaves with rust urediospores (2 mg spores plant^{-1}) mixed with pure talc (1:10, w:w) using a spore settling tower. Plants were incubated for 24 h at 20 °C in complete darkness and 100% relative humidity, and then returned to growth chamber conditions to allow disease development. Fresh urediospores were collected from leaves the same day of the bioassay. Every set of plants per isolate was maintained apart from the others in distinct growth chambers to avoid cross inoculations.

2.3. Fungal inoculations and disease assessments

2.3.1. Detached leaves experiments

A first screening evaluating the macroscopical and microscopical effects of all metabolites was performed using a detached leaf method [2,28], so that both inoculation density and incubation conditions could be more precisely controlled. Susceptible plants of pea cv. Messire and oat cv. Selma were grown under the controlled conditions mentioned above until the fifth leaf stage (pea) and the second-formed leaf (oat) were achieved. Then several fourth-formed (pea) and first leaves (oat) were excised and placed, adaxial side up, on 4% technical agar in Petri dishes. For each fungal pathogen, cut leaves were arranged in a randomized design with four replicates per treatment, each replicate

Table 1. Physic data for anthraquinones, positive and negative control tested in pea and oat pathogens inhibition assays

Compounds	MW*
Lentiquinone A	274.2
Lentiquinone B	292.3
Lentiquinone C	292.3
Lentisone	290.3
ω-Hydroxypachybasin	254.2
Phomarin	254.2
Untreated (control)	-
Water (control)	-
Water and MeOH 5% (control)	-
Isagro® (Tetraconazol 12.5%)	-
Mirador® (Azoxystrobin 25%)	-

*Molecular weight. A line has to be included in Table 1, between phomarin and untreated (control). In compounds coloumn you may put "pachybasin", and in MW column you should put 238.2.

having four leaves. The metabolites listed in Table 1 were tested at concentrations of 1 mM. Compounds were dissolved in MeOH (5%) and then brought up to the assay concentration with distilled water. The test solutions (50 µL) were applied on the adaxial leaf side together with negative controls consisting of distilled water and MeOH (5%) which were applied in the same amount (50 µL) as negative controls. Droplets (50 µL) of commercial anti-powdery mildew fungicide (Tetraconazol 12.5%, Isagro® by Sipcan Inagra S.A.), or anti-rust fungicide (Azoxystrobin 25%, Mirador® by Adama Agricultural Solutions Ltd.), were applied depending on the plant-pathogen combination as positive control (0.2 g L^{-1}). The solvent was evaporated in a laminar flow cabinet until dry. For each pathogen, the Petri dishes were inoculated in a spore settling tower spreading 50 fresh spores mm^{-2} collected from the susceptible inoculated genotypes (described in Section 2.2). Only for rust inoculation, plates were transferred during 24 h to a cabinet at 20 ± 2 °C in complete darkness and high relative humidity, and then were returned to growth chamber conditions. Twenty-four hours after inoculation (h.a.i.), four leaves per plant, treatment and replicate were cut and used to perform microscopical studies on fungal development. Leaves were fixed by placing the adaxial surface upon filter paper moistened with 1:3 (v:v) glacial acetic acid: absolute ethanol. When the chlorophyll was eliminated, the leaves were transferred to filter paper moistened with tap water (minimum 2 h) to soften the tissue and then to filter paper moistened with lactoglycerol (1:1:1 v:v:v, lactic acid: glycerol:water). Fungal structures were staining with 0.25% Trypan blue, as described by Barilli *et al*. [29] (for rust), or aniline blue in lactoglycerol (0.1%) (for

powdery mildew) [28] before examination by light microscopy using a phase contrast Leica DMLS microscope at ×20 and ×40 magnifications (Leica Microsystems, Wetzlar, Germany). On every leaf segment, germination frequency was assessed by scoring 100 spores for the presence of a germ tube. Further steps of fungal development were assessed on additional 35 randomly selected germinated spores, recording if appressoria was formed, and the number of hyphal tips and haustoria produced per colony. Finally, disease severity (DS%) was macroscopically assessed on an additional three inoculated cut leaves per repetition and treatment 2 weeks after inoculation by a visual estimation of the leaf area covered with rust pustules or with mildew mycelium. The presence or absence of necrosis indicating the phytotoxic effect of the metabolites tested was recorded.

2.3.2. Whole-plants assays

To simulate the effect of the treatments with metabolite against the different pathogens under real conditions, several experiments were carried out on whole plants of susceptible pea cv. Messire and oat cv. Selma, infected with either powdery mildew or rust under the controlled conditions mentioned in Section 2.2. Plants were arranged in a randomized design with five replicates per treatment, plant species and pathogen, each replicate having three plants. Two-weeks old plants were inoculated by dusting the whole plants with fresh collected spores of rusts or powdery mildews. Each plant-pathogen combination was inoculated apart, following procedures described in Section 2.3.1. Metabolite pachybasin, which was found to be the most effective in pea and oat detached leaves against both pathogens, was applied in whole plants at two concentrations as 1 and 2 mM. In order to study the protective effect of the metabolite, a set of plants was treated 2 days before fungal inoculation (DBI) and another set at the same time as fungal inoculation (0 DBI). In addition, to study the curative effect of the metabolites, a third set of plants was treated 2 days after fungal inoculation (DAI). Pachybasin was suspended in distilled water with MeOH (5%) and then sprayed over the canopy of the seedlings until run-off. For the negative controls, plants were untreated (Control), sprayed with distilled water only (Water) or sprayed with distilled water with MeOH at 5%. The commercial fungicides for rust or powdery mildew depending on the assay, were applied as positive control at their recommended concentrations. Disease severity was assessed 7 or 10 days after inoculation, for powdery mildews and rusts respectively, as percentage of the whole

plant covered by the symptoms. Attention was paid to observe eventual necrosis indicating the phytotoxic effect of the metabolites tested.

2.3. Statistics

For statistical analysis, percentage data were transformed to arcsine square roots (transformed value = 180/π × arcsine [√(%/100)]) to normalize data and stabilize variances throughout the data range. Transformed data were subjected to analysis of variance (ANOVA) using Statistix 8 (Analytical Software, Tallahassee, FL), after which residual plots were inspected to confirm data conformity to normality. Significance of differences between means was determined by calculating least significant difference (LSD) ($P = 0.01$).

3. Results and discussion

3.1. Detached leaves assays and microscopic assessments

In this study, four anthraquinones analogues (Fig. 1, 1–4) and three anthraquinones (Fig. 1, 5–7) previously isolated [27] from the culture filtrates and mycelium of *A. lentis* were tested for their inhibitory effect on rusts and powdery mildews at all plant penetration stages, from spore germination till colony formation and sporulation. The metabolites studied were identified comparing their ^{1}H NMR and ESI MS spectra and specific optical rotation with those previously reported [27]. Their purity was higher than 95% and was ascertained by ^{1}H NMR and HPLC analyses.

3.1.1. Plant-rust interactions

For rusts, when environmental conditions are favorable, urediospores germinate forming a germ tube that grows over the leaf surface until a stoma is recognized. Then, an appressorium is differentiated over a stoma and a substomatal vesicle is formed inside the stomatal cavity from which the fungus grows intercellularly towards the mesophyll forming infection hyphae that, after coming into contact with the mesophyll cells wall, originates the haustorial mother cells. This structure originates a nutrient-absorbing haustorium and, from this moment the pathogen's development is supported by host

Table 2. Microscopical observations on the effect achieved by seven metabolites (at 1 mM) on rusts development stages at 24 hai*

Treatments	Pea – *Uromyces pisi* Microscopical data				Oat – *Puccinia coronata* f.sp. *avenae* Microscopical data			
	% Germination	% Appressoria	Nº of hypal tips/colony	No of haustoria/ colony	% Germination	% Appressoria	Nº of hypal tips/colony	No of haustoria/ colony
Untreated	79.1 ± 4.3 a	54.9 ± 1.8 a	3.6 ± 1.1 a	1.8 ± 0.7 a	79.4 ± 3.7 a	53.6 ± 0.6 a	3.5 ± 0.6 a	1.9 ± 0.5 a
Water control	78.9 ± 3.7 a	52.5 ± 2.1 a	3.5 ± 1.1 a	1.8 ± 0.5 a	79.0 ± 3.8 a	52.8 ± 1.4 a	3.3 ± 0.5 a	1.7 ± 0.2 a
MeOH 5% control	77.1 ± 3.4 a	52.7 ± 2.8 a	3.2 ± 1 ab	1.7 ± 0.8 a	78.0 ± 4.3 a	53.4 ± 1.2 a	3.4 ± 0.9 a	1.8 ± 0.9 a
Lentiquinone A 1 mM	39.3 ± 0.5 b	16.7 ± 1.5 b	2.9 ± 0.8 bc	1.6 ± 0.9 ab	28.4 ± 3.1 b	11.8 ± 0.5 b	2.6 ± 0.6 ab	1.3 ± 0.5 ab
Lentiquinone B 1 mM	52.0 ± 4.3 ab	19.4 ± 1.2 b	3.1 ± 1 ab	1.3 ± 0.4 bc	31.2 ± 5.9 b	2.5 ± 0.3 d	2.6 ± 0.4 ab	1.4 ± 0.4 ab
Lentiquinone C 1 mM	6.4 ± 2.1 bc	2.7 ± 0.9 de	1.2 ± 0.5 d	0.5 ± 0.4 d	1.9 ± 1.1 d	0.1 ± 0.03 fg	0.8 ± 0.2 b	0.3 ± 0.15c
Lentisone 1 mM	23.2 ± 3.6 b	14.8 ± 0.8 bc	3.5 ± 0.6 a	1.9 ± 0.8 a	28.7 ± 2.9 b	2.3 ± 0.3 d	3.2 ± 0.7 a	1.6 ± 0.5 a
ω-Hydroxypachybasin 1 mM	35.5 ± 2.7 b	14.0 ± 1.0 bc	2.2 ± 0.4 c	1.2 ± 0.6 c	29.2 ± 2.8 b	7.0 ± 0.9 c	1.4 ± 0.4 b	1.2 ± 0.2 b
Phomarin 1 mM	46.0 ± 1.6 b	13.6 ± 0.7 c	2.9 ± 1.0 bc	1.3 ± 0.5 bc	22.4 ± 1.0 b	1.2 ± 0.3 e	3.0 ± 0.4 a	1.4 ± 0.6 ab
Pachybasin 1 mM	3.4 ± 1.1 c	0.4 ± 0.5 e	0 ± 0 e	0 ± 0 e	2.7 ± 0.2 e	0.3 ± 0.2 fg	0 ± 0 c	0 ± 0 d
Fungicide Azoxystrobin 25%	1.5 ± 0.4 d	0.4 ± 0.4 e	0 ± 0 e	0 ± 0 e	0.6 ± 0.2 e	0 ± 0 g	0 ± 0 c	0 ± 0 d

Experiments were performed on susceptible pea and oat genotypes.
Values, per column and treatment, followed by different letters differ significantly at $P < 0.01$.
*Hours after inoculation.

metabolism [30]. Already by 24 h after inoculation (h.a.i.) the two rust fungi studied (*Uromyces pisi* on pea and *Puccinia coronata* f.sp. *avenae* on oat) showed high percentage of spore germination (>70%) in the untreated and negative controls, with no significant differences among them. By contrary, spore germination was markedly reduced (<2.4%) by the fungicide (Azoxystrobin 25%) (Table 2), as expected since its efficacy in reducing rust germination and subsequent fungal penetration stages is well known in several crops including legumes and cereals [5,31]. Also lentiquinone C and pachybasin markedly inhibited spore germination of both rusts at similar levels than the commercial fungicide (range 1.9-6.4%) (Table 2). Remaining metabolites also significantly inhibited spore germination of rusts as compared with the untreated control (in the range of 1.5-3.4 and 2.5-3.5-fold reduction in pea and oat, respectively), but not as strongly as lentiquinone C and pachybasin (Table 2).

Germtube elongation and successful appressoria formation of germinated spores was not affected by the negative controls but were almost suppressed (<1% appressoria formation) by the commercial fungicide (Table 2), and significantly inhibited at various levels by all anthraquinones and close metabolites studied (range 0.1–19.4%). All the compounds tested reduced *U. pisi* and *P. coronata* f.sp. *avenae* appressorial formation below 20% and 11%, respectively (Table 2, Fig. 3). In particular, lentiquinone C and pachybasin were very active in reducing appressoria forma- tion in both rusts, showing a fungitoxic effect that was comparable to those obtained with the chemical at the same concentration (<2.7%) (Table 2). Microscopically, as observed for spore germination, a high number of hyphal tips per colony (values up to 3) and haustoria per colony (range

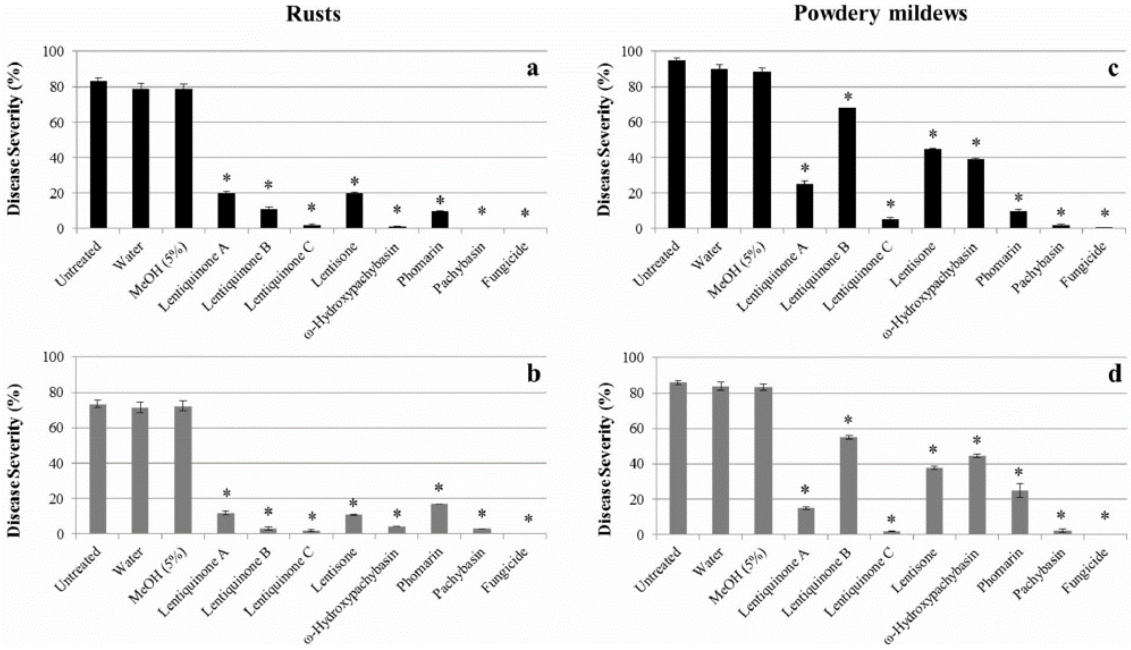

Figure 3. Disease severity (%) of pea and oat rusts (black and grey columns, respectively) (a) and pea and oat powdery mildews (black and grey columns, respectively) (b) in cut leave assays on negative (untreated, water and MeOH 5%) controls, treated leaves with metabolites and positive controls (fungicide). The compounds were tested at a concentration of 1 mM. The experiment was repeated four times. For each metabolite, the asterisk indicates that differences on fungal development due to the metabolite tested compared to the negative controls were significant ($P < 0.01$).

between 1.3 and 2) were achieved by the fungus in the negative controls (untreated, water and MeOH 5%), with no significant differences between them (Table 2). On the contrary, no colony development was observed in inoculated leaves treated with the commercial fungicide (Table 2). Lentiquinone C strongly inhibited both the number of hyphal tips and haustoria per colony in both rust pathosystems (hyphal tips per colony <1.2 and haustoria per colony <0.2), while pachybasin completely inhibited fungal expansion with no hyphal growth tips and no haustoria at all. These results were comparable with those obtained with the chemical at the same concentration. This is the first report on the inhibition effect exerted by antraquinones and close metabolites as pachybasin and lentiquinone C on rust pathogens, on early fungal development steps. This is in line with macroscopical observations on DS (% of pustules covering leaf tissue) which was about 80% in pea and 70% in oat negative controls, but underwent a strong reduction in all metabolites treated leaves (DS < 20% (Fig. 3), particularly high for lentiquinone C and pachybasin (<4%), similar to the commercial fungicide.

We did not observe any sign of phytotoxicity caused by any of the metabolites used for this study on plant hosts (both pea and oat) at the concentration tested. This is in

line with results found by Masi *et al*. [27] which highlighted the relation between the quinone skeleton of several anthraquinone analogues and their phytotoxicity.

Considering the results obtained, some structure-activity relationships could be hypothesized. Among the anthraquinone analogues (lentiquinones A-C and lentisone) the significant activity of lentiquinone C could be attributed to the different stereochemistry of the hydroxy group at C-2 comparing to the structure of lentiquinone B. However, the reduction of the ketone group at C-10 and the presence of a double bond between C-1 and C-9a also seem to be important for the activity considering that lenti- sone has the same stereochemistry of lentiquinone C for the carbons of C-ring but it does not have these substitutions. The lower activity of lentiquinone A could be explained for the presence of a pyran C-ring, which is not present in the other three analogues. Among the anthraquinones (pachybasin, ω-hydroxypachybasin and phomarin), the strong activity of pachybasin could be due to the presence of a methyl group at C-3 of the C-ring and the simultaneous absence of substituents at A-ring of the anthraquinone system which could differently affect their oxidoreductive ability [21,27].

3.1.2. Plant-powdery mildew interactions

The biological cycle of powdery mildews includes the germination of conidia on the host epidermis over which, as a difference with rusts, the pathogen grows epiphytically, invaginating haustoria into epidermal cells for feeding, but then forming secondary mycelia followed by subsequent epiphytic colony growth [32].

On pea- and oat-powdery mildew interactions studied on detached leaves, the percentage of fungal spore germination was higher than 70% and 60%, respectively, in all the negative controls (untreated, water and MeOH 5%), with no significant differences between them (Table 3). On the contrary, the commercial fungicide (Tetraconazol 12.5%) strongly reduced spore germination (<2%) showing its well-known activity on powdery mildew development [33]. Pachybasin was particularly effective reducing spore germination of both powdery mildew species (<6%). This was followed by lentiquinone C that was also very effective reducing oat powdery mildew spore germination (8.9%), although not that much for the pea pathogen (39.3%). All other metabolites also significantly reduced germination but this reduction was more modest (in the range 31.8–46.7%). There were no signs of phytotoxicity on leaves.

Table 3. Microscopical observations on the effect achieved by seven metabolites (at 1 mM) on powdery mildew development stages at 24 hai*

Treatments	Pea – *Erysiphe pisi* Microscopical data				Oat – *Blumeria graminis* f. sp. *avenae* Microscopical data			
	% Germination	% Appressoria	Nº of hypal tips/colony	No of haustoria/ colony	% Germination	% Appressoria	Nº of hypal tips/colony	No of haustoria/ colony
Untreated	77.6 ± 5 a	52.1 ± 3.7 a	11.2 ± 3.8 a	4.8 ± 1.8 a	72.8 ± 5.3 a	45.4 ± 4.3 a	6.9 ± 1.9 a	3.4 ± 0.9 a
Water control	75.8 ± 3.6 a	51.2 ± 4.3 a	10.4 ± 2.9 a	4.5 ± 1.3 a	70.2 ± 4.6 a	44.9 ± 4.6 a	6.3 ± 1.7 a	3.1 ± 0.7 a
MeOH 5% control	75.5 ± 3.7 a	51.7 ± 3.6 a	10.4 ± 2.6 a	4.3 ± 1.6 a	69.2 ± 4.7 a	44.3 ± 4.3 a	6.4 ± 1.4 a	3.1 ± 0.7 a
Lentiquinone A 1 mM	40.6 ± 2.9 bc	45.1 ± 2.9 b	2.3 ± 2.0 c	1.6 ± 0.9 b	43.0 ± 6.8 b	44.9 ± 4.1 a	3.8 ± 1.5 b	1.2 ± 0.8 bc
Lentiquinone B 1 mM	41.0 ± 5.0 bc	44.9 ± 3.9 b	3.7 ± 1.4 b	1.4 ± 0.8 b	31.8 ± 3.6 c	33.1 ± 2.9 b	3.1 ± 0.4 bc	1.5 ± 0.6 b
Lentiquinone C 1 mM	38.3 ± 1.7 c	5.8 ± 2.9 e	1.8 ± 1.2 c	0.4 ± 0.3 c	8.9 ± 3.1 d	11.0 ± 1.6 de	3.8 ± 1.3 b	0.7 ± 0.4 c
Lentisone 1 mM	46.7 ± 2.6 b	16.9 ± 4.1 d	4.4 ± 1.6 b	2.2 ± 0.8 ab	45.2 ± 2.8 b	23 ± 4.8 c	3.6 ± 1.2 b	1.7 ± 0.6 b
ω-Hydroxypachybasin 1 mM	45.9 ± 2.9 b	46.9 ± 2.9 b	3.7 ± 1.5 b	0.6 ± 0.4 bc	38.8 ± 2.4 bc	31.4 ± 3.0 b	2.2 ± 0.8 c	0.5 ± 0.4 cd
Phomarin 1 mM	40.8 ± 4.9 bc	33.7 ± 2.6 c	3.1 ± 1.4 bc	1.4 ± 0.7 b	40.9 ± 4.2 bc	31.1 ± 5.5 b	3.6 ± 1.0 b	1.5 ± 0.6 b
Pachybasin 1 mM	5.8 ± 1.6 d	7.05 ± 1.9 e	1.4 ± 0.7 cd	0.3 ± 0.2 c	4.6 ± 2 d	7.5 ± 2.1 e	1.7 ± 0.8 cd	0.3 ± 0.2 d
Fungicide Tetraconazol 12.5%	1.6 ± 0.8 e	0.5 ± 0.2 f	1.3 ± 0.6 d	0.3 ± 0.2 c	0.9 ± 0.3 e	0.8 ± 0.5f	1.4 ± 0.7 d	0.25 ± 0.2 d

Experiments were performed on susceptible pea and oat genotypes.
Values, per column and treatment, followed by different letters differ significantly at $P < 0.01$.
*Hours after inoculation.

The successive appressoria formation stage of both powdery mildews was also strongly reduced by lentiquinone C (<11%) and pachybasin (<7.4%) compared to their controls. These compounds also strongly inhibited colony growth (<1.7 hyphal tips, with <1 haustoria per colony) in both pathosystems (Table 3). No associated phytotoxicity was observed for any treatment or pathosystem, in agreement to what found in Section 3.1.1.

Table 4. Macroscopical observations: infection type (IT) and final disease severity (DS%) of rust on plant of oat cv. Selma inoculated with *Puccinia coronata* f.sp. *avenae* and of pea cv. Messire inoculated with *Uromyces pisi* mM

Treatments	Oat – *Puccinia coronata* f.sp. *avenae*						Pea – *Uromyces pisi*					
	2 DBI		**0 DBI**		**2 DAI**		**2 DBI**		**0 DBI**		**2 DAI**	
	IT	**DS%**	**IT**	**DS%**	**IT**	**DS%**	**IT**	**DS%**	**IT**	**DS%**	**IT**	**DS%**
Untreated	4	85.0 ± 2.0 a	4	90.0 ± 2.0 a	4	87.2 ± 1.6 a	4	75.5 ± 1.5 a	4	75.3 ± 1.2 a	4	78.0 ± 1.4 a
Water control	4	80.3 ± 3.2 a	4	87.8 ± 2.3 a	4	86.8 ± 3.1 a	4	73.0 ± 1.3 a	4	73.2 ± 1.2 a	4	78.3 ± 1.1 a
MeOH 5% control	4	80.4 ± 3.0 a	4	84.4 ± 3.1 a	4	88.3 ± 3.4 a	4	70.9 ± 1.2 a	4	74.9 ± 1.2 a	4	76.1 ± 1.9 a
Pachybasin 1 mM	4	27.2 ± 4.6 b	4	8.3 ± 2.6 b	4	50.5 ± 5.4 b	4	24.3 ± 2.0 b	4	13.1 ± 1.7 b	4	43.8 ± 1.3 b
Pachybasin 2 mM	4	20.7 ± 3.9 b	4	3.4 ± 2.1 c	4	44.4 ± 3.4 b	4	22.4 ± 1.7 c	4	2.5 ± 0.8 c	4	39.4 ± 1.6 c
Fungicide Azoxystrobin 25%	0	0 ± 0c	0	0 ± 0d	0	0 ± 0c	0	0 ± 0c	0	0 ± 0 d	0	0 ± 0c

DS% was assessed on whole planta 10 DAI.* Pachybasin selected metabolite, negative (untreated, water and MeOH 5%) and positive (Azoxystrobin 25% fungicide) controls were applied to plants at different times to fungal inoculation: 2 DBI,† 0 DBI and 2 DAI. Pachybasin was tested at a concentration of 1 and 2.
Values, per column and treatment, followed by different letters differ significantly at P < 0.01.
*Days after inoculation.
† Days before inoculation.

Our results show that pachybasin and lentiquinone C strongly inhibit both pre- and post-penetration stages of rusts and powdery mildews. Uredospores and conidia respond to host leaf treatment with a failure in germination and, successively, with a decrease in the proportion of normally developed appressorium competent for host penetration, which lead to a reduced andsmaller final number of fungal colonies produced. These quantitative *in vitro* data suggest that this specific morphological response is as

consequence of the direct dose-dependent contact with the compounds. This is in accordance with previous results found for pachybasin tested *in vitro* against *B. graminis* f.sp. *hordei* (the causal agent of powdery mildew in barley) at different concentrations ranging between 10^{-4} and 1 mM [34], and showing a dose-dependent efficacy in inhibit conidia germination and sub- sequent appressoria formation. In fact, Hildebrandt *et al*. showed that pachybasin only significantly affected the fungal pre-penetration processes at the higher concentration tested, what agrees with our observations. Lentiquinone C has previously showed antibiotic and herbicide effects [27], but this is the first report on its fungicide capacity.

3.2. Studies performed in planta

Pachybasin, followed by lentiquinone C appeared the most effective anthraquinones inhibiting rusts and powdery mildews, when applied at a concentration comparable with the commercial fungicide. Pachybasin was produced in larger amounts by the fungus and thus was selected for further validations on whole-plant experiments.

Table 5. Macroscopical observations: infection type (IT) and final disease severity (DS%) of powdery mildew on plants of pea cv. Messire and oat cv. Selma inoculated with *Erysiphe pisi and Blumeria graminis* f.sp. *avenae*, respectively

	Oat – *B. graminis* f.sp. *avenae*						Pea – *E. pisi*					
	2 DBI		**0 DBI**		**2 DAI**		**2 DBI**		**0 DBI**		**2 DAI**	
Treatments	**IT**	**DS%**	**IT**	**DS%**	**IT**	**DS%**	**IT**	**DS%**	**IT**	**DS%**	**IT**	**DS%**
Untreated	4	80.0 ± 2.2 a	4	84.3 ± 2.9 a	4	87.2 ± 1.28 a	4	77.6 a ± 3.5 a	4	75.3 ± 2.8 a	4	78.8 ± 2.4 a
Water control	4	83.5 ± 4.0 a	4	82.7 ± 2.7 a	4	80.7 ± 5.8 a	4	81.7 ± 2.1 a	4	77.8 ± 1.9 a	4	81.0 ± 1.5 a
MeOH 5% control	4	83.6 ± 4.2 a	4	79.7 ± 3.1 a	4	86.1 ± 5.5 a	4	81.8 ± 2.4 a	4	78.3 ± 1.5 a	4	82.2 ± 1.5 a
Pachybasin 1 mM	4	12.1 ± 2.5 b	4	6.6 ± 6.5 b	4	9.4 ± 1.8 b	4	16.5 ± 2.8 b	4	2.0 ± 0.9 b	4	6.9 ± 2.8 b
Pachybasin 2 mM	4	9.0 ± 3.0 b	4	0 ± 0 c	4	1.0 ± 0.6 c	4	13.8 ± 2.6 b	4	1.4 ± 1.0 bc	4	3.9 ± 1.8 b
Fungicide Tetraconazol 12.5%	0	0 ± 0c	0	0 ± 0c	0	0 ± 0 c	0	0.5 ± 0.1 c	0	0 ± 0 c	0	0.6 ± 0.2 c

DS% was recorded on whole plants 7 DAI.* Pachybasin selected metabolite, negative (untreated, water and MeOH 5%) and positive (Tetraconazol 12.5% fungicide) controls were applied to plants at different times to fungal inoculation: 2 DBI,† 0 DBI and 2 DAI. Pachybasin was tested at a concen- tration of 1 and 2 mM.

Values, per column and treatment, followed by different letters differ significantly at $P < 0.01$.

*Days after inoculation.

† Days before inoculation.

While the applied metabolites in *in vitro* system facilitates a more or less homogeneous and reproducible conditions with lipophilic compounds, their efficient deposition by leaf spraying is highly dependent on the properties of the plant cuticle, the efficacy of spray mixture adjuvants, as well as by the envi- ronmental conditions, which clearly affects the general comparability of *in vitro* and *in vivo* data sets. Pachybasin was sprayed on whole plants at two concentrations (1 and 2 mM), before and after fungal inoculation to test its preventive and curative effects.

In the experiments with rusts, high IT values (= 4), indicating a fully compatible interaction not associated with host cell necrosis at the infection site, were observed on both the pea and the oat accession in all treatments, showing that the metabolites did not induce hypersensitive resistance (Table 4). Treatments with pachybasin provided a significant reduction of DS for both rusts (<50%) compared with the negative controls (DS > 70%) (Table 4). Pachybasin was particularly effective when applied at the time of inoculation (0 DBI, <8% DS), followed by protective application 2 days earlier (2 DBI, <27% DS), being less effective although still significant when applied after fungal inoculation (2 DAI, <51% DS) (Fig. 4a,b). The concentration of the metabolite made little dif- ference, being the lowest dose tested equally significant than the commercial fungicide in reducing the disease. The significant curative effect exhibit by pachybasin is in agreement with the inhibitory effects on early stages of fungal infection, from spore germination to early haustoria formation that normally occurs within the first 24 h, so these effects are not seen when treating 2 DBI. However, the fact that there is still a significant DS reduction by about half, confirms a curative effect reducing further growth of already formed fungal colonies. No symptoms of phytotoxicity were already observed, confirming previous observations.

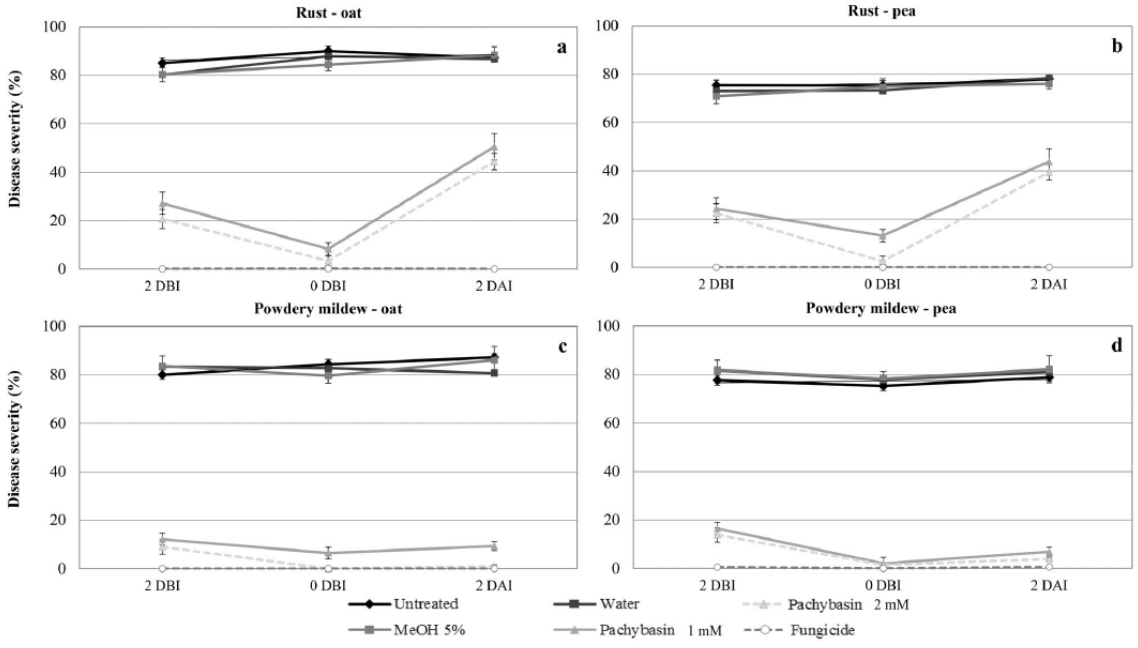

Figure 4. Disease severity (%) measured on rust-inoculated whole plants of oat (a) and pea (b) at 10 days after inoculation, and on powdery mildew-inoculated whole plants of oat (c) and pea (d) at 7 days after inoculation. Plants were treated at different time points (2 days before inoculation (2 DBI), the same day of fungal inoculation (0 DBI) and 2 days after inoculation (2 DAI)) with pachybasin at two different concentrations (1 and 2 mM), and were compared with positive controls (fungicides) and negative controls (untreated, water and MeOH 5%).

Similarly, treatments with pachybasin provided a significant control of both powdery mildews (DS% < 17) compared with the negative controls (DS% > 75) (Table 5; Fig. 4c,d). Differently than with rusts, pachybasin showed similarly good curative and protective effects (DS < 17% at 2DBI, <7% at 0DBI; <10% at 2 DAI). This can be explained by the ectotrophic growth of powdery mildew, which allowed that the metabolite enters into direct con- tact with fungal mycelia even when applied later. On the contrary rusts grow over the epidermis only until the stage of appressoria formation, with all subsequent fungal growth occurring inside substomatal vesicle or the mesophyll.

Due to the strong fungal inhibition of pachybasin, we could suppose that repeated preventive application of this anthraquinones may effectively reduce the major part of the fungal infection potential.

4. Conclusions

Biotrophic fungi as powdery mildew and rust fungi are among the most common and important plant fungal pathogens of both cereal and legume crops. Although cultural and biological practices contribute to reduce the risk of disease development, they do not provide sufficient protection. As a result, chemical control including the use of commercial fungicide from multiple chemical groups is, in practice, the most effective tool for managing these pathogens when no genetic resistance is available. Unfortunately, the risk of resistance development is high because typical protective programs include multiple applications per season. In addition, some of the most economically destructive species of biotrophic fungi are considered to be high-risk pathogens and are able to develop resistance to several chemical classes within a few years. This situation has decreased the efficacy of the major fungicide classes that are employed against both rusts and powdery mildews leading to the application of general integrated disease management strategies as dose limitation, mixtures and search of alternative molecules.

With respect to this last point, many plant pathogens, especially necrotrophic fungi, are capable of producing a broad panel of natural substances representing an unexploited source of potential biofungicides with new molecular structures and mode of actions against several crop diseases. Nevertheless, the choice of the best metabolite as well as the optimal dose and time of application varied greatly depending on the pathosystem involved. In fact, for biotrophic plant fungi of agronomic importance as rusts

and powdery mildews, a successful initial spore germination and fungal penetration are crucial phases for disease development. Here, seven fungal metabolites originate from *A. lentis* and belonging to the anthraquinone-analogues natural compounds were tested with a general success, for their fungitoxic activity against several powdery mildew and rusts of agronomic importance. Lentiquinone C and pachybasin were both promising compounds reducing *in vitro* the early developmental stages of *U. pisi*, *P. coronata* f. sp. *avenae*, *E. pisi* and *B. graminis* f.sp. *avenae*, as well as their post-penetration development at values comparable to those obtained by chemical protection. Large amount of pachybasin is produced by *A. lentis* mycelium (7 g kg^{-1}) while only 0.4 mg L^{-1} of lentiquinone C could be obtained from its culture filtrate [27].

For this reason, the former compound was the most interesting for *in planta* studies, where inhibition of fungal growth at pre- and post-penetration stages, with consequent interesting preventive and curative effects against fungal infection, were exhibited. Due to the pronounced similarities with respect the limitation in germ tube formation and following steps of pathogen development between all biotrophic fungi tested here, it is tempting to hypothesize that pachybasin might exert their antifungal activity with a direct antifungal activity and/or also by modifying in a certain manner the host perception from the pathogen. In fact, the germ tubes of biotrophic fungi possess high capability for sensing host topographical signals [35] and to recognize host-derived vola- tiles as signals to stimulate and regulate certain following steps of fungal development process. It has been demonstrated that other molecules such as farnesyl acetate acted as an antagonistic signal and strongly regulated the rate of appressoria differentiation and colony development in several rust species [36]. Additional studies in this direction may be of interest to better understand the biological properties of pachybasin, which will lead us to an adequate use of this compound in agriculture against biotrophic pathogens of agronomic interest.

In addition, further ecotoxicological studies will be performed in the near future as required to realize the scale-up of pachybasin production in big fermenters and its bioformulation is feasible in order to make the compound safe and economical for its use in agriculture.

Acknowledgments: The authors thank Dr. Vurro M. CNR Bari (Italy), for supplying culture filtrates and mycelium of *Ascochyta lentis*. Prof. Evidente is associated with the Istituto di Chimica Biomolecolare, CNR, Pozzuoli, Italy. Financial support by projects

PID2020-11466RB-100 of Spanish AEI and P20 00986 of Plan Andaluz Investigación are acknowledged.

Data available statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

1. Savary S, Ficke A, Aubertot JN and Hollier C, Crop losses due to diseases and their implications for global food production losses and food security. *Food Secur* 4:519–537 (2012).
2. Sillero JC, Fondevilla S, Davidson J, Patto MC, Warkentin TD, Thomas J *et al*., Screening techniques and sources of resistance to rusts and mildews in grain legumes. *Euphytica* 147:255–272 (2006).
3. Van der Merwe M, Ericson L, Walker J, Thrall PH and Burdon JJ, Evolu- tionary relationships among species of *Puccinia* and *Uromyces* (*Pucciniaceae*, *Uredinales*) inferred from partial protein coding gene phylogenies. *Micol Res* 111:163–175 (2007).
4. Sánchez-Martín J, Rubiales D and Prats E, Resistance to powdery mil- dew (*Blumeria graminis* f.sp. *avenae*) in oat seedlings and adult plants. *Plant Pathol* 60:846–856 (2011).
5. Carmona M, Sautua F, Pérez-Hérnandez O and Reis E, Role of fungicide applications on the integrated management of wheat stripe rust. *Front Plant Sci* 11:733 (2020).
6. Sulima SS and Zhukov VA, War and peas: molecular bases of resistance to powdery mildew in pea (*Pisum sativum* L.) and other legumes. *Plan Theory* 11:339 (2022).
7. Oliver RP, A reassessment of the risk of rust fungi developing resistance to fungicides. *Pest Manag Sci* 70:1641–1645 (2014).
8. Cimmino A, Andolfi A, Avolio F, Ali A, Tabanca N, Khan IA *et al*., Cyclopaldic acid, seiridin, and sphaeropsidin A as fungal phytotoxin, and larvicidal and biting deterrents against *Aedes aegypti* (Diptera: Culicidae): structure-activity relationships. *Chem Biodivers* 10:1239–1251 (2013).

9. Cimmino A, Andolfi A and Evidente A, Phytotoxic terpenes produced by phytopathogenic fungi and allelopathic plants. *Nat Prod Commun* 9:401–408 (2014).
10. Cimmino A, Masi M, Evidente M, Superchi S and Evidente A, Fungal phytotoxins with potential herbicidal activity: chemical and biological characterization. *Nat Prod Rep* 32:1629–1653 (2015).
11. Wang W, Ben-Daniel BH and Cohen Y, Control of plant diseases by extracts of *Inula viscosa*. *Phytopathology* 94:1042–1047 (2004).
12. Andolfi A, Zermane N, Cimmino A, Avolio F, Boari A, Vurro M *et al*., Phytotoxic bi-and tri-cyclic sesquiterpene lactones produced by *Inula viscosa*: potential for broomrapes and field dodder management. *Phytochemistry* 86:112–120 (2013).
13. Graniti A, Sparapano L and Evidente A, Cyclopaldic acid, a major phytotoxic metabolite of *Seiridium cupressi*, the pathogen of a canker disease of cypress. *Plant Pathol* 41:563–568 (1992).
14. Andolfi A, Maddau L, Basso S, Linaldeddu BT, Cimmino A, Scanu B *et al*., Diplopimarane, a 20-*nor*-*ent*-pimarane produced by the oak pathogen *Diplodia quercivora*. *Nat Prod Rep* 77:2352–2360 (2014).
15. Barilli E, Cimmino A, Masi M, Evidente M, Rubiales D and Evidente A, Inhibition of spore germination and appressoria formation of rust species by plant and fungal metabolites. *Nat Prod Commun* 11: 1343–1347 (2016).
16. Barilli E, Cimmino A, Masi M, Evidente M, Rubiales D and Evidente A, Inhibition of early development stages of rust fungi by the two fungal metabolites cyclopaldic acid and *epi*-epoformin. *Pest Manag Sci* 73:1161–1168 (2017).
17. Evidente A, Randazzo G, Iacobellis NS and Bottalico A, Structure of cavoxin, a new phytotoxin from *Phoma cava* and cavoxone, its related chroman-4-one. *J Nat Prod* 48:916–923 (1985).
18. Evidente A, Sparapano L, Motta A, Giordano F, Fierro O and Frisullo S, A phytotoxic pimarane diterpene of *Sphaeropsis sapinea* f. sp. *cupressi*, the pathogen of a canker of cypress. *Phytochemistry* 42:1541–1546 (1996).
19. Barilli E, González-Bernal MJ, Cimmino A, Agudo-Jurado FJ, Masi M, Rubiales D *et al*., Impact of fungal and plant metabolites application on early development stages of pea powdery mildew. *Pest Manag Sci* 75:2464–2473 (2019).
20. Dewick PM, *Medicinal Natural Products: A Biosynthetic Approach*, 3rd edn. John Wiley & Sons, Oxford, England (2009).

21. Masi M and Evidente A, Fungal bioactive anthraquinones and analogues. *Toxins* 12:714 (2020).
22. Evidente A, Cimmino A and Masi M, Phytotoxins produced by pathogenic fungi of agrarian plants. *Phytochem Rev* 18:843–870 (2019).
23. Masi M, Maddau L, Linaldeddu BT, Scanu B, Evidente A and Cimmino A, Bioactive metabolites from pathogenic and endophytic fungi of forest trees. *Curr Med Chem* 25:208–252 (2018a).
24. Gessler NN, Egorova AS and Belozerskaya TA, Fungal anthraquinones. *Appl Biochem Microbial* 49:85–99 (2013).
25. Sugawara K, Igeta E, Amano Y, Hyuga M and Sugano Y, Degradation of antifungal anthraquinone compounds is a probable physiological role of DyP secreted by *Bjerkandera adusta*. *AMB Exp* 9:56 (2019).
26. Liu SY, Lo CT, Shibu MA, Leu YL, Jen BY and Peng KC, Study on the anthraquinones separated from the cultivation of *Trichoderma harzianum* strain Th-R16 and their biological activity. *J Agr Food Chem* 57:7288–7292 (2009).
27. Masi M, Nocera P, Zonno MC, Tuzi A, Pescitelli G, Cimmino A *et al*., Lentiquinones A, B, and C, phytotoxic anthraquinone derivatives isolated from *Ascochyta lentis*, a pathogen of lentil. *J Natural Prod* 81: 2700–2709 (2018b).
28. Rubiales D and Carver TLW, Cellular responses of *Hordeum chilense* genotypes to inappropriate *formae specialis* of the cereals powdery mildew fungus. *Can J Bot* 78:1561–1570 (2000).
29. Barilli E, Sillero JC, Moral A and Rubiales D, Characterization of resis- tance response of pea (*Pisum* spp.) against rusts (*Uromyces pisi* (Pers) Wint). *Plant Breed* 128:665–670 (2009).
30. Emeran AA, Sillero JC, Niks RE and Rubiales D, Infection structures of host-specialized isolates of *Uromyces viciae-fabae* and of other species of *Uromyces* infecting leguminous crops. *Plant Dis* 89:17–22 (2005).
31. Shah DA and Dillard HR, Yield loss in sweet corn caused by *Puccinia sorghi*: a metaanalysis. *Plant Dis* 90:1413–1418 (2006).
32. Barilli E, Rubiales D, Gjetting T and Lyngkjaer MF, Differential gene transcript accumulation in peas in response to powdery mildew (*Erysiphe pisi*) attack. *Euphytica* 198:13–28 (2014).

33. Bellón-Gómez D, Vela-Corcía D, Pérez-García A and Torésa JA, Sensitivity of *Podosphaera xanthii* populations to anti-powdery-mildew fungicides in Spain. *Pest Manag Sci* 71:1407–1413 (2015).
34. Hildebrandt U, Marsell A and Riederer M, Direct effects of physcion, chrysophanol, emodin, and pachybasin on germination and appressorium formation of the barley (*Hordeum vulgare* L.) powdery mil- dew fungus *Blumeria graminis* f.sp. *hordei* (DC.) Speer. *J Agric Food Chem* 66:3393–3401 (2018).
35. Read ND, Kellock LJ, Collins TJ and Nd Gundlach AM, Role of topography sensing for infection-structure differentiation in cereal rust fungi. *Planta* 202:163–170 (1997).
36. Mendgen K, Wirsel SGR, Jux A, Hovmann J and Boland W, Volatiles modulate the development of plant pathogenic rust fungi. *Planta* 224:1353–1361 (2006).

CHAPTER V

Impact of fungal and plant metabolites application on early development stages of pea powdery mildew

Impact of fungal and plant metabolites application on early development stages of pea powdery mildew

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This chapter is published in

Pest Management Science 2019, 75(9), 2464-2473

DOI: https://doi.org/10.1002/ps.5351

Background: Pea powdery mildew incited by *Erysiphe pisi* represents a major constraint for pea crops worldwide. Crop protection is largely based on chemical control, although recently a renewed interest in the discovery of natural products as alternatives to synthetic fungicides application has emerged. Thus, 12 bioactive plant and fungal metabolites belonging to different class of natural compounds were evaluated, together with a commercial fungicide, at different concentrations on detached pea leaves for their potential to inhibit spore germination and subsequent stages of fungal growth. The most effective metabolites were tested at different concentrations *in planta* under controlled conditions to evaluate the level of control achieved by treatments before, concurrently and after pathogen inoculation. Pathogen development was macroscopically scored on whole plants as percentage of disease severity and area under the disease progress curve. **Results**: Cavoxin, inuloxin C and sphaeropsidin A strongly inhibited *E. pisi* germination and haustoria formation and reduced colony size. This effect was dose dependent. These results were further confirmed in whole plants by spraying the metabolites on plant leaves

for preventive or curative control, which reduced fungal developmental of *E. pisi* at levels comparable with those obtained by application of the fungicide.

Conclusions: Cavoxin, inuloxin C and sphaeropsidin A have potential as alternatives to synthetic fungicides for the control of crop pathogens of economic importance such as powdery mildew.

Keywords: *Erysiphe pisi*; *Pisum sativum*; biocontrol; fungal and plant metabolites

1.Introduction

Pea (*Pisum sativum* L.) is an important grain legume crop worldwide that is mainly used for consumption as green vegetables and dry seeds [1]. Pea is cultivated in 7.3 million hectares with a total production of 600 metric tons per year globally [2]. Productivity of pea is affected by many diseases and insect-pests. Among the various diseases affecting pea, powdery mildew, caused by *Erysiphe pisi*, is of great significance [3]. The biological cycle of *E. pisi* includes germination of conidia (asexual spores) or ascospores (sexual spores) on host epidermis and direct penetration of epidermal cells by invagination of haustoria into epidermal cells for feeding, followed by subsequent epiphytic colony growth [4]. *Erysiphe* species are obligate parasites feeding on living plant cells that cannot be cultured on artificial nutrient medias. Therefore, bioassays for these pathogens are very complicated.

Owing to their economic importance, a large number of methods to control powdery mildew have been proposed, including cultural practices, the use of resistant varieties and fungicide application. Despite the substantial effort to generate resistant pea varieties, the use of synthetic chemical fungicides is the mainstay method for *E. pisi* control [5]. However, their widespread application has caused many environmental concerns due to low specificity and slow biodegradability. These include pollution of soil and groundwater, negative impact on flora and fauna, and risks to human and animal health. Furthermore, the high application costs and the ability of the pathogen to evolve resistance to chemical fungicides have prompted efforts to discover natural products as templates for the development of fungicidal biochemicals with new molecular structures and mode of actions. Many plant pathogens, especially necrotrophic or hemibiotrophic fungi, produce novel antimicrobial compounds that can be a source of such useful metabolites [6–8]. Similarly, active metabolites isolated from weeds and cultivated plants

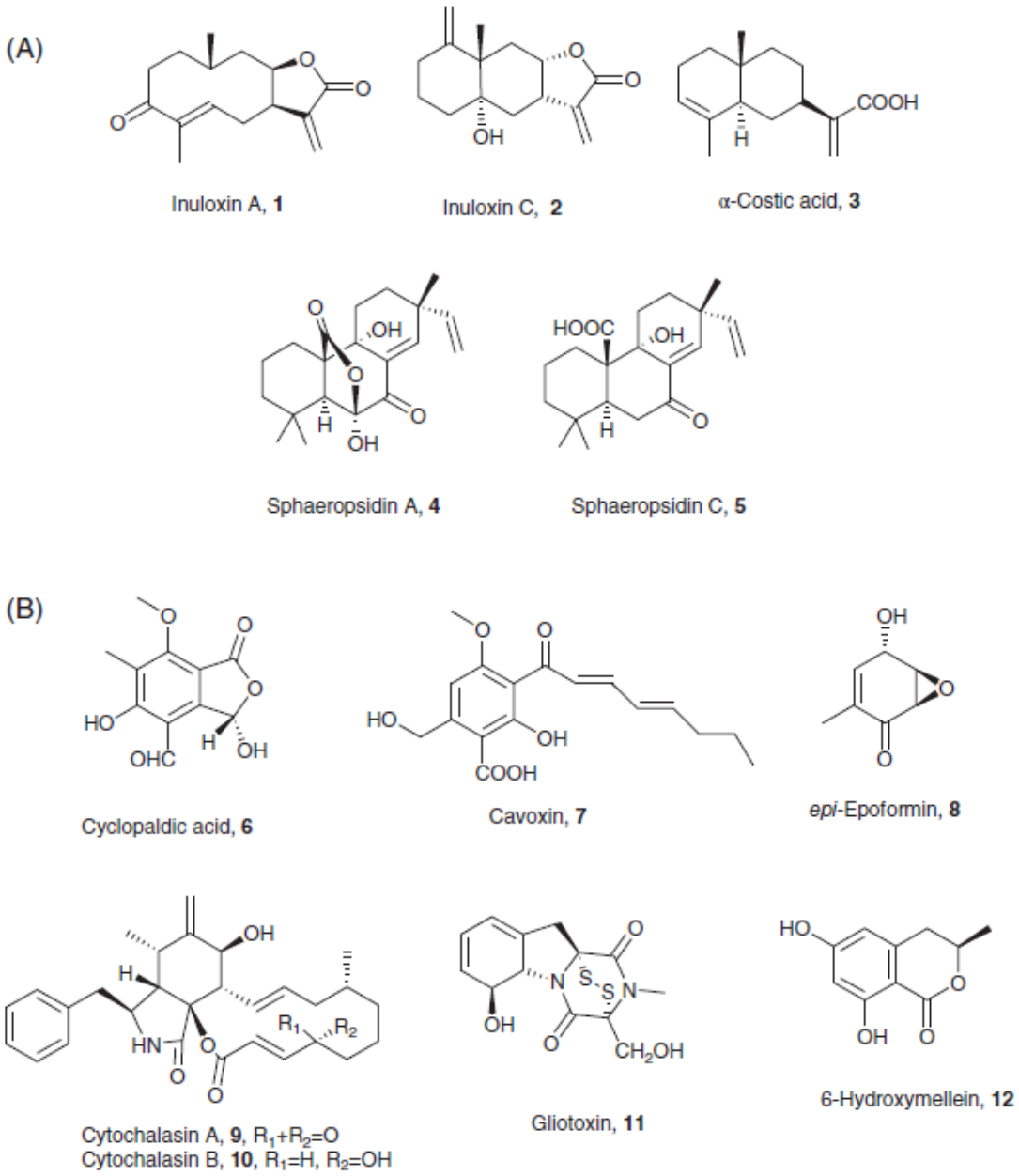


Figure 1. (A) Structures of the plant (1 – 3) and fungal (4 and 5) terpenes tested against pea powdery mildew. (B) Structures of the fungal (6 – 12) metabolites belonging to different classes of natural compounds tested against pea powdery mildew.

have a number of potentially useful biological activities [9,10] As spore germination, haustoria formation and subsequent colony growth are key phases for the initial development of biotrophic plant pathogens such as powdery mildews, an approach for their management could be the use of natural plant or fungal metabolites as inhibitors in these early stages of infection.

In this manuscript the potential of nine fungal and three plant toxins was tested as natural fungicides assaying their inhibitory effects on early stages of development of

E. pisi in detached leaf bioassays. The three most effective metabolites were also tested in whole plants at several application times to determine their protective and/or curative effects against powdery mildew.

2. Materials and Methods

2.1. Plant and fungal bioactives metabolites

The toxins used for the study (Fig. 1, Table 1) belong to different classes of natural compounds as follows. The sesquiterpenes inuloxins A and C, and α-costic acid (**1** – **3**, Fig. 1(A)) were purified from the organic extract of *Inula viscosa* (L.) Ait [10]. The diterpenes sphaeropsidins A and C (**4** and **5**, Fig. 1(B)) and cyclopaldic acid (**6**) were purified from the culture filtrates of *Diplodia cupressi* A.J.L. Phillips and A. Alves [11,12] and *Seiridium cupressi* (Guba) Boesewinkel [13], respectively. Cavoxin (**7**) was isolated from the liquid culture of *Phoma cava* Schulzer [14], while *epi*-epoformin (**8**) was purified from the organic extract of *Diplodia quercivora* Linaldeddu & A.J.L. Phillips [15]. Cytochalasins A (**9**) and B (**10**) were isolated from the solid culture of *Pyrenophora semeniperda* (Brittleb. & D.B. Adam) Shoemaker [16], (Fig. 1(B), Table 1). Finally, gliotoxin (**11**) was isolated from the solid culture of *Neosartorya pseudofischeri* S.W. Peterson [17] and 6-hydroxymellein (**12**) was isolated from the liquid culture of *Phoma chenopodiicola* Gruyter, Noordel. & Boerema (Fig. 1(b), Table 1) [18]. Metabolites **1** – **3** were from plant origin, while metabolites **4** – **12** were from fungal origin (Table 1).

2.2. Plant growth and pathogen multiplication

P. sativum subsp. *sativum* susceptible cv. Messire was used both for pathogen multiplication and assays. Plants were raised from seeds in pots (6 × 6 × 10 cm) filled with a potting mixture (sand/perlite, 1:1 vol/vol), then were grown under standard conditions in growth chamber at 20 ± 2 °C and 65% relative humidity under a photoperiod at 14 h light/10 h dark with light intensity of 150 μmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes. As its condition as a biotrophic fungi requires, the pea powdery mildew (*E. pisi*) isolate CO-01 derived from mildew population collected at Córdoba (southern Spain) was maintained on Messire seedlings under the conditions described above. Leaves with heavy sporulation were shaken 1 day before

inoculation of selected accessions for the time course study to ensure a supply of young conidia (Fig. 2).

2.3. Detached leaves assays

For histological studies, detached leaves were used so that both inoculation density and incubation conditions were controlled precisely. Susceptible plants of cv. Messire were grown under the controlled conditions mentioned above until the fifth leaf stage and then several fourth-formed leaves were excised and placed, adaxial side up, on 4% technical agar in Petri dishes as described by Rubiales and Carver [19]. Cut leaves were arranged in a randomized design with four replicates per treatment, each replicate having four leaves. The metabolites listed in Table 1 were tested at concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 0.5×10^{-4} and 0.25×10^{-4} M. Compounds were dissolved in MeOH (5%) and then brought up to the assay concentration with distilled water. The test solutions (50 μL) were applied on the adaxial leaf side. Droplets (50 μL) of distilled water and MeOH (5%) were applied as negative controls. Droplets (50 μL) of commercial anti-powdery mildew fungicide (Nimrod Quattro® (ADAMA), bupirimate 25%, the fungicide 5-butyl-2-ethylamino-6-methylpyrimidin-4-yldimethylsulphamate at 0.8 M) were applied as positive control (0.2 g L^{-1}). The solvent was evaporated in a laminar flow cabinet, and then the Petri dishes were inoculated in a spore settling tower with 50 fresh conidiospores mm^{-2} (Fig. 2). Twenty-four hours after inoculation (h.a.i.), four leaves per plant, treatment and replicate were used to assess the percentage of fungal spore germination, number of hyphae per colony and number of fungal tips per colony. Leaves were fixed by placing the adaxial surface upon filter paper moistened with 1:3 (v/v) glacial acetic acid:absolute ethanol. When the chlorophyll was eliminated, the leaves were transferred to filter paper moistened with tap water (minimum 2 h) to soften the tissue and then to filter paper moistened with lactoglycerol (1:1:1 v/v/v, lactic acid:glycerol:water). Fungal development was examined by light microscopy after staining the fungus with 0.25% Trypan blue, as described by Barilli et al [20]. On every leaf fixed, germination frequency was assessed by scoring 100 conidiospores for the presence of a germ tube. The size of the colonies was also measured for another 35 randomly selected colonies in terms of number of hyphal tips and number of haustoria produced per colony. In addition, the presence of necrosis indicating the phytotoxic effect of the metabolites tested was also assessed macroscopically.

Table 1. Metabolites and controls tested for pea powdery mildew inhibition

Compound	Compound	Source	MW*
Control	Water	Water	--
Control	Water and methanol 5%	Methanol 5%	--
Fungicide	Nimrod Quattro® (ADAMA)	Bupirimate 25% (p:v)(5-butyl-2-ethylamino-6-methylpyrimidin-4-yldimethylsulphamate at 0.8 M)	--
1	Inuloxin A	Inula viscosa (plant)	248
2	Inuloxin C	I. viscosa (plant)	248
3	α-Costic acid	I. viscosa (plant)	234
4	Sphaeropsidin A	Diplodia cupressi (fungus)	348
5	Sphaeropsidin C	D. cupressi (fungus)	332
6	Cyclopaldic acid	Seridium cupressi (fungus)	238
7	Cavoxin	Phoma cava (fungus)	320
8	epi-Epoformin	Diplodia quercivora (fungus)	154
9	Cytochalasin A	Pyrenophora semeniperda (fungus)	477
10	Cytochalasin B	P. semeniperda (fungus)	479
11	Gliotoxin	Neosartorya pseudofisheri (fungus)	326
12	6-Hydroxymellein	Phoma chenopodiicola (fungus)	194

The effective concentration for 50% inhibition (EC_{50}) was calculated according to Alexander et al [21] using the inhibition rate of the fungal isolate in the presence of each compound. Spore germination for each of the replicates was converted to percentage inhibition compared with the untreated control by: 100 – (percentage germination of fungicide-compound/mean percentage germination of control). The concentration of compounds that effectively inhibited spore germination by 50% of the untreated control (EC_{50}) was determined by linear interpolation.

2.4. Whole-plant assays

To simulate the effect of the treatments with metabolites on the pathogen under real conditions, several experiments were carried out on whole young plants of the powdery mildew-susceptible pea cv. Messire under the controlled conditions mentioned in Section 2.2. Plants were arranged in a randomized design with five replicates per treatment, each replicate having three plants. Pea plants were inoculated at the third leaf stage by dusting the whole plants with fresh collected conidiospores of *E. pisi*. Fungal inoculation has already been described in Section 2.3. Metabolites cavoxin, inuloxin C and sphaeropsidin A were applied in whole plants at two concentrations found to be effective in Section 2.3, with no plant phytotoxicity. In order to study the protective effect of the metabolites, a set of plants was treated 5 days before fungal inoculation (DBI) and

Figure 2. (A) Seedling of *Pisum sativum* cv. Messire placed in a spore settling tower for *E. pisi* inoculation and (B) pathogen sporulation 8 days after plant inoculation. (C) Petri dishes with pea cv. Messire detached leaves treated with phytotoxins at different concentrations.

another set at the same time as fungal inoculation (0 DBI). In addition, to study the curative effect of the metabolites, a third set of plants was treated 2 days after fungal inoculation (DAI). Metabolites **2**, **4** and **7** were suspended in distilled water with MeOH (5%) and then sprayed over the canopy of the seedlings until run-off. For the negative controls, plants were untreated (Control), sprayed with distilled water (Water) and sprayed with distilled water with MeOH (5%). The commercial powdery mildew fungicide (Bupirimato 25%, ADAMA) was applied as positive control at its recommended concentration of 0.2 g L^{-1}. When the fungus macroscopically appeared, disease severity was assessed daily as the percentage of the whole plant covered by fungal mycelium until its growth becomes stabilized. These data were used to calculate the area under the disease progress curve (AUDPC) using the following formula:

$$AUDPC = \sum_{i=1}^{k} \frac{1}{2}[(S_i + S_i + 1)(t_i + 1 - t_i)]$$

where *Si* is the fungal severity at assessment date *i*, *ti* is the number of days after the first observation on assessment date *i* and *k* is the number of successive observations. Finally, the associated necrosis as plant phytotoxicity was assessed.

2.5. Statistics

For statistical analysis, average data were subjected to analysis of variance (ANOVA) to test the effect of each phytotoxin and treatment, after which residual plots were inspected to confirm data conformity to normality. The significance of differences between means was determined by calculating the honestly significant difference (HSD) to test the differences between treatments for each phytotoxin evaluated. All statistical analyses were performed by R software [22].

3. Results and discussion

3.1. Chemical nature of fungal and plant metabolites tested

The phytotoxins used for the study belong to different classes of natural compounds. Inuloxins A and C and α-costic acid (**1–3**, Fig. 1) are three sesquiterpenoids belonging to the germacrenolide, eudesmane and custunolide subgroups, respectively [10]. Sphaeropsidins A and C (**4** and **5**, Fig. 1(A)) are tetra- and tri-cyclic pimarane diterpenes [11,12], cyclopaldic acid and *epi*-epoformin (**6** and **8**, Fig. 1(B)), are a pentasubstituted benzofuranone [13] and a cyclohexen oxide [15], respectively. Cytochalasins A and B (**9** and **10**, Fig. 1(B)) are two 24-oxa[14]-cytochalasans [16], and cavoxin and 6-hydroxymellein (**7** and **12**, Fig. 1(B)) are a pentasubstituted benzoic acid [14] and isocoumarin [18], respectively. Finally, gliotoxin (**11**, Fig. 1(B)) is a representative member of the epipolythio-dioxopiperazine (ETP) class, which is well known for its potent cytotoxicity toward cancer cell lines [23].

Many of these metabolites are isolated as phytotoxins produced by fungi pathogenic to forest plants (Italian cypress and cork oak) as sphaeropsidins A and C, cyclopaldic acid and *epi*-epoformin (**4–6** and **8**) but they also show other interesting biological activity [24]. In fact, metabolite **4** showed bactericide activity as well as significant anticancer activity associated with a new mode of action, while **5** and **8** showed

insecticide and antirust activities. Cychalasins A and B and 6-hydroxymellein (**9**, **10** and **12**) were isolated from fungi proposed as mycoherbicides for the control of noxious weeds such as cheatgrass (*Bromus tectorum*) and fat hen that infest very important agronomic crops [8], while cavoxin was reported as a phytotoxin of a fungus pathogenic for chestnut [14] and showed a strong antifungal activity against the causal agents of mold (*Aspergillus niger* and *Penicillium roqueforti*) in preserved food [25]. In addition, it was also formulated in a biofilm (PBS, poly- butylene succinate) to improve food packaging [25] Inuloxins A and C and α-costic acid (**1** – **3**) were isolated from an alleopathic plant that showed phytotoxic activity against some parasitic weeds such as broomrapes (*Orobanche* and *Pelipanche*, spp.) and dodder (*Cuscuta campestris*) [10]. Finally, gliotoxin (**11**) has antifungal activity [26].

3.2. Inhibition of powdery mildew spore germination

On the pea-powdery mildew pathosystem studied on detached leaves, the percentage of fungal spore germination was higher than 80% in all the negative controls (control untreated, water and MeOH 5%), with no significant differences between them (Table 2). This suggests that either water or methanol at 5% (necessary to dissolve the metabolites mentioned above in water) did not modify normal spore development and did not exert any fungitoxic activity on the pathogen tested. On the contrary, spore germination lower than 15% was observed in inoculated leaves treated with the fungicide as positive control, as expected. In fact, this chemical is a well-known powdery mildew fungicide that prevents powdery mildew sporulation and suppresses new subsequent fungal infections [27]. Most of the metabolites tested significantly reduced powdery mildew spore germination (Table 2), with large differences between the effects of the different com- pounds. Metabolites **1**, **2** and **3** were effective in reducing spore germination, with an effect that was dose dependent (Table 2). Both metabolites **1** and **2** are well known for their seed germination inhibition of parasitic weeds such as *Orobanche crenata* Forssk. and *Cuscutacampestris* Yunck [10], while metabolite **3** showed antimicrobial activity against *Pennicillium roqueforti* Thom. and *Aspergillus niger* P.E.L. van Tieghem [28]. On *E. pisi*, metabolite **2** was particularly effective when applied at concentrations higher than 10^{-3} M, with a reduction in spore germination comparable to the fungicide. Metabolites **3**, **4** and **6** showed lower antifungal activity,

although still significant. Metabolite **5** was less effective, with only some slight but significant inhibition on germination at 10^{-2} M. Metabolite **7** reduced spore germination at all concentrations, reaching reductions similar to the fungicide when applied at 10^{-2} M (Table 2), confirming previous results on plant pathogenic fungi such as rusts [29], *Colletotrichum* and *Phomopsis* [30]. Effects on *E. pisi* spore germination by metabolites **8** – **12** were moderate to low, not comparable to the fungicide at any concentration.

The effective concentration for 50% inhibition (EC_{50}) was calculated to compare the fungal sensitivity to the 12 natural compounds and the chemical on the basis of spore germination inhibition (Table 3). The chemical fungicide is a 5-butyl-2-ethylamino-6-methylpyrimidin-4-yldimethylsulphamate belonging to the demethylation inhibitors (DMI) group of ergosterol-biosynthesis-inhibiting (EBI) fungicides, which showed systemic curative and protectant actions. Because DMI fungicides are powerful inhibitors that operate during the early stages of powdery mildew development (Fungicide Resistance Action Committee [FRAC] group 8), an assay based on spore germination is likely to be a better method for determining sensitivity of powdery mildew to natural compounds compared with the synthetic fungicide. The EC_{50} value for the fungicide was significantly lower (range 0.092 – 1.458, average 1.03 g L^{-1}) than those reported for natural metabolites (Table 3). Regarding the natural metabolites tested, differences in sensitivity were found for *E. pisi* to metabolites **2** (range 1.022 – 2.532, mean EC_{50} = 1.86), **4** (range 0.985 – 2.751, mean EC_{50} = 1.78) and **7** (range 1.678 – 2.548, mean EC_{50} = 1.98), which were comparable with the fungicide (Table 2).

3.3. Inhibition of powdery mildew colony development

As observed for spore germination, a high number of hyphal tips per colony (values up to 3) and haustoria per colony (up to 2.5) were achieved by the fungus in the untreated negative controls (water and 5% MeOH), with no significant differences between them (Supplementary Table 1). On the contrary, no or strongly reduced colony development was observed in inoculated leaves treated with the positive control, while several compounds strongly inhibited both the number of hyphal tips and haustoria per colony (Supplementary Table 1). This is very significant from a fungal control point of view, as the metabolites enhanced a penetration resistance mechanism which mainly inhibits the fungal infection process after the fungus has formed secondary haustoria,

Table 2. Percentage of spore germination achieved by 12 metabolites at different concentrations (0.25×10^{-4}, 0.5×10^{-4}, 10^{-4}, 10^{-3} and 10^{-2} M) tested on pea detached leaves inoculated with powdery mildew

Treatment	0.25×10^{-4} M	0.5×10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M	Treatment	0.25×10^{-4} M	0.5×10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M
		Metabolite 1: Inuloxin A						*Metabolite 2: Inuloxin C*			
Negative control	100 a	100 a	100 a	100 a	100 a	Negative control	100 a	100 a	100 a	100 a	100 a
Water	95.3 a	89.2 a	87.8 a	94.5a	95.4 a	Water	95.7 a	91.6 ab	93.5 a	92.3 a	98 a
MeOH	95.7 a	91.2 a	86.7 a	88.7a	89.9 a	MeOH	95.8 a	88.9 ab	88.2 ab	91.6 a	95.5 a
Phytotoxin	63.9 b	47.9 b	59.5 b	59.2b	53.6 b	Phytotoxin	84.4 a	70.9 b	65.2 b	28 bc	25.9 bc
Positive control	11.7 c	11.5 c	9.9c	14.4c	12.8c	Positive control	16.2 b	9.0 c	11.9 c	10.7 c	12 c
		Metabolite 3: α-Costic acid						*Metabolite 4: Sphaeropsidin A*			
Negative control	100 a	100 a	100 a	100 a	100 a	Negative control	100 a	100 a	100 a	100 a	100 a
Water	96.2 a	90.1 a	97.5 ab	97.8 a	96.5 a	Water	95.2 a	89.2 a	87.8 a	94.5 a	95.4 a
MeOH	91 ab	85.7 ab	91.6 ab	97 a	95.3 a	MeOH	95.7 a	91.2 a	86.7 a	88.7 a	89.9 a
Phytotoxin	82.5 b	72.3 b	70.9 b	72.1 b	61.2 b	Phytotoxin	63.9 b	47.9 b	48.5 b	43.9 b	31.3 b
Positive control	10c	14 c	12.3 c	10.7 c	14 c	Positive control	11.7 c	12.7 c	9.9 c	14.4 c	16.8 c
		Metabolite 5: Sphaeropsidin C						*Metabolite 6: Cyclopaldic acid*			
Negative control	100 a	100 a	100 a	100 a	100 a	Negative control	100 a	100 a	100 a	100 a	100 a
Water	96.8 a	98.3 a	96 a	94.6 a	93.5 a	Water	88.3 a	89.3 ab	88.7a	98.3 a	97.2 a
MeOH	93.1 a	89.5 a	95.6 a	96.6 a	96.2 a	MeOH	90 a	93 ab	88 a	85 ab	92 a
Phytotoxin	85.2 a	89.2 a	91.9 a	92.2 a	74.9 b	Phytotoxin	56.5 b	66.1 b	52.7 b	55.7 b	72 b
Positive control	15.3 b	15 b	14 b	8.3 b	4.7 c	Positive control	13 b	12.7 c	14 b	11 c	12 c
		Metabolite 7: Cavoxin						*Metabolite 8:* epi-*Epoformin*			
Negative control	100 a	100 a	100 a	100 a	100 a	Negative control	100 a	100 a	100 a	100 a	100 a
Water	89.3 a	95.6 a	91.7 a	91.6 a	89.7 a	Water	88 a	87.7 ab	88 ab	88 a	92.7 ab
MeOH	94.3 a	91.3 ab	90.4 a	88.2 a	89 a	MeOH	91 a	87 ab	85 ab	85 a	84.4 b
Phytotoxin	58.3 b	53.9 b	51.1 b	30.6 b	21.7 b	Phytotoxin	78.8 a	68.8 b	63.5 b	54.2 b	86.3 b
Positive control	13 c	9.3 c	13.7 c	14.7 c	12 b	Positive control	11.3 b	9.3 c	15.3 c	11 c	9.6 c
		Metabolite 9: Cytochalasin A						*Metabolite 10: Cytochalasin B*			
Negative control	100 a	100 a	100 a	100 a	100 a	Negative control	100 a	100 a	100 a	100 a	100 a
Water	89.3 a	95.4 ab	87.7 a	97.3 a	87.3 a	Water	90.2 ab	91.1 ab	94.7 a	96.7 ab	87.5 a
MeOH	92.2 a	88 ab	89.7 a	90.7 a	89 a	MeOH	89.3 ab	92 ab	94.7 a	89.8 ab	90.7 a
Phytotoxin	86 a	76 b	77.3 a	77.7 a	71.8 b	Phytotoxin	72 b	73.7 b	85.3 a	81.8 b	77.3 a
Positive control	13.6 b	13.4 c	14.3 b	12.1 b	10.4 c	Positive control	15 c	11 c	13 b	12.4 c	11.6 b
		Metabolite 11: Gliotoxin						*Metabolite 12: 6-Hydroxymellein*			
Negative control	100 a	100 a	100 a	100 a	100 a	Negative control	100 a	100 a	100 a	100 a	100 a
Water	90 a	94.3 a	87.2 a	95.2 a	96.8 a	Water	95.5 a	91.9 a	96.4 a	90.9 a	88.9 a
MeOH	89 ab	92.8 a	86.7 ab	87 ab	93.5 b	MeOH	91.9 ab	90.9 a	82.6 ab	89.6 a	88.9 a
Phytotoxin	68 b	55.1 b	62.6 b	68.1 b	79.9 b	Phytotoxin	78.3 b	76.9 a	66.6 b	64.7 b	57.7 b
Positive control	12.3 c	13.2 c	14.4 c	13.8 c	11.7 c	Positive control	13 c	15.66 b	13.1 c	15 c	12.9 c

Experiment was performed at 24 h after inoculation (h.a.i.). Values, per column and treatment, followed by different letters differ significantly at $P = 0.05$.

resulting in a lower subsequent fungal expansion. In particular, metabolites **2**, **4** and **7** were the most effective for controlling further steps of *E. pisi* development since values lower than 1.5 hyphal tips per colony were observed, even at the lowest concentration applied (0.25×10^{-4} M) (Supplementary Table 1). The same tendency was observed for haustoria formation, where values were lower than 1 for metabolite **2** when applied at concentrations of 10^{-3} and 10^{-2} M, and for metabolites **4** and **7** at any concentration applied. These results were comparable with those obtained with the chemical at the same concentration. No phytotoxicity was observed 24 h after innoculation with any metabolite and at any concentrations tested. As a consequence, inuloxin C, sphaeropsidin A and cavoxin are promising compounds because of their general high efficacy reducing fungal development in the pea-*E. pisi* pathosystem included in the study under *in vitro* conditions. Nevertheless, larger time frames between metabolite application and fungal growth measurements need to be considered in further assays in order to evaluate their long-lasting efficacy. For these reasons, *in planta* assays have been performed under controlled conditions.

3.4. Studies performed in planta

Inuloxin C (**2**), sphaeropsidin A (**4**) and cavoxin (**7**) were also tested in whole-

Table 3. Effective concentration for 50% inhibition (EC_{50}) of different metabolites (g L^{-1}) tested on spore growth inhibition of pea powdery mildew

	EC50 spore germination (%)	
Compound	**Range**	**Mean**
1	2.138 – 4.344	3.52 ab
2	1.022 – 2.532	1.86 c
3	2.253 – 3.963	3.08 ab
4	0.985 – 2.751	1.78 c
5	2.233 – 5.001	3.7 4ab
6	2.106 – 6.702	4.65 a
7	1.678 – 2.548	1.98 c
8	2.683 – 5.888	4.31 ab
9	1.957 – 10.08	4.58 a
10	3.246 – 5.121	3.94 ab
11	2.402 – 3.713	3.11 ab
12	2.002 – 2.762	2.47 bc
Positive control	0.092 – 1.458	1.03 c

Values followed by different letters differ significantly at $P = 0.05$.

plant studies under controlled conditions. Metabolites were sprayed on pea susceptible plants at the two lowest concentrations found to be effective under *in vitro* conditions (10^{-3} and 10^{-2} M) at three different time points to fungal inoculation. Visible disease was macroscopically daily evaluated until 12 days after fungal inoculation (Fig. 3). Treatment with metabolites **2**, **4** and **7** provided a significant reduction in the percentage of final disease severity (DS%) (<45%) and AUDPC (<288) ($P < 0.001$) at any time of application compared with the negative controls (DS > 90%; AUDPC > 455) (Table 4), with no significant differences between compounds for each time of application considered. The detailed microscopic study performed *in vitro* showed that this protection provided by the metabolites acted both before and after fungal penetration to plant leaves. No symptoms of phytotoxicity were observed on plants treated with metabolites **2** or **4**, confirming previous results of microscopic assessment, while some necrotic spots were observed on leaves treated with metabolite **7** at 5 DBI. This is not surprising, as some phytotoxic effects on pea plants have been observed in a study conducted against rust fungi [31]. Authors observed brown lesions on leaves 48 h after their treatment with cavoxin. This is probably the reason why we did not microscopically find any symptom, as leaves for histological studies were removed earlier in our study (24 h) when the metabolite had not affected the plant tissues. The protective effect reached with all metabolites significantly improved when they were applied at the same time as fungal inoculation (0 DBI) compared to when they were applied 5 DBI ($P < 0.01$), similar to the effects of the commercial fungicide (Table 4, Figs. 4 – 6). Here we also found that significantly reduced symptoms compared with untreated controls were strongly maintained when all metabolites were applied 2 DAI (curative effect) (Table 4, Figs. 4 – 6). In fact, fungal development measured as diary evaluation of disease severity (DS%), as well as the AUDPC, was significantly decreased in plants treated with natural compounds compared with the controls (Table 4, Figs. 4 – 6), with a consequent delay in fungal growth and sporulation. In particular, results on plants treated with metabolites **4** and **7** were comparable with those provided by the fungicide (Table 4, Supplementary Fig. S1). A study on the effects at early and late stages (early aborted colonies and/or primarily mycelial growth impairment) of fungal infection should be considered for further study.

A fully compatible reaction not associated with necrosis was observed in all treatments (Supplementary Fig. S1). As a consequence, the observed resistance after treatment with metabolites was never complete, as in the case of all experiments performed under controlled conditions, where high inoculum loads were used in

Table 4. Disease severity (DS%) and area under the disease progress curve (AUDPC) of *Erysiphe pisi* development on plants of pea cv. Messire

		Metabolite 2		Metabolite 4		Metabolite 7	
Time	Treatment	DS%	AUDPC	DS%	AUDPC	DS%	AUDPC
5 DBI	Negative control	95 a	522 a	95 a	522 a	96 a	522.5 a
	Water	90 a	470 a	90 a	465 a	90 a	465 a
	MeOH 5%	90 a	535 a	90 a	535 a	90 a	505 a
	Positive control	30 c	169 c	28 c	174 c	30 c	171 d
	10^{-3} M	43.3 b	288 b	44 b	249.1 b	44.3 b	251.6 b
	10^{-2} M	35.8 c	198 c	35.9 c	195.6 c	42.3 b	213.6 c
0 DBI	Negative control	95 a	492.5 a	95 a	522.5 a	95 a	521.5 a
	Water	90 a	455 a	90 a	457 a	90 a	455 a
	MeOH 5%	95 a	457 a	95 a	462 a	95 a	467 a
	Positive control	0 d	0 c	0 c	0 c	0 c	0 d
	10^{-3} M	30.8 b	115.1 b	22.9 b	109.9 b	29.1 b	133.9 b
	10^{-2} M	22.2 c	91.9 b	21 b	91.4 b	28.6 b	107.9 c
2 DAI	Negative control	90 a	510 a	90 a	510 a	90 a	510 a
	Water	90 a	485 a	90 a	485 a	90 a	485 a
	MeOH 5%	90 a	465 a	90 a	465 a	90 a	465 a
	Positive control	0 c	0 c	0 b	0 b	0 c	0 b
	10^{-3} M	17 b	86.6 b	8 b	31.35 b	12 bc	54.1 b
	10^{-2} M	14.2 b	80.9 b	7.7 b	29.6 b	9.4 c	44.4 b

DS% is expressed as the percentage of disease severity covering the whole plant 12 days after infection (DAI). Metabolites **2**, **4** and **7**, negative (untreated, water and MeOH 5%) and positive (bupirimate 25%) controls were applied to plants at different times to fungal inoculation: 5 days before inoculation (DBI), 0 DBI and 2 days after inoculation (DAI). Compounds were tested at concentrations of 10^{-3} and 10^{-2} M.

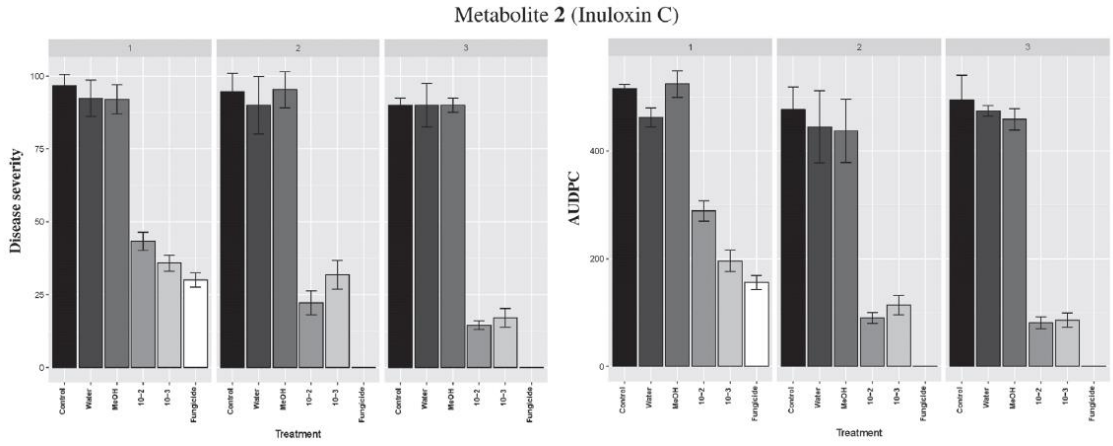


Figure 4. Powdery mildew symptoms on plants of pea cv. Messire treated with metabolite 2 (Inuloxin C). DS% is expressed as the percentage of canopy covered by mildew 12 DAI. With daily assessments of DS%, the AUDPC was calculated. Metabolite **2** (inuloxin C), negative (untreated, water and MeOH 5%) and positive (fungicide) controls were applied to plants at different times to fungal inoculation: (**1**)5 DBI, (**2**)0 DBI and (**3**) 2 DAI). Compounds were tested at concentrations of 10^{-3} and 10^{-2} M.

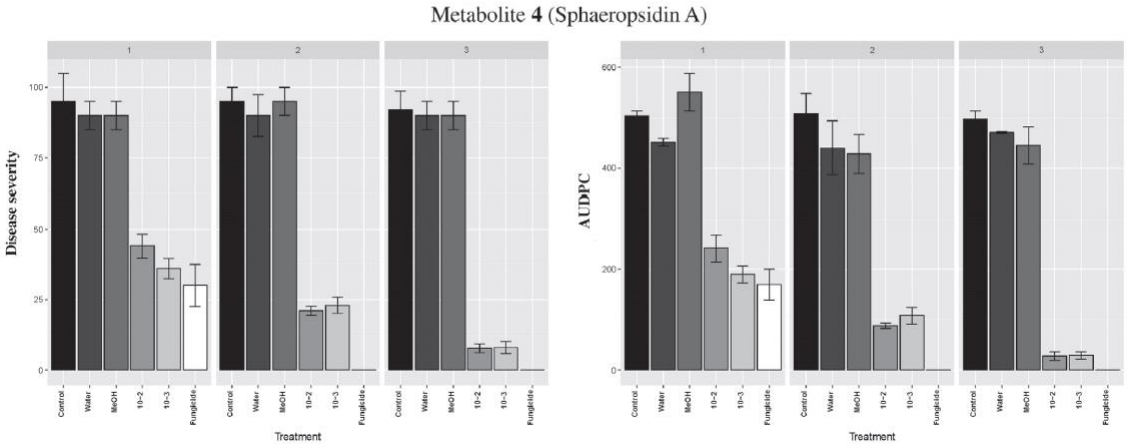


Figure 5. Powdery mildew symptoms on plants of pea cv. Messire treated with metabolite **4** (sphaeropsidin A). DS% is expressed as the percentage of canopy covered by mildew 12 DAI. With daily assessments of DS%, the AUDPC was calculated. Metabolite **4** (sphaeropsidin A), negative (untreated, water and MeOH 5%) and positive (fungicide) controls were applied to plants at different times to fungal inoculation: (**1**)5 DBI), (**2**)0 DBI and (**3**)2 DAI. Compounds were tested at concentrations of 10^{-3} and 10^{-2} M.

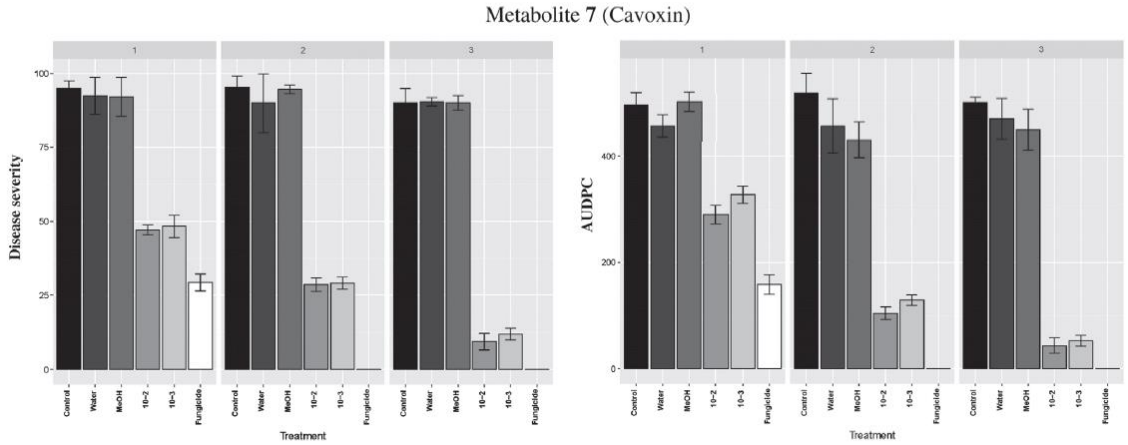


Figure 6. Powdery mildew symptoms on plants of pea cv. Messire treated with metabolite **7** (cavoxin). DS% is expressed as the percentage of canopy covered by mildew 12 DAI. With daily assessments of DS%, the AUDPC was calculated. Metabolite **7** (cavoxin), negative (untreated, water and MeOH 5%) and positive (fungicide) controls were applied to plants at different times to fungal inoculation: (**1**)5 DBI), (**2**)0 DBI and (**3**) 2 DAI. Compounds were tested at concentrations of 10^{-3} and 10^{-2} M.

situations favoring pathogen development. However, treatment at least at 10^{-3} M with any metabolite tested effectively reduced by 50% the disease severity, with this reduction being higher when applied at 0 DBI and 2 DAI. This reduction could be attributed to the toxic effect on the fungus, as all of them showed fungistatic activity against the conidiospores. Information on the mode of action is crucial for the successful development and adoption of any control strategy. Such information is critical to optimize application methods and correct timing of field sprays.

4. Conclusions

Biotrophic fungi such as powdery mildews are regularly managed with fungicides, especially when no genetic resistance is available [5]. When pathogen control focuses on the deployment of cultivars carrying dominant resistance genes, fungicides contribute about equally to genetics in the control of the disease. As a result, resistance to chemicals evolved by fungal isolates has emerged as one of the most complicated issues threatening food security, leading to the application of general integrated disease management strategies such as dose limitation, mixtures and the search for alternative molecules.

In the present study, 12 fungal metabolites from different sources belonging to different classes of natural compounds were tested with general success for their fungitoxic activity against a disease of agronomic importance, pea powdery mildew. In *in vitro* studies, inuloxin C, sphaeropsidin A and cavoxin were promising compounds, all reducing the early developmental stages of *E. pisi*. At concentrations 10^{-3} M and higher the fungal inhibition was comparable to that of positive control. Furthermore, additional studies performed *in planta* also showed that the compounds, when applied at the same moment of fungal infection (0 DBI) or later (5 DAI), inhibited fungal growth at pre- and post-penetration stages with consequent preventive and curative effects against powdery mildew infection similar to those achieved by positive control. As cavoxin was phytotoxic to pea, this metabolite is unsuitable for further studies. The effect of these metabolites against other biotrophic fungi has also been previously proven on leguminous and cereal rusts [29]. In particular, cavoxin was highly effective against two isolates belonging to *Puccinia* spp. and four isolates belonging to *Uromyces* spp. Thus, inuloxin C and sphaeropsidin A warrant further attention to gain a better understanding of their biological properties for practical applications in agriculture against powdery mildew and other possible pathogens of agronomic interest. It is also important to note that spharopsidin A is produced in good yield (560 mg L^{-1}) from the liquid culture filtrates of *D. cupressi* and is easily crystallized as white needles (250 mg L^{-1}) [11]. Scale-up of production in large fermenters to produce large amounts of sphaeropsidin A could make the compound economical for use in agricutlture. Similarly, inuloxin C was obtained in dried *I. viscosa* plants as pure oil (80 mg kg^{-1}) [10]. This is a common Mediterranean basin plant that is easy to collect, transport and process, reducing the cost of obtaining abundant amounts of inuloxin C.

Acknowledgments: This paper was given at the workshop on Natural Products in Pest Management: Innovative approaches for increasing their use which took place in Bellagio, Italy on 25-29 September 2018, and which was sponsored by the OECD Cooperative Research Programme: Biological Resource Management for Sustainable Agricultural Systems whose financial support made it possible for the author to participate in the workshop.

The opinions expressed and arguments employed in this paper are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.

The authors thanks Prof. L. Maddau, Prof. L. Sparapano and Dr. M. Vurro from, respectively, University of Sassari, University of Bari and CNR (Italy) for supplying cultures of *D. cupressi*, *D. quer- civora*, *S. cupressi*, *N. pseudofisheri*, *P. cava*, *P. chenopodiicola* and *P. semeniperda*. We also thank Dr. M. Vurro and Mr. N. Monte- murro for supplying *Inula viscosa* plants. Research was supported by Programme STAR 2017 (M.M.) (UniNa and Compagnia di San Paolo, Italy). Dr. E. Barilli and M.J. González Bernal are also greatly indebted to the AGL2017-82907-R project co-financed by FEDER for financial support.

References

1 Smy´kal P, Aubert G, Burstin J, Coyne CJ, Ellis NTH, Flavell AJ, et al., Pea (*Pisum sativum* L.) in the Genomic Era. *Agronomy* **2**:74 – 115 (2012).

2 FAOSTAT. *Food and Agriculture Organization Corporate Statistical Database*. Available: http://faostat.fao.org [20 December 2017].

3 Kraft JM and Pfleger FL, *Compendium of Pea Diseases and Pests*. American Phytopathological Society Press, St. Paul, MN, pp. 14 – 17 (2001).

4 Iglesias-García R, Rubiales D and Fondevilla S, Penetration resistance to *Erysiphe pisi* in pea mediated by *er1* gene is associated with protein cross-linking but not with callose apposition or hypersensitive response. *Euphytica* **201**:381 – 387 (2015).

5 Fondevilla S and Rubiales D, Powdery mildew control in pea. A review. *Agron Sustain Devel* **32**:401 – 409 (2012).

6 Cimmino A, Andolfi A, Avolio F, Ali A, Tabanca N, Khan IA et al., Cyclopaldic acid, seiridin, and sphaeropsidin A as fungal phytotoxin, and larvicidal and biting

deterrents against *Aedes aegypti* (Diptera: Culicidae): structure-activity relationships. *Chem Biodivers* **10**: 1239 – 1251 (2013).

7 Cimmino A, Andolfi A and Evidente A, Phytotoxic terpenes produced by phytopathogenic fungi and allelopathic plants. *Nat Prod Commun* **9**:401 – 408 (2014).

8 Cimmino A, Masi M, Evidente M, Superchi S and Evidente A, Fungal phytotoxins with potential herbicidal acivity: chemical and biological characterization. *Nat Prod Rep* **32**:1629 – 1653 (2015).

9 Wang W, Ben-Daniel BH and Cohen Y, Control of plant diseases by extracts of *Inula viscosa*. *Phytopathology* **94**:1042 – 1047 (2004).

10 Andolfi A, Zermane N, Cimmino A, Avolio F, Boari A, Vurro M et al., Inuloxins A-D, phytotoxic bi-and tri-cyclic sesquiterpene lactones produced by *Inula viscosa*: potential for broomrapes and field dodder management. *Phytochemistry* **86**: 112 – 120 (2013).

11 Evidente A, Sparapano L, Motta A, Giordano F, Fierro O and Frisullo S, A phytotoxic pimarane diterpene of *Sphaeropsis sapinea* f. sp. *cupressi*, the pathogen of a canker of cypress. *Phytochemistry* **42**:1541 – 1546 (1996).

12 Evidente A, Sparapano L, Fierro O, Bruno G, Giordano F and Motta A, Sphaeropsidins B and C, phytotoxic pimarane diterpenes from *Sphaeropsis sapinea* f. sp. *cupressi* and *Diplodia mutila*. Phytochemistry **45**:705 – 713 (1997).

13 Graniti A, Sparapano L and Evidente A, Cyclopaldic acid, a major phytotoxic metabolite of *Seiridium cupressi*, the pathogen of a canker disease of cypress. *Plant Pathol* **41**:563 – 568 (1992).

14 Evidente A, Randazzo G, Iacobellis NS and Bottalico A, Structure of cavoxin, a new phytotoxin from *Phoma cava* and cavoxone, its related chroman-4-one. *J Nat Prod* **48**:916 – 923 (1985).

15 Andolfi A, Maddau L, Basso S, Linaldeddu BT, Cimmino A, Scanu B, et al., Diplopimarane, a 20-*nor*-*ent*-pimarane *produced* by the oak pathogen *Diplodia quercivora*. *J Nat Prod* **77**: 2352 – 2360 (2014).

16 Evidente A, Andolfi A, Vurro M, Zonno MC and Motta A, Cytochalasins Z1, Z2 and Z3, three 24-oxa [14] cytochalasans produced by *Pyrenophora semeniperda*. *Phytochemistry* **60**:45 – 53 (2002).

17 Masi M, Andolfi A, Mathieu V, Boari A, Cimmino A, Moreno Y Banuls L, et al., Fischerindoline, a pyrroloindole sesquiterpenoid isolated from *Neosartorya*

pseudofischeri, with in vitro growth inhibitory activity in human cancer cell lines. *Tetrahedron* **69**: 7466 – 7470 (2013).

18 Cimmino A, Andolfi A, Zonno MC, Berestetskyi A, Vurro M and Evidente A, Chenopodolans A– C: phytotoxic furopyrans produced by *Phoma chenopodiicola*, a fungal pathogen of *Chenopodium album*. *Phytochemistry* **96**:208 – 213 (2013).

19 Rubiales D and Carver TLW, Defence reactions of *Hordeum chilense* accessions to three formae speciales of cereal powdery mildew fungi. *Can J Bot* 78:1561 – 1570 (2000).

20 Barilli E, Rubiales D, Gjetting T and Lyngkjaer MF, Differential gene transcript accumulation in peas in response to powdery mildew (*Erysiphe pisi*) attack. *Euphytica* **198**:13 – 28 (2014).

21 Alexander B, Browse DJ, Reading SJ and Benjamin IS, A simple and accurate mathematical method for calculation of the EC50. *J Pharmacol Toxicol Meth* **41**:55 – 58 (1999).

22 R Development Core Team, *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna (2016).

23 Sun Y, Takada K, Takemoto Y, Yoshida M, Nogi Y, Okada S et al., Gliotoxin analogues from a marine-derived fungus, *Penicillium* sp., and their cytotoxic and histone methyltransferase inhibitory activities. *J Nat Prod* **75**: 11 – 114 (2011).

24 Masi M, Maddau L, Linaldeddu BT, Scanu B, Evidente A and Cimmino A, Bioactive metabolites from pathogenic and endophytic fungi of forest trees. *Curr Med Chem* **25**:208 – 252 (2018).

25 Santagata G, Valerio, F, Cimmino A, Dal Poggetto G, Masi M, Di Biase M, et al., Chemico-physical and antifungal properties of poly (butylene succinate)/cavoxin blend: study of a novel bioactive polymeric based system. *Eur Polym J* **94**: 230 – 247 (2017).

26 Kaouadji M, Steiman R, Seigle-Murandi F, Krivobok S and Sage L, Gliotoxin: uncommon ^{1}H couplings and revised ^{1}H-and ^{13}C-NMR assignments. *J Nat Prod* **53**:717 – 719 (1990).

27 Bellón-Gómez D, Vela-Corcía D, Pérez-García A and Torésa JA, Sensi- tivity of *Podosphaera xanthii* populations to anti-powdery-mildew fungicides in Spain. *Pest Manag Sci* **71**:1407 – 1413 (2015).

28 Valerio F, Masi M, Cimmino A, Moeini SA, Lavermicocca P and Evidente A, Antimould microbial and plant metabolites with potential use in intelligent food

packaging. *Nat Prod Res* **32**:1605 – 1610 (2018).

29 Barilli E, Cimmino A, Masi M, Evidente M, Rubiales D and Evidente A, Inhibition of spore germination and appressoria formation of rust species by plant and fungal metabolites. *Nat Prod Commun* **11**:1343 – 1347 (2016).

30 Schrader KK, Andolfi A, Cantrell, CL, Cimmino A, Duke, SO, Osbrink W, et al., A survey of phytotoxic microbial and plant metabolites as potential products for pest management. *Chem Biodivers* **7**: 221 – 2280 (2010).

31 Barilli E, Cimmino A, Masi M, Evidente M, Rubiales D and Evidente A, Inhibition of early development stages of rust fungi by the two fungal metabolites cyclopaldic acid and *epi*-epoformin. *Pest Manag Sci* **73**:1161 – 1168 (2017).

Supplementary Materials:

Impact of fungal and plant metabolites application on early development stages of pea powdery mildew

Supplementary material

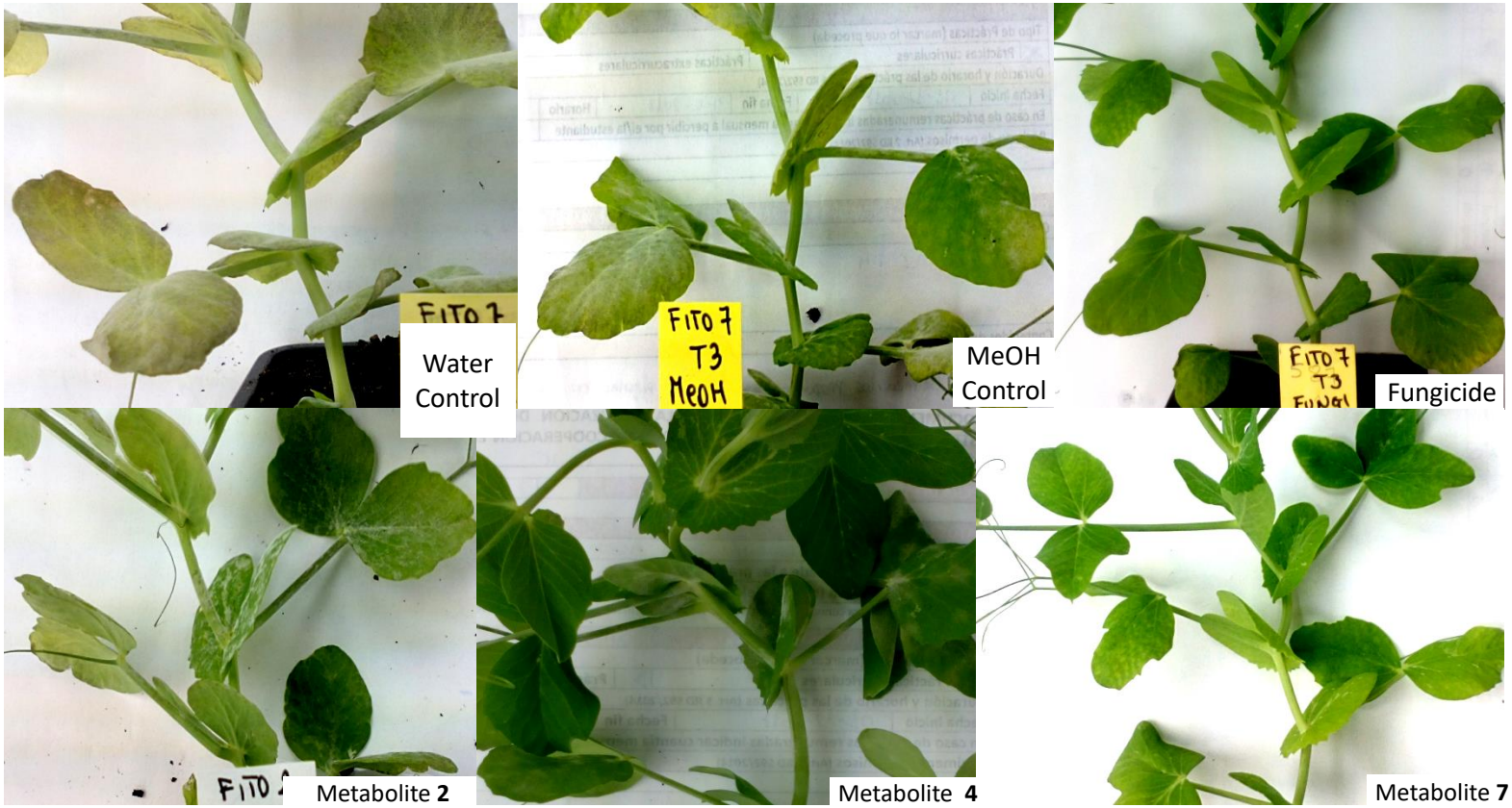
Figure S1. Symptoms of *Erysiphe pisi* on susceptible pea plants cv. Messire on negative controls (water and MeOH 5%) and positive control (fungicide), and treated with inuloxin C (**2**), sphaeropsidin A (**4**) and cavoxin (**7**). Disease was scored 12 days after inoculation.

Table S1. Effect on *Erysiphe pisi* hyphal tips development and haustoria formation per colony in Petri dish assays at 24 h after inoculation (24 h.a.i.) on negative (untreated control, water and MeOH 5%) controls, treated leaves with inuloxin C (**2**), sphaeropsidin A (**4**) and cavoxin (**7**) tested at different concentrations (0.25×10^{-4}, 0.5×10^{-4}, 10^{-4}, 10^{-3} and 10^{-2} M) and positive control (fungicide Bupirimato 25%).

Supplementary Table 1. Effect on *Erysiphe pisi* hyphal tips development and haustoria formation per colony in Petri dish assays at 24 hours after inoculation (24 h.a.i.) on negative (untreated control, water and MeOH 5%) controls, treated leaves with inuloxin C (**2**), sphaeropsidin A (**4**) and cavoxin (**7**) tested at different concentrations (0.25×10^{-4}, 0.5×10^{-4}, 10^{-4}, 10^{-3} and 10^{-2} M) and positive control (fungicide Bupirimato 25%).

PHYTOTOXIN 2: INULOXIN C										
	0.25×10^{-4} M		**0.5×10^{-4} M**		**10^{-4} M**		**10^{-3} M**		**10^{-2} M**	
Treatment	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*
Negative control	3,37 a	3,51 a	3,31 a	3,58 a	3,07 a	3,3 a	3,07 a	3,51 a	3,07 a	3,36 a
Water	3,24 a	3,52 a	3,07 a	3,41 a	3,22 a	3,4 a	3,13 a	3,42 a	3,3 a	3,48 a
MeOH	3,24 a	3,41 a	3,06 a	3,46 a	3,08 a	3,02 a	3,29 a	3,53 a	3,19 a	3,18 a
Phytotoxin	1,63 b	1,37 b	1,66 b	1,17 b	1,45 b	1,21 b	1,27 b	0,26 b	1,06 b	0,32 b
Positive control	0,84 c	0,32 c	1,06 c	0,36 c	1,09 c	0,26 c	1,13 b	0,34 b	0,85 b	0,34 b
PHYTOTOXIN 4: SPHAEROSIDIN A										
	0.25×10^{-4} M		**0.5×10^{-4} M**		**10^{-4} M**		**10^{-3} M**		**10^{-2} M**	
Treatment	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*
Negative control	2,72 a	2,79 a	2,7 a	2,84 a	2,5 a	2,82 a	2,59 a	2,78 a	2,61 a	2,9 a
Water	2,76 a	2,86 a	2,98 a	2,91 a	2,44 a	2,59 a	2,69 a	2,58 a	2,85 a	2,68 a
MeOH	2,75 a	2,86 a	2,74 a	2,61 a	2,58 a	2,71 a	2,3 a	2,6 a	2,84 a	2,63 a
Phytotoxin	1,28 b	0,54 b	1,1 b	0,45 b	1,09 b	0,41 b	1,24 b	0,51 b	1,28 b	0,47 b
Positive control	0,86 c	0,36 c	0,77 c	0,39 b	0,75 b	0,3 b	0,7 c	0,15 c	0,89 c	0,44 b
PHYTOTOXIN 7: CAVOXIN										
	0.25×10^{-4} M		**0.5×10^{-4} M**		**10^{-4} M**		**10^{-3} M**		**10^{-2} M**	
Treatment	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*	*Hyphaes*	*Haustoria*
Negative control	3,3 a	2,79 a	2,98 a	2,6 a	3,37 a	2,89 a	3,36 a	2,86 a	3,4 a	3,06 a
Water	2,86 a	2,64 a	2,92 a	2,37 a	2,83 a	2,71 a	3,12 a	2,53 a	3,2 a	2,88 a
MeOH	2,82 a	2,53 a	2,82 a	2,33 a	2,82 a	2,64 a	2,96 a	2,69 a	3,03 a	2,75 a
Phytotoxin	1,57 b	0,93 b	1,41 b	1,02 b	1,34 b	0,91 b	1,17 b	0,71 b	1,1 b	0,97 b
Positive control	0,77 c	0,33 c	0,82 c	0,39 c	0,78 c	0,41 c	0,76 c	0,36 b	0,76 c	0,3 c

Supplementary Fig. 1 Symptoms of *Erysiphe pisi* on susceptible pea plants cv. Messire on negative controls (water and MeOH 5%), positive control (fungicide), and treated with inuloxin C (**2**), sphaeropsidin A (**4**) and cavoxin (**7**). Disease was scored 9 days after inoculation.



CHAPTER VI

CONCLUSIONS

From the analysis carried out with the fungus *Ascochyta fabae*, the following conclusions can be drawn (CHAPTER II):

1) Our research has provided information about the phytotoxic secondary metabolites produced by this pathogen under different *in vitro* growth conditions, revealing a rich diversity of compounds with differential phytotoxic effects.
2) Seven metabolites belonging to different classes of natural compounds (those produced in greater quantities by the fungus) have been identified and isolated from each of the growth media.
3) The exudate of the fungus that presents the greatest phytotoxicity in the bioassay on cut leaves is that derived from growth in Czapek-Dox medium. Furthermore, the most susceptible crops were those belonging to the genus *Vicia* spp. (specifically *Vicia faba*, *V. narbonensis* and *V. sativa*).
4) A different behavior of each of the compounds has been observed in the legumes tested, but at higher concentrations the compounds that present a notable activity in the *Vicias* are ascosalitoxin (presents notable activity in each of the legumes except lentils), benzoic acid (the most notable activity in vetch occurs at the highest concentration) and ascofuranol (it is dependent on the concentration in faba bean and pea and has greater activity in narbon.

From the analysis carried out with the three fungal isolates of the genus *Colletotrichum*, the following conclusions were obtained (CHAPTER III):

5) A very complete metabolomic profile has been obtained with around 84 metabolites separated as follows: 9 compounds were validated with pure standards (level A), 20 were putatively annotated (levels B(i) and B(ii)), and for 43 compounds it was possible to assign only the natural product class (level C(i) and C(ii)), while 12 remained unknown.
6) VIP score indicates that each medium has a different metabolomic profile and when compared with each isolate it is observed that the *Colletotrichum truncatum* isolates remain close while *Colletotrichum trifolii* remains further away.

7) The test with the organic extract of each of the isolates presents different activities depending on the fungal isolate, the growth medium, the tested concentration and the host plant.

Regarding the search for a possible compound with a biofungicidal capacity for a necrotrophic fungus such as *Ascochyta lentis*, the conclusions have been the following (CHAPTER IV):

8) When a treatment with pachybasin is performed in both cereals and legumes against powdery mildew, it is observed a clear protective effect (since it reduces the severity of the disease when treated and inoculated at the same time) as well as a curative effect (since it reduces the severity of the disease when treated just after the disease has already established) against both pathogens.
9) When treating with pachybasin against rust in both cereals and legumes, it is observed that it has a protective effect (since it reduces the severity of the disease when treated and inoculated at the same time) against the attack of these pathogens.

Finally, regarding the search for metabolites with biofungal capacity from other potential sources such as a plant or other fungi, the results have been the following (CHAPTER V):

10) Inuloxin C (extracted from the *Inula viscosa*), sphaeropsidin A (extracted from *Diplodia cupressi*) and cavoxin (extracted from *Phoma cava*) showed the most fungicidal activity against the pea powdery mildew incited by the pathogen *Erysiphe pisi*. However, since cavoxin also showed a certain associated phytotoxic activity in plant, both inuloxin C and sphaeropsidin A have potential as alternatives to synthetic fungicides for the control of crop pathogens of economic importance such as powdery mildew.

Del análisis realizado con el hongo *Ascochyta fabae* se pueden extraer las siguientes conclusiones (CHAPTER II):

1) Nuestra investigación ha proporcionado información acerca de los metabolitos secundarios fitotóxicos producidos por este patógeno en diferentes condiciones de crecimiento *in vitro*, revelando una rica diversidad de compuestos con efectos fitotóxicos diferenciales.
2) Siete metabolitos pertenecientes a diferentes clases de compuestos naturales (los producidos en mayores cantidades por el hongo) han sido identificados y aislado a partir de cada uno de los medios de crecimiento.
3) El exudado del hongo que presenta mayor fitotoxicidad en el bioensayo en hojas cortadas es el derivado por el crecimiento en medio Czapek-Dox. Además, los cultivos más susceptibles fueron los pertenecientes al genéro *Vicia* spp. (especificamente *Vicia faba*, *V. narbonensis* y *V. sativa*).
4) Se ha observado un comportamiento diferente de cada uno de los compuestos en las leguminosas testadas, pero a mayor concentración los compuestos que presentan una actividad destacable en las *Vicias* son el ascosalitoxin (presenta actividad destacable en cada una de las leguminosas excepto en lenteja) , el ácido benzoico (la actividad más destacable en veza se da a la concentración más alta) y el ascofuranol (es dependiente de la concentración en haba y guisante y presenta mayor actividad en narbón.

Del análisis realizado con los tres aislados de hongos del género *Colletotrichum* se obtuvieron las siguientes conclusiones (CHAPTER III):

5) Se ha obtenido un perfil metabolómico muy completo con alrededor de 84 metabolitos separados de la siguiente manera: 9 compuestos fueron validados con estándares puros (nivel A), 20 fueron supuestamente anotados (niveles B(i) y B(ii)), y para 43 A los compuestos sólo se les pudo asignar la clase de producto natural (nivel C(i) y C(ii)), mientras que 12 quedaron desconocidos.
6) El análisis VIP indica que cada medio tiene un perfil metabolómico diferente y al comparar con cada aislado se observa que los aislados de *Colletotrichum truncatum* permanecen cerca mientras que *Colletotrichum trifolii* permanece más alejado.

7) El ensayo con el extracto orgánico de cada uno de los aislados presenta actividades diferentes dependiendo del aislado fúngico, el medio de crecimiento, la concentración probada y la planta huésped.

Respecto a la búsqueda de posibles compuestos derivados del hongo necrotrofo *Ascochyta lentis*, que muestren capacidad fungicida hacia hongos biotrófoso (roya y oidio) tanto en legumbres como en cereales, las conclusiones han sido las siguientes (CHAPTER IV):

8) Al tratar con pachybasyn frente al oídio tanto en cereales como en legumbres, se observa que tiene tanto un efecto protectivo (ya que reduce la gravedad de la enfermedad al tratarse al mismo tiempo del establecimiento de la enfermedad) como curativo (ya que reduce la gravedad de la enfermedad cuando se trata después de que la enfermedad ya se haya establecido) contra el ataque de estos patógenos.
9) Al tratar con pachybasin frente a la roya tanto en cereales como en leguminosas, se observa que tiene un efecto protector (ya que reduce la gravedad de la enfermedad al tratarse e inocularse al mismo tiempo) frente al ataque de estos patógenos.

Finalmente, en cuanto a la búsqueda de metabolitos con capacidad biofúngica a partir de otras fuentes potenciales como una planta u otros hongos, los resultados han sido los siguientes (CHAPTER V):

10) Inuloxin C (extraído de la planta *Inula viscosa*), sphaeropsidin A (extraído de *Diplodia cupressi*) y cavoxin mostraron la mayor actividad fungicida contra el mildiú polvoriento de los guisantes incitado por el patógeno *Erysiphe pisi*. Sin embargo, dado que el cavoxin también mostró una cierta actividad fitotóxica asociada en las plantas, tanto el inuloxin C como el sphaeropsidin A tienen potencial como alternativas a los fungicidas sintéticos para el control de patógenos de cultivos de importancia económica como el oídio.

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