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TESIS DOCTORAL:

SÍNTESIS DE NUEVOS ANTIFÚNGICOS DE ORIGEN NATURAL PARA SU USO EN AGRICULTURA

Presentada por Laura Buzón Durán para optar al grado de Doctora por la Universidad de Valladolid

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Memoria selectiva para recordar lo bueno,
prudencia lógica para no arruinar el presente,
y optimismo desafiante para encarar el futuro.

Isabel Allende

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RESUMEN

La presente Tesis Doctoral es una aportación enmarcada en el área multidisciplinar constituida por la interrelación de la Ingeniería Agroforestal con la Ingeniería de Materiales y la Microbiología, en tanto que las principales contribuciones de la investigación realizada son relativas a nuevas formulaciones con actividad antimicrobiana con aplicaciones en Viticultura y en la protección de otros cultivos.

Aspectos fundamentales del trabajo realizado han sido la extracción de especies bioactivas de diversas plantas, la preparación de complejos conjugados con una solubilidad y biodisponibilidad mejoradas gracias al uso de oligómeros de quitosano y glicósidos, su ensayo *in vitro* e *in vivo* contra diversos fitopatógenos, y la transferencia de la tecnología desarrollada al sector mediante la preparación y tramitación de patentes.

Los nuevos preparados han demostrado la sinergia preconizada para la combinación de los extractos naturales con los oligómeros de quitosano o con el esteviósido, alcanzado eficacias comparables a las de algunos fungicidas convencionales frente a hongos de la familia Botryosphaeriaceae, *Fusarium culmorum* y *Phytophthora cinnamomi*, y frente a bacterias como *Erwinia amylovora*.

Los productos desarrollados, respetuosos con el medio ambiente y aptos para su uso en producción integrada, son altamente prometedores para abordar el reto de disminuir el uso abusivo de tratamientos fitosanitarios de origen químico en Agricultura, cumpliendo con los requerimientos de la Directiva europea 2009/128/CE (Art. 14) y su transposición al RD 1311/2012.

ABSTRACT

This PhD Thesis is a contribution framed in the interdisciplinary area constituted by the interrelation between Agroforestry Engineering, Materials Engineering and Microbiology, insofar as the main results of the conducted research are related to new formulations with antimicrobial activity aimed at applications in Viticulture and in the protection of other crops.

Key aspects of this work have been the extraction of bioactive species from various plants, the preparation of conjugated complexes with improved solubility and bioavailability (thanks to the use of chitosan oligomers and glycosides), their *in vitro* and *in vivo* testing against various phytopathogens, and the transfer of the developed technology to the sector through the drafting and filing of patents.

The new preparations have demonstrated the synergy preconized for the combination of natural extracts with chitosan oligomers or stevioside, achieving efficacies comparable to those of some conventional fungicides against fungi of the Botryosphaeriaceae family, *Fusarium culmorum* and *Phytophthora cinnamomi*, and against bacteria such as *Erwinia amylovora*.

The developed products, environmentally friendly and suitable for use in integrated production, are highly promising to address the challenge of reducing the abusive use of chemical phytosanitary treatments in Agriculture, complying with the requirements of the European Directive 2009/128/EC (Art. 14) and its transposition to Spanish Royal Decree 1311/2012.

INTRODUCCIÓN

ÍNDICE

1. MARCO DE REFERENCIA

Las estrategias tradicionales de protección de las plantas resultan a menudo insuficientes, y la aplicación de plaguicidas químicos provoca diversos problemas medioambientales y de salud humana. Además, la resistencia de algunos patógenos y plagas de las plantas contra el uso indiscriminado de plaguicidas se está convirtiendo rápidamente en un problema grave [1]. De este modo, el diseño y evaluación de formulaciones alternativas que sean seguras, efectivas y sostenibles desde un punto de vista ambiental ha llegado a ser un objetivo prioritario de alto interés.

La aplicación de mezclas de fungicidas a los cultivos, con sus inherentes y distintos modos de acción, es una práctica recomendada e incluso comercializada por Bayer® y Syngenta®, que da lugar a efectos aditivos en la inhibición de los hongos (Brent y Hollomon, 2007). Las mezclas en las que los fungicidas pueden actuar de forma sinérgica poseen el atractivo añadido de permitir el uso de dosis reducidas de cada compuesto para un nivel deseado de inhibición, lo que puede reducir los costes y el impacto medioambiental. Dado que el uso de fungicidas químicos sigue siendo un componente importante de las estrategias de protección de cultivos disponibles [2], la explotación de las sinergias de los fungicidas ayuda a abordar la demanda de reducción del uso de productos químicos al tiempo que conserva las ventajas de una potente protección contra los hongos. Sin embargo, esta estrategia debe utilizarse con cuidado, ya que la resistencia desarrollada a un compuesto anulará el efecto de la combinación, es decir, el segundo agente puede estar a una concentración demasiado baja para inhibir el crecimiento de los hongos por sí mismo. Estos riesgos pueden ser compensados por la incorporación de tales mezclas en los programas de fungicidas rotativos. Las combinaciones de agentes que actúan de forma sinérgica contra los fitopatógenos fúngicos han sido referidas en la literatura [3,4] pero la implementación de las aplicaciones de campos se hace , a todas luces, necesaria.

La Directiva europea 2009/128/CE establece en su artículo 14 las bases para la utilización de pesticidas de modo sostenible, destacando como aspecto fundamental la reducción de su uso, particularmente el cobre en viticultura (Piwi, 2016). Hay que destacar el reciente cambio normativo a nivel europeo de los nuevos preparados, que según el Reglamento (UE) 2019/1009 del Parlamento Europeo y del Consejo, de 5 de junio de 2019, por su carácter de *bioestimulantes*, pasan a ser recalificados en el CFP 6 como fertilizantes (y no como fitosanitarios). También debe tenerse en consideración que, según el Reglamento 834/2007, sobre producción y etiquetado ecológico, así como el Reglamento 889/2008 (por el que se establecen disposiciones de aplicación del Reglamento 834/2007) y sus posteriores modificaciones, los productos y subproductos de origen vegetal son considerados como sustancias aptas para su uso en Agricultura Ecológica. Por ejemplo, el quitosano y los extractos vegetales de cola de caballo (*Equisetum arvense*) y ortiga (*Urtica dioica*), ricos en compuestos polifenólicos, son de uso extendido como productos bioestimulantes agrícolas.

En cuanto a las enfermedades de la madera de la vid, en la Figura 1, se reflejan los avances realizados en los últimos 15 años junto con los retos que aún quedan pendientes de abordar.

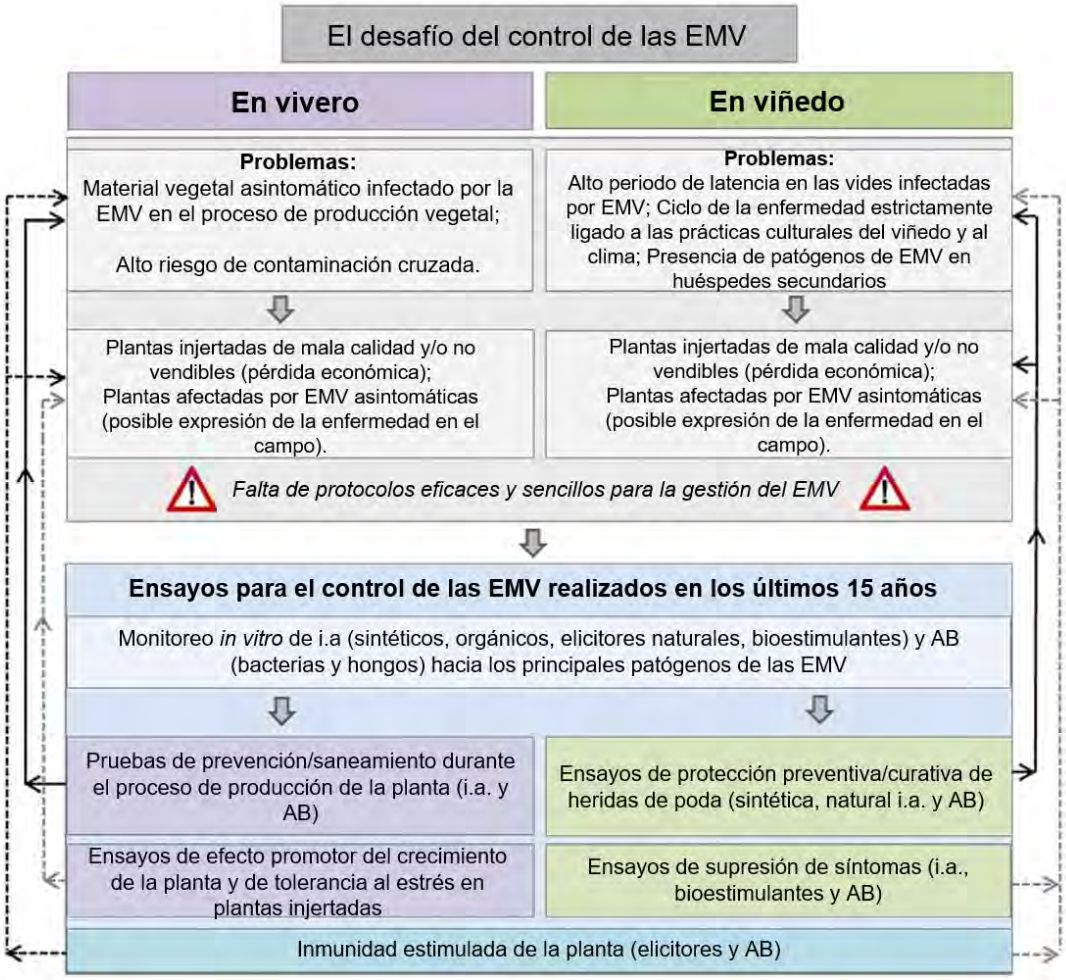

Figura 1. Esquema que refleja los problemas relacionados con la presencia de las enfermedades de la madera de la vid (EMV), tanto en vivero como en viñedo (en recuadros grises), informando de las diferentes estrategias (recuadros de color) puestas en marcha para solucionar la falta de protocolos adecuados y eficientes de control de la enfermedad hasta ahora existentes. i.a.: ingrediente activo; AB: agente de biocontrol. Adaptado de Mondello, *et al.* [5].

2. ENFERMEDADES AGRÍCOLAS QUE SUPONEN UN RIESGO PARA LA SALUD PÚBLICA

2.1. Botryosphaeriaceae, un reto para la fitopatología en un mundo cambiante

2.1.1. *Introducción general*

Las especies de Botryosphaeriaceae (Botryosphaeriales, Ascomycetes), distribuidas por todo el mundo, poseen diferentes funciones ecológicas. Estos hongos pueden actuar como saprobios endofíticos o patógenos latentes [6,7]. Algunos miembros de esta familia son reconocidos como agresivos patógenos de plantas en diferentes tipos de huéspedes. Desde los cultivos agrícolas hasta los huéspedes forestales, estos hongos no tienen fronteras [8,9]. Su amplia distribución, su capacidad de persistir endofíticamente; su capacidad de convertirse en patógenos sólo cuando sus huéspedes están bajo estrés, causando enfermedades que eventualmente pueden llevar a la muerte del huésped; y la capacidad de adaptarse y colonizar nuevos hospedadores [6,10] hace que estos organismos sean un gran reto en la actualidad [11].

Varias especies de Botryosphaeriaceae actualmente aceptadas han sido descritas en el siglo XIX, como por ejemplo *Diplodia mutila* (1834), *Diplodia seriata* (1845) y *Botryosphaeria dothidea* (1863), entre otras. A lo largo de los años la posición taxonómica de estos organismos ha venido experimentando múltiples clasificaciones (para una visión histórica, véase Phillips, et al. [7]).

2.1.2. *Distribución mundial en función de los países y la diversidad donde existen aislados*

En las colecciones de "*Nucleotide—GenBank*" el 80% todos los aislados de Botrryospheriacea se encuentra distribuido en sólo 11 países: China (1810 aislados), Estados Unidos de América (1310), Sudáfrica (1141), Brasil (1077), Australia (796) Italia (622) Irán (439), India (412), España (347), Malasia (324) y Portugal (311). De forma similar, la diversidad tiende a aumentar con el esfuerzo de muestreo: China (72 especies), Estados Unidos de América (55), Sudáfrica (62), Brasil (42), Australia (57) Italia (51) Irán (35), India (28), España (31), Malasia (17) y Portugal (23) [12].

Es sabido que dentro de la familia Botryosphaeriaceae solo unas pocas especies como *Botryosphaeria dothidea*, *Diplodia sapinea*, *Diplodia seriata*, *Dothiorella sarmentorum*, *Neofusicoccum parvum* y *Lasiodiplodia theobromae* tienen una distribución mundial [7,13,14].

En cuanto a la distribución en función de la diversidad de especies, hasta ahora sólo *Diplodia corticola* y *Neofusicoccum mangiferae* no se encuentran en todos los continentes. Esta observación confirma la capacidad de las especies restantes para extenderse a nivel mundial. *D. sarmentorum*, *D. corticola*, *D. mutila*, *N. australe*, *D. seriata* y *D. sapinea* se encuentran sólo en ecosistemas templados y mediterráneos, mientras que *Neofusicoccum parvum* parece ser el organismo más adaptado, detectándose desde el norte hasta el sur, con la excepción de los bosques boreales y los pastizales montanos. Los bosques boreales parecen ser el lugar más improbable para encontrar especies de Botryosphaeriaceae: hasta ahora, sólo se ha registrado *D. sapinea* en estas regiones [12].

2.1.3. *Especificidad del hospedador*

Respecto a la especificidad del hospedador, en general, *L. theobromae* es, con diferencia, el organismo con el mayor número de hospedadores conocidos (666 de los 749 hospedadores notificados para el género Lasiodiplodia), el mayor número de aislados en el GenBank (1944), el mayor número de apariciones en los países (97) y el mayor número de informes sobre la interacción hospedador-hongo (365). Para el resto de especies, es posible observar, que algunas posen la capacidad de colonizar un mayor número de huéspedes que otras (por ejemplo *N. parvum* con 223 hospedadores en 50 países y *B. dothidea* con 403 hospedadores en 66 países) mientras otras, suficientemente establecidas a nivel mundial presentan un bajo número de hospedadores (por ejemplo, *D. sapinea* fue reportada en 62 países pero sólo en 102 hospedadores, o *D. seriata* establecida en 46 países con 121 hospedadores y *Lasiodiplodia pseudotheobromae* con 124 huéspedes en 44 países) [12].

2.1.4. *Factores que influyen en la patogenicidad*

La patogenicidad de los aislados de Botryosphaeriaceae que conducen a la mortalidad de las plantas ha sido constatada principalmente en plántulas y en condiciones de estrés por sequía [10,15,16]. Sin embargo, la mortalidad de las plantas en la naturaleza es a menudo una combinación de múltiples estreses bióticos y abióticos.

Las enfermedades fúngicas pueden ser devastadoras debido a los siguientes factores *(i)* tensiones abióticas, como el estrés térmico, la sequía y las inundaciones, que pueden alterar el rendimiento fisiológico de la planta y predisponen a los árboles a la colonización por varios patógenos. De hecho, una reducción de la tolerancia al estrés biótico conduce a mayores tasas de mortalidad

(ii) la temperatura y la humedad, que afectan a la esporulación y la dispersión de los patógenos, y muy probablemente, a la prevalencia de determinados patógenos [17-19]. La temperatura modula la fitotoxicidad y la citotoxicidad de los hongos Botryosphaeriaceae que, en general, se cultivan a 25°C. No obstante, *B. dothidea*, *D. corticola* y *N. parvum* inducen una elevada mortalidad celular cuando se cultivan a 37 °C, dato que indica que temperaturas ambientales más altas pueden conducir a una mayor virulencia [20];

(iii) la migración de los patógenos provocada por el cambio climático puede aumentar la incidencia de la enfermedad o su alcance geográfico, cuando los patógenos encuentran nuevos huéspedes y (o) nuevos vectores potenciales; y pueden aparecer nuevas amenazas, ya sea por un cambio en la composición de las especies arbóreas o por especies invasoras. Los patógenos que han sido importantes en el sur de Europa pueden extenderse hacia el norte y también hacia las montañas [17];

(iv) la falta de carbono, que induce la mortalidad cuando se agotan los recursos de carbohidratos no estructurales, afectando al mantenimiento normal de la planta, al crecimiento y a los mecanismos de defensa de la planta [21]; y

(v) el efecto tóxico de los metabolitos fúngicos es otro factor que induce la mortalidad [22].

En la Figura 2 se observa el ciclo de la enfermedad decaimiento por Botryosphaeria y en la Figura 3, los factores de estrés abiótico y biótico que contribuyen al desarrollo del hongo de la enfermedad del tronco de la vid y a la expresión de los síntomas.

Figura 2. Ciclo de la enfermedad decaimiento por Botryosphaeria [23].

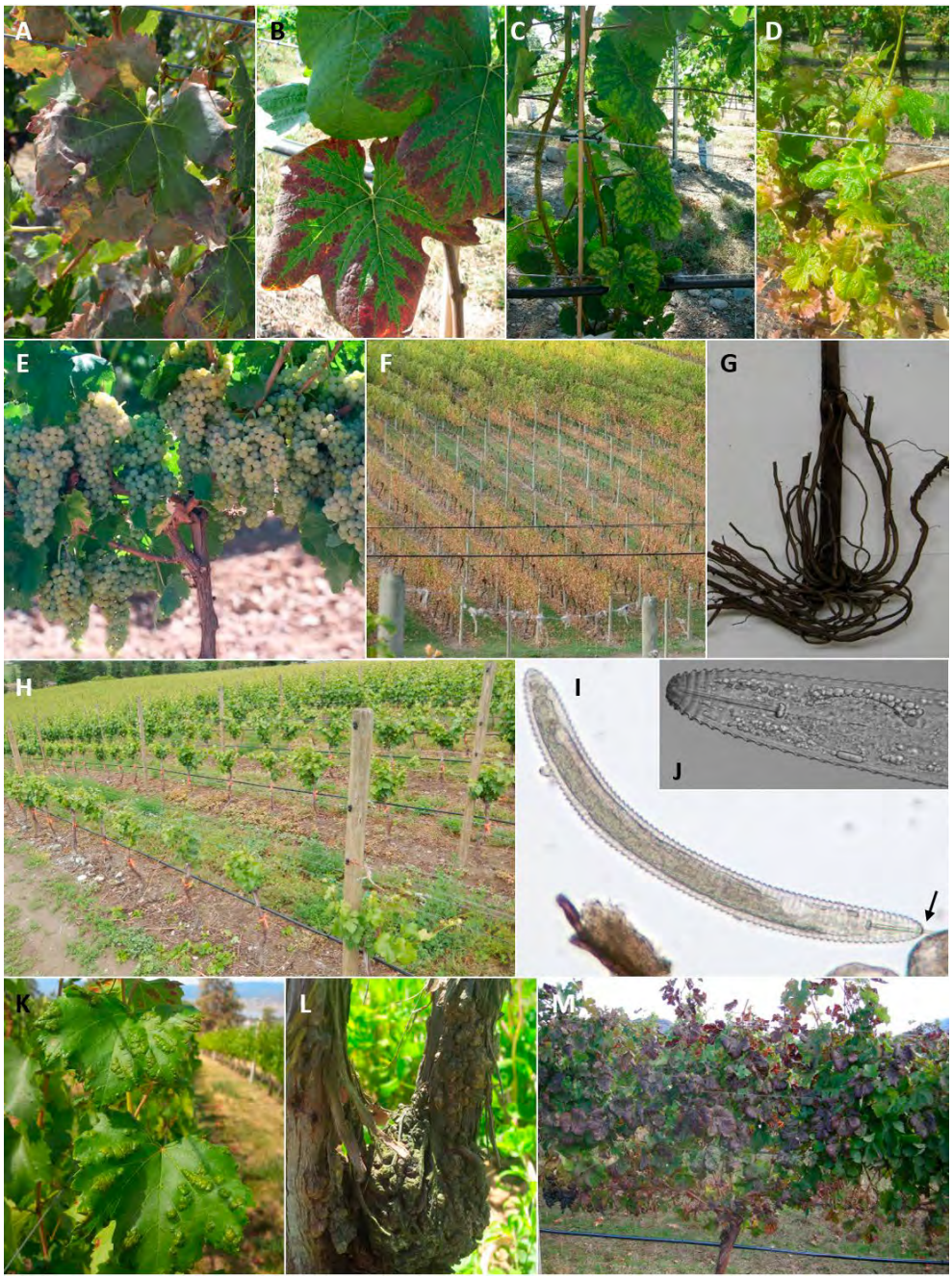

Figura 3. Factores de estrés abiótico y biótico que contribuyen al desarrollo del hongo de la enfermedad del tronco de la vid y a la expresión de los síntomas. **A**. Quemado de la hoja como resultado de un severo estrés hídrico de la vid. **B** y **C**. Hojas de vides jóvenes que muestran deficiencia de nutrientes. **D**. Deformidades de las hojas causadas por daños causados por herbicidas. **E**. Sobrecultivo severo durante los primeros años de establecimiento del viñedo (cortesía de D. Gramaje, ICVV, Logroño, España). **F**. Los daños invernales y/o las heladas primaverales pueden favorecer el desarrollo de la enfermedad. **G**. Enraizamiento en J como resultado de las malas condiciones de plantación. H. Vinos de bajo vigor causados por una infestación de nematodos. **I**. Nematodo del anillo (*Mesocriconema xenoplax*) alimentándose de una raíz introduciendo el estilete (flecha negra). J. Detalle de la cabeza del nematodo del anillo. **K**. Agallas en las hojas causadas por el ácaro erineum (*Colomerus vitis*). Los altos niveles de daños causados por el insecto podrían aumentar el estrés de la vid. **L**. Agalla de la vid causada por *Agrobacterium vitis*. **M**. Vid con síntomas de enrollamiento de la hoja de la vid causados por el virus asociado al enrollamiento de la hoja de la vid. Las vides afectadas por factores de estrés biótico (enfermedades fúngicas, bacterianas o víricas) pueden favorecer las infecciones por hongos GTD, la colonización de la madera y la expresión de los síntomas. Adaptado de Hrycan, *et al.* [24].

2.2. *Erwinia amylovora*, especie en cuarentena

2.2.1. *Introducción general*

Erwinia amylovora es un patógeno vegetal no obligado, estrechamente relacionado con otras especies bacterianas de importancia agrícola y clínica [25]. Esta bacteria es el agente etiológico del fuego bacteriano, una de las enfermedades más devastadoras que afectan a plantas rosáceas como el peral (*Pyrus communis* L.), el manzano (*Malus domestica* Borkh.) y otros árboles frutales de importancia económica y especies de plantas ornamentales y silvestres, principalmente de la subfamilia Spiraeoideae [26], siendo responsable de grandes descensos de rendimiento, largos confinamientos fitosanitarios y elevadas pérdidas económicas. El ciclo de vida de *E. amylovora* es complejo y está estrechamente vinculado al desarrollo estacional del huésped. Algunos parámetros que afectan al desarrollo de las epidemias de fuego bacteriano son las condiciones meteorológicas, las temperaturas y la humedad ambientales, la presencia de vectores, la virulencia de la cepa que produce la infección, el estado fenológico del hospedador y la resistencia al patógeno, así como la supervivencia de *E. amylovora* en entornos relacionados con las plantas [27,28]. El crecimiento de *E. amylovora*, la producción de factores de virulencia en un amplio rango de temperaturas junto con su baja especificidad de especies hospedadoras [28-30] y el cultivo generalizado de sus hospedadores están probablemente correlacionados con la continua propagación del patógeno a nuevas regiones en diferentes zonas climáticas [31,32] y nuevos hospedadores [33,34].

2.2.2. *Distribución a nivel mundial*

El fuego bacteriano es originario de América del Norte y se ha extendido a numerosos países de diferentes continentes, como Europa, Oceanía y Asia occidental. La enfermedad se registró por primera vez en Europa en 1957 en Inglaterra, y se ha extendido rápidamente a otros países europeos, como Francia, España, Serbia, Croacia, Túnez, Hungría y Suiza [35]. En España se identificó por primera vez en 1995 en Guipúzcoa: la enfermedad se detectó en un huerto de manzanas de sidra cercano a la frontera atlántica francesa [36]. La distribución mundial de genotipos de *E. amylovora* puede haberse visto favorecida por el comercio de plantones infectados de plantas hospedadoras, en las que la enfermedad pasa fácilmente desapercibida, ya que la bacteria puede vivir como endófita o epífita [35].

2.2.3. *Invasión del huésped*

En la Figura 4 se resumen los principales focos de infección de la bacteria. Casi todos los tejidos de la planta huésped son susceptibles de ser infectados por *E. amylovora* y, durante los brotes de fuego bacteriano es fácil observar síntomas característicos como necrosis, exudación y/o cancros en las flores, los brotes, las hojas, los frutos, las ramas o el tronco [27,30]. Por lo tanto, el fuego bacteriano se considera principalmente una enfermedad de la parte aérea de la planta y hay abundante literatura que describe la infección de *E. amylovora* y las vías de migración dentro de los tejidos aéreos del huésped [30,37,38]. Por el contrario, las raíces que muestran síntomas de fuego bacteriano apenas se observan en el campo [30,39].

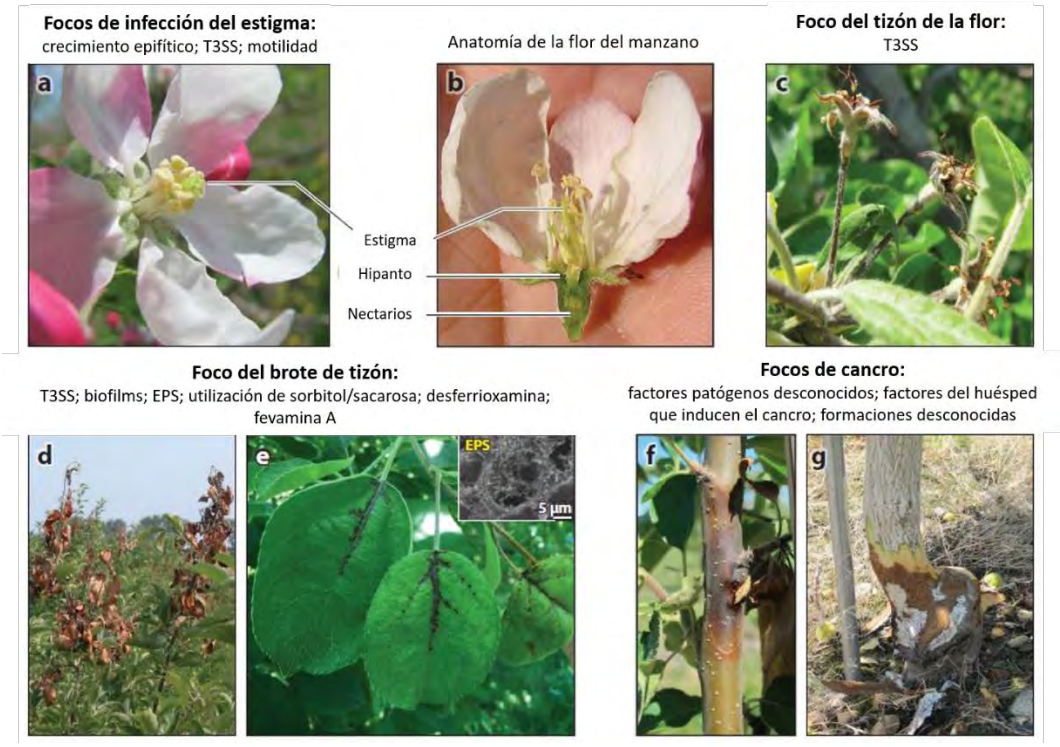

Figura 4. Principales focos de infección del ciclo de la enfermedad de *Erwinia amylovora* y factores críticos de virulencia y patogenicidad. (**a**,**b**) Flor de manzana. Las células de *E. amylovora* crecen en los estigmas antes de utilizar la motilidad para migrar por el estilo hasta el hipanto, iniciando la infección tras la entrada en los nectarios de las flores. (**c**) La infección del tizón de la flor está mediada principalmente por el sistema de secreción de tipo III (T3SS). (**d**) La infección del tizón de los brotes implica múltiples fases, incluyendo la infección mediada por el T3SS y una fase de biofilm que muestra (**e**) la necrosis venosa en las hojas infectadas. (Inset) una micrografía electrónica de barrido muestra un biofilm de *E. amylovora* dentro del xilema de la hoja. xilema de la hoja. Los cancros, que se forman en el tronco de un (**f**) manzano o en la (**g**) unión entre la púa y el portainjerto, representan la fase menos estudiada de la enfermedad de *E. amylovora*. Abreviatura: EPS, exopolisacárido. Adaptado de Kharadi, *et al.* [40]

2.2.4. *Mecanismos de transmisión de la bacteria*

E. amylovora entra a través de aberturas y heridas naturales en las plantas del huésped, colonizando los espacios intercelulares migrando al sistema vascular y produciendo biopelículas que bloquean los vasos de la xilema e inhiben el transporte de agua. Esta producción excesiva de biofilms puede llevar a originar exudados bacterianos. Estos exudados son reconocidos como las principales fuentes de diseminación de *E. amylovora* a otros huéspedes cercanos a través del viento, el agua, los insectos y las prácticas culturales, como la poda con herramientas contaminadas [35].

Diferentes estudios han informado de su supervivencia en el suelo, el agua, los insectos que habitan en el suelo y los nematodos durante períodos variables (días-semanas-meses) dependiendo de las condiciones ensayadas [28,41,42]. Por lo tanto, es factible que, en condiciones propicias, *E. amylovora* pueda infectar las raíces de la planta huésped a través del suelo contaminado, el agua o a través de insectos y nematodos que dañan las raíces.

2.2.5. *Protección de plantas*

Para proteger las plantas del fuego bacteriano, se utiliza un enfoque sistemático global que incluye diversas medidas químicas, biológicas, agrícolas y de cuarentena. Ninguna medida es suficiente por sí sola [30]. Al mismo tiempo, el principal componente del conjunto de medidas de protección es la identificación de las plantas infectadas que suponen un peligro para toda la comunidad vegetal cultivada.

Los métodos de diagnóstico incluyen la inspección visual y los métodos específicos que pueden confirmar de forma fiable la presencia de un patógeno en plantas sintomáticas o en las primeras fases de la enfermedad en ausencia de síntomas. Los métodos de detección de *E. amylovora* más utilizados son la siembra de bacterias en medios semiselectivos, los ensayos inmunoenzimáticos (ELISA), la microscopía inmunofluorescente y la reacción en cadena de la polimerasa (PCR) [43].

2.2.6. *Situación actual*

La falta de medidas fitosanitarias eficaces para controlar y prevenir los brotes de fuego bacteriano, que resultan esenciales para evitar la diseminación de esta enfermedad, han llevado a la clasificación de *E. amylovora* como especie de cuarentena, clasificada en la lista de los diez patógenos vegetales bacterianos más importantes de las plantas junto con otros fitopatógenos [44,45].

Desde 1992 la EPPO (Organización Europea para la Protección de las Plantas) lo considera un organismo de cuarentena debido a su gran potencial de daño en caso de introducción en el territorio europeo, por lo que está incluido en el Anexo IIB de la Directiva 2000/29/CE (traspuesta a la legislación nacional en el RD 58/2005).

Actualmente, existe legislación específica sobre medidas preventivas contra su introducción y difusión (RD 58/2005), así como un programa nacional de erradicación y control del fuego bacteriano de las rosáceas (RD 1201/1999).

Un reto importante al que se enfrentan las autoridades fitosanitarias en relación con el fuego bacteriano es la dificultad para detectar a tiempo la entrada de *E. amylovora* en una nueva zona/país, y aplicar medidas de contención científicamente fundamentadas [46].

2.3. La fusariosis

2.3.1. *Introducción general*

La fusariosis del trigo y otros cereales de grano pequeño ha recibido una atención considerable en las últimas décadas, pero ciertamente no es una enfermedad nueva. Smith (1884) hizo una descripción temprana de los síntomas de la enfermedad de Fusarium en las cabezas de trigo y cebada causada por *Fusisporium culmorum*, un sinónimo de *Fusarium culmorum*. Arthur (1891) informó de las diferentes reacciones de los cultivares de trigo en cuanto a la gravedad de la sarna del trigo, nombre que se le dio inicialmente la enfermedad causada por las infecciones de *Fusarium* [47].

Como se ha dicho, la fusariosis se detectó por primera vez en 1884 en Inglaterra y desde entonces se han producido brotes episódicos en diferentes países del mundo En los últimos 25 años las epidemias se han vuelto más frecuentes y más graves, y se han

intensificado hasta convertirse en una importante amenaza para la seguridad alimentaria mundial. Se han registrado grandes pérdidas en Estados Unidos, Canadá, Europa, China y Sudamérica.

Cepas de otras especies de Fusarium como *F. acuminatum*, *F. avenaceum*, *F. cerealis*, *F. chlamydosporum*, *F. culmorum*, *F. equiseti*, *F. langsethiae*, *F. poae*, *F. sporotrichiodes* y *F. tricinctum* también pueden producir la enfermedad [48].

2.3.2. *Importancia de la fusariosis del trigo*

Aunque se han realizado grandes esfuerzos para combatir la fusariosis en las últimas décadas, sigue siendo una de las enfermedades vegetales más importantes y más ampliamente estudiadas en el trigo y los cereales pequeños. Savary, *et al.* [49] llevaron a cabo un estudio mundial sobre las pérdidas de rendimiento en base a patógenos y plagas individuales y estimaron una pérdida de rendimiento global del 21,5% para el trigo. Las pérdidas de rendimiento debidas a la fusariosis ocuparon el segundo lugar después de la roya de la hoja y fueron particularmente altas en China, el Medio Oeste de Estados Unidos, Canadá, el sur de Brasil, Paraguay, Uruguay y Argentina. La fusariosis no sólo origina graves pérdidas de rendimiento y calidad, sino que además es de especial importancia para la seguridad de los alimentos y piensos, ya que los patógenos de la fusariosis contaminan el grano con tricotecenos y otros metabolitos fúngicos tóxicos que suponen un riesgo para la salud de las personas y los animales [50].

2.3.3. *Severidad y factores que influyen en el desarrollo del microorganismo*

Las ascosporas y conidios de las cepas de Fusarium persisten en los residuos de los cultivos durante largos períodos de tiempo, hasta que las temperaturas sean propicias y se disponga de suficiente humedad para que el hongo pueda crecer y esporular. Las espigas de trigo son susceptibles de ser colonizadas por el hongo durante la antesis, un momento en el que los peritecios fúngicos suelen expulsar ascosporas. Las espigas infectadas se blanquean prematuramente y aparecen descoloridas y arrugadas, lo que puede reducir el rendimiento y la calidad del grano hasta un 80% [51].

La severidad de la fusariosis en los campos de los agricultores puede alcanzar proporciones epidémicas, desarrollarse de forma moderadamente severa o permanecer baja y en gran medida no detectada. *Fusarium spp.* sobrevive bien como saprofito en los restos de la planta o puede vivir en la superficie de la planta sin causar la enfermedad. Los mismos hongos pueden convertirse en patógenos oportunistas destructivos con una ventana de infección bastante estrecha y un periodo parasitario relativamente corto en el caso de la fusariosis (Shaner, 2003).

Para la infección y el desarrollo de la fusariosis son fundamentales *(a)* la abundancia y la agresividad del inóculo durante la etapa de planta vulnerable, que abarca esencialmente varios días alrededor de la antesis, *(b)* las condiciones ambientales durante este período crítico y *(c)* la susceptibilidad o el estado de resistencia de la planta. El cultivo de variedades resistentes desempeña un papel crucial en el control eficaz y fiable de la fusariosis. Se ha demostrado en múltiples ocasiones que la eficiencia de la aplicación de fungicidas para reducir la severidad de la FHB y la principal micotoxina de Fusarium, el deoxinivalenol (DON), es mayor en los cultivares moderadamente resistentes que en los susceptibles [52,53].

2.3.4. *Estrategias para reducir la fusariosis y las micotoxinas*

Aparte de la toma en consideración de las condiciones climáticas a la hora de realizar la siembra, se consideran estrategias previas a la cosecha la utilización de métodos agronómicos como la rotación de cultivos, el laboreo y la fertilización, Las buenas prácticas agrícolas en el manejo de los cultivos pueden conseguir que aumente su vigor y su resistencia a los factores de estrés abióticos y bióticos, incluyendo la susceptibilidad a los hongos productores de toxinas [54].

En lo que respecta a la protección biológica de las plantas, hasta la fecha, se conocen muchas interacciones beneficiosas entre la planta y los microorganismos que pueden utilizarse potencialmente en la agricultura. Es extremadamente importante caracterizar los endófitos y su papel en la agricultura, especialmente en la resistencia a las enfermedades de las plantas. Los agentes biológicos son una alternativa a los productos químicos. Son factores de origen natural, que principalmente incluyen microorganismos antagonistas [55].

Cabe señalar que aun cuando los métodos preventivos no químicos sean insuficientes para limitar la aparición de los hongos Fusarium, su combinación con los métodos químicos, en acciones integradas, permite reducir con mayor eficacia la aparición de hongos patógenos y sus metabolitos secundarios. La investigación científica muestra que la integración de la resistencia de los cultivos con los fungicidas puede ser una estrategia eficaz para el control de FHB y DON en el trigo de invierno [53,56].

Entre los métodos químicos, la aplicación de fungicidas es el método más común de gestión del FHB. Sin embargo, esta estrategia no siempre es eficaz [57]. Se han utilizado muchos fungicidas para reducir el FHB, incluyendo triazoles, carbendazim, mancozeb, benomilo, procloraz, propiconazol y triadiamenol. Sin embargo, ninguno de estos productos químicos basta por sí solo para controlar completamente el FHB en el trigo [48]

El uso continuado de fungicidas que comparten el mismo modo de acción (por ejemplo, inhibidores de la biosíntesis de esteroles) puede inducir una presión selectiva sobre las poblaciones de hongos patógenos, dando lugar a inestabilidad y brotes de la enfermedad [49]. Además, pueden producirse aumentos en la producción de micotoxinas cuando los fungicidas se aplican por debajo de la dosis recomendada o si difieren en su actividad contra diferentes patógenos de la fusariosis. En consecuencia, existe una necesidad urgente de desarrollar herramientas alternativas para el manejo del FHB de una manera manera eficiente y sostenible [57].

También es importante el tratamiento de las materias primas agrícolas después de la cosecha. Dichas acciones deben estar destinadas a limitar el riesgo de contaminación con hongos patógenos y micotoxinas. Los métodos físicos, biológicos y químicos sirven para este propósito.

En las estrategias de pos cosecha, la educación y la formación de los productores agrícolas en la aplicación de diversos métodos, entre los que destacan las buenas prácticas agrícolas, resultan fundamentales para minimizar el efecto de los hongos patógenos y las micotoxinas en los productos agrícolas [58].

3. SUSTANCIAS PROMETEDORAS PARA LA LUCHA CONTRA ENFERMEDADES AGRÍCOLAS

3.1. Quitosano

El quitosano, el derivado desacetilado de la quitina, es un polisacárido lineal lineal de β-(1,4)-enlace de N-acetilglucosamina, que se obtiene principalmente de los residuos de los caparazones de cangrejos y camarones. Se ha mostrado que el quitosano tiene una actividad antimicrobiana de amplio espectro capaz de inhibir el crecimiento de una gran variedad de hongos, bacterias y levaduras [59,60]. No cabe duda de que el quitosano es una sustancia potencialmente antimicrobiana debido a su buena biocompatibilidad, biodegradabilidad, no toxicidad y su disponibilidad en abundancia. Ya en 1979 se demostró que el quitosano posee una actividad de amplio espectro contra varios hongos [61]. En general, se ha informado de que el quitosano es muy eficaz en la inhibición de la germinación de esporas, la elongación del tubo germinal y el crecimiento crecimiento radial [59]. Lo que es más interesante es la opinión generalizada de que tanto los hongos como las levaduras y los mohos son más sensibles al quitosano que las bacterias Gram-negativas y Gram-positivas [62,63]. Esto puede ser debido a que el mecanismo de acción varía en función del tipo de microorganismo. En la Figura 5 se pueden observar los mecanismos de acción del quitosano frente a bacterias Gram-positivas y Gram-negativas.

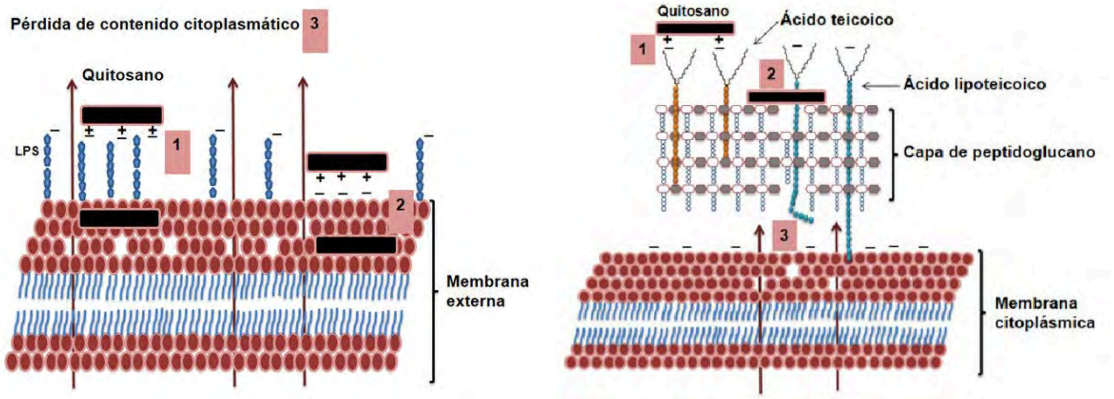

Figura 5. Mecanismo de acción del quitosano sobre bacterias Gram negativas (izquierza) [1) Atracción electrostática entre el quitosano (carga +) y los lipopolisacáridos (LPS) de la membrana externa (carga -). 2) Unión de moléculas a la membrana externa. 3) Alteración de la membrana y salida de moléculas esenciales] y mecanismo de acción de quitosano sobre bacterias Gram positivas (derecha) [1) Atracción electrostática entre el quitosano (carga +) y el ácido teicoico y lipoteicoico de la pared celular (carga -). 2) Unión de quitosano a la pared celular. 3) Se altera la interacción entre la membrana y la pared, lo que lleva a la salida de moléculas esenciales [64].

La actividad antifúngica de quitosano se considera principalmente fungistática, más que fungicida. Por ejemplo, se ha informado de que el mecanismo antifúngico para *A. parasiticus* es principalmente fungistático, y se atribuye al alto peso molecular de la muestra de quitosano utilizada [65]. Sin embargo, esta opinión sigue siendo controvertida ya que algunos investigadores afirmaron que el quitosano tiene actividad fungicida y puede inhibir completamente ciertos hongos como *F. oxysporum, R. stolonifer, Penicillium digitatum y C. albicans* [66,67]. Actualmente se admite que la interacción electrostática de grupos amino protonados del quitosano en la superficie celular del

hongo es la clave para dotarlo de la capacidad de unirse, alterar o destruir la superficie celular microbiana.

La mayor diferencia entre los dos mecanismos puede ser que el efecto fungistático es recuperable y reversible, mientras que el efecto fungicida es destructivo e irreversible, lo que se diferencia de los tradicionales antimicrobianos de bajo peso molecular [68]. En la Figura 6 se muestran las interacciones principales que ocurren entre el quitosano y una célula fúngica.

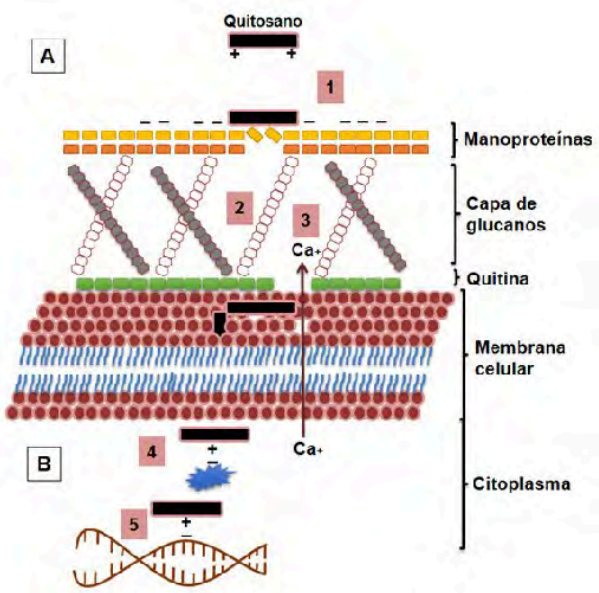

Figura 6. A) Mecanismo de acción antifúngico del quitosano a nivel extracelular. 1) Unión del quitosano a la pared celular mediante atracción electrostática. 2) Alteración de la pared y la membrana celular y formación de poros. 3) Salida de componentes esenciales como el Ca+. **B)** Mecanismo de acción intracelular. 1) Unión a la pared celular. 2) Alteración de la pared y la membrana celular y formación de poros. 4) Ingreso del quitosano al citoplasma e interacción con enzimas esenciales. 5) Interacción con ácidos nucleicos [64].

El quitosano se considera un candidato antifúngico ideal debido a su mecanismo no específico de supresión de patógenos. Se dirige principalmente a las paredes microbianas y muestra una menor tendencia a desencadenar el desarrollo de resistencia a los medicamentos [60,68], pues el desarrollo de tal resistencia puede requerir que los hongos alteren drásticamente las estructuras de su superficie celular. En este sentido, han surgido diversas estrategias antimicrobianas basadas en el quitosano, que pueden proporcionar soluciones prometedoras a la problemática que se considera en la presente memoria.

Afortunadamente, es fácil modificar químicamente el quitosano para mejorar sus características físicas y propiedades debido a la presencia de sitios reactivos atractivos, como los grupos hidroxilo y amino, susceptibles de reacción en procesos de conjugación. Curiosamente, la modificación estructural generalmente no cambia las propiedades básicas del quitosano, pero sí introduce nuevas propiedades. La estructura molecular del quitosano le permite someterse a muchas reacciones, como la oxidación, reducción, halogenación, fosforilación, acilación, etc., dando lugar a diferentes derivados [69-72]. Un gran número de estudios han demostrado que la mejora de las propiedades catiónicas del quitosano no sólo puede mejorar su solubilidad, sino también aumentar su actividad y ampliar sus aplicaciones [73,74]. Se cree que los derivados catiónicos del quitosano con

carga positiva permanente suelen tener una mejor capacidad antimicrobiana que el propio quitosano.

En particular, es evidente que las estrategias y los métodos de modificación del quitosano para su uso como antifúngico no han sido suficientemente desarrolladas. Por lo tanto, es necesario continuar profundizando en la investigación de las propiedades antifúngicas de los derivados del quitosano.

3.2. Estevióside

El estevióside se extrae de la planta *Stevia rebaudiana* (Bertoni), un arbusto herbáceo perenne de la familia Asteraceae (Compositae), originario de Brasil y Paraguay, y cultivado por sus hojas dulces [75]. Además de aportar dulzor, el estevióside también ofrece beneficios terapéuticos [76], actuando como antihiperglucémico [77], antihipertensivo [78], antiinflamatorio [79], antitumoral [80], o inmunomodulador [81].

La composición de los extractos de hoja de estevia y la concentración de los compuestos bioactivos difieren según los disolventes utilizados y los diferentes tipos de métodos de extracción empleados [82,83]. Los fitoquímicos de esta planta son glucósidos, alcaloides, saponinas, taninos y azúcares [84], incluida la vitamina soluble del ácido fólico, las vitaminas C, B12 y B6, la niacina y la tiamina [85]. Los glucósidos presentes en *S. rebaudiana* son el estevióside (9,1%), el rebaudiósido A (3,8%), el rebaudiósido C (0,6%) y el dulcósido (0,3%) [86]. Además de los glucósidos, la estevia también es una buena fuente de minerales, aminoácidos esenciales, ácidos grasos y otros compuestos bioactivos beneficiosos para la salud, como los diterpenos no glicosídicos, flavonoides, compuestos fenólicos, fibra bruta, fitosteroles, ácidos clorogénicos y triterpenos [87]. Esta planta consta de fitoquímicos que tienen efectos biológicos más allá de los referidos en el párrafo anterior, por ejemplo, actividad antioxidante [88], actividad antibacteriana y antifúngica [89,90], actividad antirretrovírica [91] y actividad antidiabética [92].

Múltiples estudios han puesto de manifiesto la actividad antimicrobiana de diferentes extractos preparados a partir de hojas de estevia. El extracto crudo de hoja de estevia en metanol y cloroformo ha presentado propiedades antibacterianas contra *Escherichia coli*, *Streptococcus mutans*, *Bacillus subtilis* y *Staphylococcus aureus*, y antifúngicas contra *Sclerotonia minor* y *Curvularia lunata* [93]. Los extractos de acetona, cloroformo y acetato de etilo han mostrado propiedades antimicrobianas contra *Aeromonas hydrophila*, *Candida albicans*, *Salmonella typhii*, *Vibrio cholera*, *Cryptococcus neoformans*, *E. coli*, *B. subtilis* y *S. aureus* [94], si bien la inhibición es fuertemente dependiente de la dosis contra todas las especies probadas [95,96].

3.3. Nanopartículas

Recientemente, la nanotecnología viene desempeñando un papel cada vez más importante en la Agricultura, por sus aplicaciones en seguridad alimentaria, inocuidad de los alimentos y productividad. Las nanopartículas (NPs) han sido utilizadas para gestionar diferentes enfermedades y plagas de las plantas [97], y los nanomateriales pueden emplearse como plataforma para la vehiculación de plaguicidas mediante tecnologías de microencapsulación, susceptibles de proporcionar una liberación controlada. La nanotecnología también puede mejorar la solubilidad en agua, reduciendo el uso de disolventes orgánicos en la formulación de los plaguicidas [98].

Además, la biosíntesis de NPs por medio de microorganismos y plantas es un enfoque respetuoso con el medio ambiente, rentable, biocompatible, seguro y ecológico [99,100].

Las nanopartículas poseen una elevada relación superficie/volumen y características peculiares en cuanto a propiedades físicas, químicas, estructurales, mecánicas, eléctricas y ópticas, lo cual las confiere aplicaciones multifacéticas. Además se caracterizan por ser biológicamente inertes, poseer toxicidad baja, y ser específicas en su acción [101].

Las actividades antimicrobianas, antifúngicas y anticancerígenas pueden observarse en varios tipos de nanopartículas (de plata (AgNPs), oro, sílice, óxidos de zinc, titanio, magnesio, etc.), así como en los nanotubos de carbono [101].

En el caso concreto de las AgNPs, autores como Khan, *et al.* [102] han demostrado su efectividad contra *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* y *Klebsiella pneumonia*. Además, se ha comprobado que las nanopartículas actúan contra *B. subtilis*, *A. niger*, *F. oxysporum*, *A. fumigatus*, *Candida albicans* [103], *Erwinia amylovora* [104], *Neofusicoccum parvum* [105] y *D. seriata* [106].

En la Figura 7 se muestran los principales mecanismos de acción de AgNPs frente a bacterias y hongos.

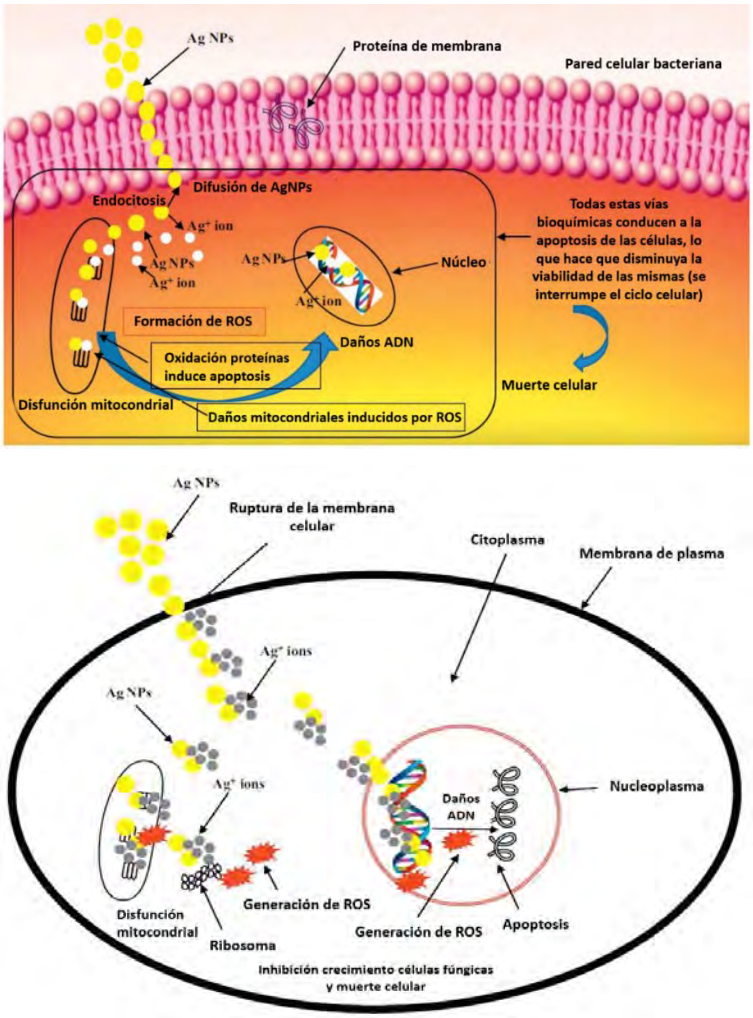

Figura 7. Mecanismos de acción de las nanopartículas de plata que inducen la muerte celular de bacterias (arriba) y de hongos (abajo). AgNPs: nanopartículas de plata; ROS ("Reactive Oxygen Species") especies reactivas del oxígeno. Adaptado de Koduru, *et al.* [107].

4. OBJETIVOS DE LA TESIS DOCTORAL

4.1. Objetivos generales

La presente Tesis Doctoral tiene como objetivo general abordar el reto de disminuir el uso abusivo de tratamientos fitosanitarios de origen químico en Agricultura, a fin de cumplir con los requerimientos de la Directiva europea 2009/128/CE (Art. 14) y su transposición al RD 1311/2012, en un contexto en el que la incidencia de las enfermedades de origen fúngico de la vid está aumentando como consecuencia del Cambio Climático. Esto solo puede conseguirse con un uso limitado y más eficaz de fitosanitarios y, en todo caso, desplazando los antifúngicos más agresivos al medio ambiente por nuevos preparados a partir de productos naturales.

4.2. Objetivos específicos

Los objetivos específicos se recogen a continuación:

- Síntesis y caracterización de nuevas formulaciones antifúngicas basadas en especies bioactivas (terpernos, polifenoles, aceites, aminoácidos azufrados, metabolitos secundarios de actinomicetos, etc.), con una solubilidad y biodisponibilidad mejoradas.
- Ensayo de dichas combinaciones a escala de laboratorio, en cultivos *in vitro* o *in vivo*, según se trate de parásitos facultativos y obligados, a fin de verificar la preconizada optimización de los tratamientos derivada de la existencia de sinergias entre los distintos agentes con actividad antifúngica.
- Ensayos en campo de las composiciones antifúngicas optimizadas sobre distintos cultivos, preferentemente cereales y vid, por su importancia en la producción agrícola de Castilla y León.
- Transferencia de tecnología: preparación y tramitación de patentes sobre los resultados.
- Divulgación de resultados a la comunidad científica, en revistas indexadas y conferencias nacionales e internacionales.

5. COHERENCIA TEMÁTICA Y METODOLÓGICA ENTRE LAS DISTINTAS PUBLICACIONES QUE FORMAN EL CUERPO PRINCIPAL DE LA MEMORIA DE TESIS DOCTORAL

La presente Tesis Doctoral es una compilación de 1 capítulo de libro, 9 artículos científicos (7 publicados y 2 pendientes de publicación) y 4 patentes sobre el objetivo general y los objetivos específicos planteados en la sección anterior.

Desde un punto de vista temático, la Tesis Doctoral parte del desarrollo de formulaciones basadas en productos naturales para el control de las EMV (enmarcadas en el proyecto de la Junta de Castilla y León con referencia VA258P18, centrado en la lucha contra la yesca). En vista de la prometedora actividad antifúngica de los productos desarrollados, se ha procedido a ampliar su ámbito de aplicación, evaluando su eficacia contra otros hongos fitopatógenos, como *Fusarium culmorum* y *Phytophtora cinnamomi*, y bacterias como *Erwinia amylovora*.

En relación con los productos naturales ensayados, la utilidad de los metabolitos secundarios producidos por especies del género *Streptomyces* spp. en el ámbito de la Agricultura es introducida por un capítulo de libro, con carácter de revisión, sobre las aplicaciones de *Streptomyces* spp. en la mejora del compost y su utilización como biofertilizante para aumentar el crecimiento y el rendimiento de las plantas, y como agentes de biocontrol. Directamente relacionado con esta revisión se encuentran la patente #1 (P201930554) y el artículo #1, relativo a la efectividad de metabolitos secundarios de dos especies de *Streptomyces* spp. (*S. rochei* y *S. lavendofoliae*) frente a tres hongos asociados a EMVs (*N. parvum, D. seriata* y *B. dothidea*).

Los artículos #2, #3, #4, #8 y #9 pertenecen a la misma línea de trabajo, sobre el control de hongos de la familia Botryosphaeriaceae, pero hacen uso de otras especies bioactivas procedentes de extractos vegetales: aminoácidos (artículo #2), extractos de zanahoria y cenoyo de mar (artículo #3), extractos de ortiga y cola de caballo (artículo #4), extractos de cardo mariano (artículo #9) o un flavonoide (artículo #8).

La referida ampliación del ámbito de aplicación de los productos desarrollados se refleja en los artículos #5, #6 y #7. En los dos primeros se aborda la problemática de la fusariosis, empleando los extractos de cardo mariano (planta también estudiada en el artículo #9) y los aminoácidos (estudiados en el artículo #2) en ensayos in vitro contra *Fusarium culmorum*, completados con ensayos de protección del grano de trigo y de espiga. Los resultados más optimizados han sido incorporados a la patente #2 (P201931118). En el artículo #7 se ensayan cuatro polifenoles (de los cuales tres -el ácido ferúlico, el ácido gálico y la silimarina- aparecen en otros artículos y en la patente #4, P202030273) contra *Phytophtora cinnamomi*, incorporando a las preparaciones las nanopartículas de plata (que sin embargo no han conducido a mejoras significativas en términos de efectividad). La explotación de uno de los resultados de este artículo se recoge en la patente #3 (P202030007).

El tratamiento metodológico común a estos artículos incluye procedimientos de síntesis que aseguran la mejora de la solubilidad y biodisponibilidad a través del uso de los oligómeros de quitosano (artículos #1, #2, #3, #4, #6 y #7) o de glicósidos de esteviol (artículos #7, #8 y #9). El uso de técnicas de Química Verde ha sido común a todos los artículos, en la fase la extracción de los principios activos (asistida por ultrasonicación) y/o en la formación de complejos conjugados (vía microondas, sonicación, uso de líquidos iónicos, etc.). La caracterización del principio/principios mayoritarios en los extractos de plantas se ha realizado por medio de cromatografía de gases-espectrometría de masas (GC-MS). Métodos complementarios de caracterización utilizados han sido la espectroscopía de infrarrojos por transformada de Fourier (FTIR), los análisis termogravimétricos (TGA) y la calorimetría de barrido diferencial (DSC). Para la cuantificación por colorimetría del contenido total de polifenoles se ha hecho uso del espectrofotómetro UV–vis. Los análisis elementales se han llevado a cabo mediante LECO. En cuanto a los ensayos in vitro, se ha utilizado el método de dilución en agar, conforme a los procedimientos estandarizados del Comité Europeo de Pruebas de Susceptibilidad a los Antimicrobianos (EUCAST). Para los ensayos in planta, se ha recurrido a la inoculación artificial del patógeno (bien a través de heridas, para las plantas-injerto de vid, o mediante aplicaciones de suspensiones conidiales en espray, para las espigas de espelta) y a su tratamiento simultáneo o secuencial con las formulaciones bioactivas.

6. REFERENCIAS

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CAPÍTULOS DE LIBRO

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Chapter 13
Applications of *Streptomyces* spp. Enhanced Compost in Sustainable Agriculture

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Abstract *Streptomyces* is the most abundant genus among actinomycetes and holds great potential for sustainable agriculture, both now and in the future, given its role as a source of antibiotics, bioactive compounds, and enzymes. This mini-review discusses the role of these microorganisms in the degradation by enzymatic hydrolysis of lignocellulosic residues during the composting process. Examples of soil amendment with compost bioaugmentated with populations of *Streptomyces* are reviewed. The advantages derived from the combined use of organic compost and microorganisms of this genus (and other members of Actinobacteria phylum) as biofertilizers to increase plants growth and yield are also presented. Finally, strategies aimed at biocontrol or at the improvement of the capacity for suppression of diseases through an increase in organic matter and Actinobacteria levels are discussed.

Keywords Actinobacteria · Bioaugmentation · Biocontrol · Biofertilizer · Bioremediation · Plant growth promotion · Secondary metabolites · Soil amendment

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13.1 Introduction

Actinobacteria are widely distributed in the water, soil, and surfaces of colonized plants and have an enormous importance in composting processes, provided that they can degrade a wide variety of biopolymers by hydrolytic enzymes (Diaz et al. 2007). The presence of organic matter makes them dominant in dry, humic, and calcareous soils, and the physical characteristics of those soils conform the size of the population (20–60%) and their composition (Araragi 1979).

The Actinomycetes phylum encompasses 6 classes, 19 orders, 50 families, and 221 genera, but new taxa are still being discovered. Actinomycetes have morphologies that range from cocci to mycelia and differentiated spores (Timková et al. 2018). The *Streptomyces* genus is the most abundant among soil bacteria and actinomycetes, and they are aerobic microorganisms that feature a high content of G+C (75%) in their DNA and large genomes in comparison with those of other microorganisms (Sanglier et al. 1993).

The *Streptomyces* genus has been exploited in the pharmaceutical industry and in commercial biocontrol products for agriculture, but their importance and the mechanisms that govern their complicated interactions with plants and other organisms are still a very active area of research (Yu et al. 2011).

Actinomycetes degrade organic matter and produce secondary metabolites, and have the ability to solubilize phosphate, to produce organic acids, siderophores, and phytohormones (Locatelli et al. 2016).

Actinobacteria, particularly those of the *Streptomyces*, *Pseudomonas*, *Agrobacterium*, and *Bacillus* genera, can be used for the biocontrol of phytopathogens in fertile soils (Wang et al. 2013b) and have shown their efficacy in controlling plant diseases both in vitro and in vivo (Virolle et al. 2015). The main mechanisms of biological control include the parasitism of hyphae, the production of secondary metabolites, the production of siderophores, and the production of extracellular enzymes such as cellulases, amylase, and chitinase. Extracellular enzymes (e.g., β-1,3-glucanases and chitinases) are responsible for the mycoparasitism exerted by certain strains of *Streptomyces* and the suppression of plant diseases (Singh and Gaur 2016). Moreover, they can produce other agro-active compounds with relevant antimicrobial potential: terpenoids, vitamins, pigments, etc. (Franco-Correa et al. 2010).

Aldesuquy et al. (1998) were among the first to report that endophytic actinomycetes could also improve the growth, vigor, and yield of wheat crops, in different environments and ecological conditions (Marques et al. 2010; Zhang et al. 2012). For instance, *Streptomyces* isolated from rotten wheat straw (e.g., *Streptomyces* sp. UU15 and *Streptomyces vinaceusdrappus* UU11) offer enormous potential for plant growth promotion (PGP) and the formation of agro-active compounds (Singh et al. 2019).

Another relevant area of application of actinobacteria is related to their potential to be applied in biostimulation, bioaugmentation, cellular immobilization, and bioremediation of organic and inorganic contaminants techniques. By means of

multi-omics analysis, it is possible to know the mechanisms involved in the formation of bioactive and biosurfactant films, the processes associated with the recycling and degradation of complex substances or pesticides and in the recovery of soils contaminated by heavy metals. Through the design of mixed specific crops and the use of microorganism consortium systems (including *Streptomyces*), new biocontrol strategies for the toxicity of contaminants may be devised. However, further studies are still necessary to optimize the role of these microorganisms in removing pollutants from contaminated environments (Timková et al. 2018).

A recent Special Issue on Plant–Microbe Interaction (Schirawski and Perlin 2018) has summarized and discussed the current understanding of plant–microbe interactions, providing a general overview of soil microbes that positively affect plant growth, and the elucidating mechanistic strategies of plant pathogens and microbiomes of seeds and roots. However, a mini-review of the most recent developments focused only on the applications of the genus *Streptomyces* would be of use to academic and industrial researchers, undergraduates and postgraduates alike, working in this field. This chapter aims to deliver a panorama of the extensive and rapidly growing applications of these microorganisms in sustainable agriculture.

13.2 Composting Process Enhancement with *Streptomyces* spp.

In spite of the spontaneous microbiological nature of the composting process, the addition of selected microbial starters can speed up the process and improve its quality. Thus, the inoculation of such selected microorganisms as part of the preparation of multifunctional biofertilizers can be a promising approach to reduce the amounts of synthetic fertilizers used in agriculture.

Thermophilic and highly cellulolytic *Streptomyces* can be isolated either from soil (Ramírez and Coha 2003), solid waste compost (Strom 1985), compost of agricultural wastes (Jang and Chang 2005), or compost-treated soils (Feng et al. 2014). In 2003, Ramírez and Coha (2003) isolated 145 cellulolytic thermophilic actinomycete strains from over 70 compost, hay, dung, and soil samples. They found 10 cellulolytic actinomycete strains with a high yield in cellulases (endoglucanase, β-glucosidase, and exoglucanase activities), and concluded that *Streptomyces* sp. 7CMC10 was the strain that featured highest activity levels (corresponding to 20.14, 5.40, and 2.61 UI·mg^{-1} of protein, respectively).

Cellulases production by a strain isolated from a Brazilian forest soil, *Streptomyces drozdowiczii*, was studied using agro-industrial products (Grigorevski de Lima et al. 2005; Semedo 2004), and that from *S. malaysiensis* was evaluated by Nascimento et al. (2009) using submerged fermentation for a mixture of brewer's spent grain and corn steep liquor. *S. viridobrunneus* strain has been shown to be cellulolytic too, due to its ability to decompose cellulose from agro-industrial residues (Da Vinha et al. 2010). Moreover, Ventorino et al. (2016) highlighted the

potential of *S. argenteolus* AE58P from a biotechnology perspective, since its behavior as a biocatalyst-producing bacterium makes it an exciting candidate for lignocellulose conversion and composting processes.

Other cellulolytic streptomycetes for composting purposes are *S. celluloflavus*, *Streptomyces* sp. SirexAA-E, *S. reticuli*, *Streptomyces* sp. Amel2xE9, *Streptomyces* sp. LamerLS-31b, *Streptomyces* sp. DpondAA-B6, *Streptomyces* sp. KhCrAH-340, *Streptomyces* sp. LaPpAH 95, and *Streptomyces* sp. ATexAB-D23 (Hillis et al. 2016). This study showed that the ability of *Streptomyces* spp. to degrade cellulose in a rapid manner would be restricted to two clades of host-associated strains, and that, although plant biomass-degrading genes (CAZy) are common in microorganisms of this genus, crucial enzyme families would be enriched in highly cellulolytic strains. The authors verified the importance of the CebR transcriptional repressor and a highly expressed cellulase (viz. GH6 family cellobiohydrolase) to the cellulolytic phenotype. Their evolutionary analyses identified intricate genomic modifications—which include the acquisition and selective retention of CAZy genes and transcriptional regulators—that would drive plant biomass deconstruction in *Streptomyces* spp. Thus, they suggested that some symbiotic streptomycetes have been selected in host-associated niches because of their increased cellulose degrading activity, which makes those strains the most relevant for composting processes enhancement. The distribution of the cellulolytic ability in *Streptomyces* genus is shown in Fig. 13.1.

According to the line of action proposed by Pugliese et al. (2008), in the following sections, we summarize a selection of *Streptomyces* spp. strains (for the most part coming from compost) for remediation purposes, as plant growth promoters and to control plant pathogens.

13.3 Soils and Water Bioremediation with *Streptomyces* spp.

Phytoremediation is a technology that uses plants to restore soils contaminated with trace elements or pesticides (Cao et al. 2016). This technique depends on the host plant–microorganisms interaction, and the bioremediation with actinobacteria is among the most popular options for the cleaning of contaminated sites (Alvarez et al. 2017). Such actinobacteria biomass used in soil remediation may be produced, for example, from cultures based on sugarcane vinasse (Aparicio et al. 2017).

Streptomyces spp. possess very interesting properties in terms of metabolic diversity, ability to rapidly colonize substrates, formation of mycelia, and production of spores in unfavorable conditions (Alexander 1991). These microorganisms have different metabolic pathways in which toxic compounds are used as an energy source for cell processes through fermentation, respiration, and co-metabolism. So as to survive in toxic environments, they have developed resistance to heavy metals

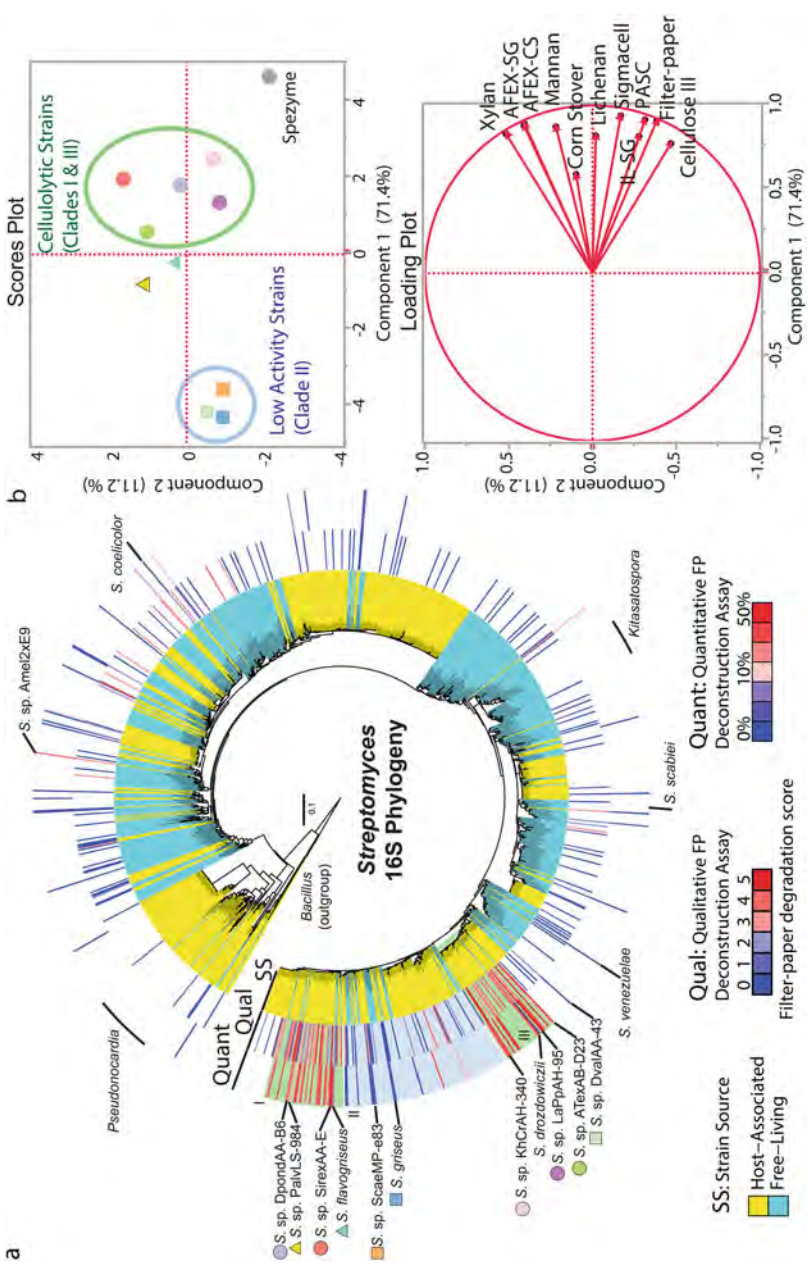

Fig. 13.1 Distribution of cellulolytic ability in *Streptomyces* genus. (**a**) 16S rRNA gene phylogenetic tree of 1141 *Streptomyces* strains from free-living (cyan) and host-associated (yellow) environments. The tree is annotated with qualitative cellulose (filter paper) degradation scores (0: no growth in 3 weeks, 5: filter

and mechanisms aimed at maintaining homeostasis (Brar et al. 2006). These combinations of these mechanisms could lead to an extraordinarily resistant bacterium, as noted by Timková et al. (2018). Some success cases of their application to bioremediation are illustrated below.

13.3.1 Trace Elements Bioremediation

From an ecological perspective, it has been proven that the inoculation of *Robinia pseudoacacia* L. (black locust) rhizosphere with the appropriate rhizobia (especially those from the *Streptomyces, Mesorhizobium*, *Rhodococcus*, and *Variovorax* genera) can provide an environmentally friendly strategy to improve heavy metal contaminated soils by phytoremediation (Fan et al. 2018).

The remediation of mining sites is a particularly complex process and depends on the physicochemical conditions of the soil (Ma et al. 2015). The role of *S. pactum* in the phytoremediation of trace elements by *Brassica juncea* (L.) Czern (brown mustard) in mine polluted soils has been studied by Ali et al. (2017c). The effects of compost amendments on the concentrations of metals in a mine floor with the same plant have also been assessed by Forján et al. (2018).

The same *Streptomyces* species has also been used in the phytoremediation of trace elements by other hyperaccumulating plants such as *Sorghum bicolor* (L.) Moench (i.e., sorghum) (Ali et al. 2017b). Moreover, its combination with wood biochar has been reported to promote phytoremediation in soils contaminated by trace elements, observing a positive impact on enzymatic activities in the smelter-contaminated soil as well as in the sorghum leaves (Ali et al. 2017a).

S. mirabilis has been found to increase sorghum productivity in soils contaminated with metals too (Schütze et al. 2014). Bacterial amendments with *S. acidiscabies* E13 and *S. tendae* F4, and mycorrhiza with *Rhizophagus irregularis* have also been assayed for phytoremediation by Phieler et al. (2015), investigating the accumulation of metals in sorghum.

Streptomyces sp. CG252 has been reported to tolerate heavy metals and remove Cr(VI) by reduction to Cr(III) (Morales et al. 2007). Likewise, soil bioaugmentation by *Streptomyces* sp. R25 and *Bacillus* sp. ZAN-044 can reduce cadmium deposited in plants (Jezequel and Lebeau 2008).

Fig. 13.1 (continued) paper deconstruction in 1 week) and quantitative cellulose degrading activities (% filter paper degraded in 10 days). Shading indicates highly cellulolytic clades I and III (green) and related low-activity clade II (blue). (**b**) PCA analysis of the lignocellulosic biomass-degrading activity of *Streptomyces* secretomes. Strains are identified by colored shapes on the tree in panel A. The scores plot shows similarity of polysaccharide degrading activity, and the loading plot indicates which substrates influence components 1 and 2 of the scores plot. Reprinted from Hillis et al. (2016) under Creative Commons Attribution License, CC BY 4.0

13.3.2 Pesticides Bioremediation

The application of a consortium of *Streptomyces* spp. to eliminate mixtures of pesticides in different soil systems has been the subject of recent attention (Fuentes et al. 2017). The successful elimination of multiple organochlorine pesticides with strains of *Streptomyces* sp. A5 and their influence on the cytotoxicity of the treated systems was recently reported by Fuentes et al. (2018).

Streptomyces spp. have been studied for the controlled elimination of a mixture of organophosphorus pesticides (diazinon and chlorpyrifos insecticides) by Briceño et al. (2017). In a similar fashion, a remarkably high degradation efficiency of *Streptomyces* sp. AH-B strain against quinclorac has been reported by Lang et al. (2018): in liquid medium, it achieved 97.2% removal after 18 days, and in soil, it attained a degradation of 87.5% after 42 days.

Even recalcitrant chlorinated pesticides like lindane may be degraded by this genus: the elimination of high concentrations of lindane in soils has been demonstrated using stable microemulsions and *Streptomyces* sp. M7. In the work by Saez et al. (2017), a soybean oil-based microemulsion allowed to solubilize 66% of the lindane present in the aqueous medium, i.e., a 4.5 times higher amount than when a surfactant was used. The authors recommended the use of microemulsions formed with soybean oil, Tween 80 (polysorbate 80) and 1-pentanol as a soil washing technology and for the ex situ bioremediation of wastewater polluted with lindane or with other hydrophobic organic compounds.

Streptomyces sp. M7, MC1, A5, and *Amycolatopsis tucumanensis* DSM 45259 have also been tested for the simultaneous elimination of lindane and chromium from different contaminated systems (Aparicio et al. 2018a, b). The viability of these strains was confirmed after the bioremediation process, so these studies pose an approximation to what may be carried out at field scale.

13.4 *Streptomyces* spp. as Plant-Growth Promoters and Biofertilizers

In agricultural practices, it should be emphasized that lignocellulosic wastes should be valorized as substrates, and the conversion of agricultural residues with microorganisms by composting processes for the preparation of multifunctional biofertilizers should thus be encouraged, replacing the fertilizers of synthetic origin. Moreover, the inclusion of species with the ability to control plant pathogens should be a priority. During the initial stages of composting, the decomposition of phytotoxic organic substances is carried out due to the presence of organic acids, ammonia, and ethylene oxide (Mehta et al. 2014). The maturity of the compost can be determined by several physical–chemical and biological or microbiological

parameters (discussed in Chap. 8 of this book), while the phytotoxicity tests involve the determination of the seed germination index (GI) of the compost extract. If GI $>$ 101, the substrate is considered as a phytostimulant and it is suitable for application as a fertilizer (Rashad et al. 2010).

In the last few years, Actinobacteria—especially the *Streptomyces* genus because of its soil dominant saprophytic nature and strong antimicrobial potential—have attracted attention as efficient plant growth promoters (Franco-Correa et al. 2010) and have inspired their application as biofertilizers to boost plant productivity. The applications in terms of PGP (and also in biocontrol of phytopathogens) of *Streptomyces* spp. have been discussed in a recent review paper by Vurukonda et al. (2018). Another recent review paper by Sathya et al. (2017), focused on grain legumes sustainable production and protection, also explains how actinobacteria can promote plant growth.

The genus *Streptomyces* comprises many species that have different functions in the promotion of plants growth, and whose characterization can be attained through different methods (Table 13.1). In 2016, Passari et al. (2016) proposed *Streptomyces* sp. strain DBT204 isolates (recovered from *Solanum lycopersicum* L., i.e., tomato) as biofertilizers to improve tomato and chili seedlings growth, observing an increase in the productivity of the crop thanks to the phytohormone producing potential and the metabolites with phytohormone mimicking activity of this strain. The strain *S. roseoflavus* NKZ-259 has also been demonstrated to be a promoter of plant growth for pepper and tomato, both in greenhouse and field tests, and simultaneously behaved as a biological agent and biopesticide agent, inhibiting pathogenic fungi (tomato gray mold) (Shi et al. 2018).

Endophytic *S. olivaceoviridis, S. viridis, S. atrovirens, S. rochei*, and *S. rimosus* have been shown to improve seed germination as well as root elongation and growth (El-Tarabily 2008; Khamna et al. 2010). Also in 2016, Tamreihao et al. (2016) reported the biocontrol and PGP activities of an *S. corchorusii* strain (UCR3-16) upon its application to rice as a biofertilizer agent. In 2018, Chaiharn et al. (2018) reported that rice root length was significantly increased by *Streptomyces* isolate KT 6-4-1. According to a study by Wang (2018), conducted in field conditions, *S. griseoplanus* (namely PSA1) in combination with P fertilizer increased soil available P content, enhanced plant growth, and enhanced grain yield of maize by 11%. In a similar fashion, Singh et al. (2016) reported that metabolites of *Streptomyces* sp. significantly enhanced biomass yield (3.58-fold increase versus control plants). In 2019, Singh et al. (2019) reported a seedling assay and antagonism test of *S. rochei* UU07 (a strain that grows well at pH 4.5–9.4 and that shows tolerance to salt), revealing it as an excellent PGP and a potential antagonist for *Rhizoctonia solani*. They confirmed that rotten wheat straw can be a source for the isolation of actinobacteria with PGP traits, which may be used as consortia for the composting of different agriculture waste materials and to increase crop yields.

The synergistic effects of the joint inoculation of the endophyte bacterium *S. griseoflavus* P4 and *Bradyrhizobium japonicum* (*B. diazoefficiens*) SAY3-7 on nitrogen fixation, nutrient uptake, plant growth, nodulation, and seed yield of

Table 13.1 Characterization and functions related to plants growth promotion of different *Streptomyces* spp.

Streptomyces spp.	Sample	Plant	Characterization	Function in PGP	References
S. humidis	Sandy soil with debris of *Diplotaxis tenuifolia*	Cucumber	Quantification of total bacteria: real time-PCR (polymerase chain reaction) *Streptomyces* spp.-specific population patterns: PCR-DGGE (denaturing gradient gel electrophoresis)	Disease suppression in the cucumber–*F. oxysporum* f. sp. *radicis-cucumerinum* system	Klein et al. (2013)
S. kumnigenesis *S. mutabilis* *S. enissocaesilis* *S. djakartensis* *S. nobilis* *S. kunmingenesis* *S. enissocaesilis*	Soil samples collected from the rhizhosphere	Wheat and tomato	Morphological, biochemical, and physiological characterization: melanin pigment, decomposition of oxalic acid and other organic acids, cell wall type, tyrosine hydrolysis by actinomycetes, xanthine and hypoxanthine utilization, starch hydrolysis. Genomic DNA isolation, PCR amplification and sequencing of the 16s rRNA gen	IAA production, phosphate solubilization, siderophores,, HCN production and 1-aminocyclopropane-1-carboxylate deaminase production	Anwar et al. (2016)
Streptomyces sp. strain DBT204	Seeds (IARI, Pusa Campus, New Delhi, India)	Chili and tomato	Morphological: primary identification of endophytic bacteria. Spore chain morphology: field emission gun/scanning electron microscope (FEG-SEM) Phylogenetic analysis: PCR	Growth promotion: significant increase in shoot length, root length and plant weight	Passari et al. (2016)
Streptomyces sp.	Vertisol soil samples (Icrisar, Pachanteru, India)	Seeds of chickpea variety ICCV 2	Identification by 16S rDNA sequencing	Improved soil health and crop growth	Sathya et al. (2016)

(continued)

Table 13.1 (continued)

Streptomyces spp.	Sample	Plant	Characterization	Function in PGP	References
Streptomyces isolate L3 *Streptomyces* isolate KT 6-4-1 *Streptomyces* isolate ST 3	Soil samples	Rice seeds (variety RD-6)	Morphological and cultural characteristics: scanning electron microscope Chemotaxonomic and physiological characteristics: ISP method Secondary identification: 16S rDNA sequence analysis	Long-term root disease control. Phosphate solubilization, through excretion of siderophores Plant growth promotion	Kpomblekou-A and Tabatabai (1994)

soybean crops have been investigated by Htwe et al. (2018) and Htwe and Yamakawa (2016). Analogous studies were conducted for *S. griseoflavus* P4 with various *Bradyrhizobium* strains (Aung and Takeo 2015) and different crops (cereals, horticultural crops, and leguminous plants), finding a beneficial effect of *S. griseoflavus* P4 in all cases.

The PGP behavior of Actinobacteria (particularly of *Streptomyces* spp.) discussed above is accomplished by different mechanisms (Passari et al. 2015), which include the production of secondary metabolites like antibiotics (e.g., chloramphenicol, fluconazole, nalidixic acid, trimethoprim, streptomycin, and rifamycin) (Doumbou et al. 2001), 1-aminocyclopropane-1-carboxylate deaminase (El-Tarabily et al. 2008), phytohormones (e.g., indole acetic acid, kinetin) (Rashad et al. 2015), desferrioxamine and coelichelin siderophores (Khamna et al. 2008); and phosphate solubilization (Mohandas et al. 2013).

Aforementioned secondary metabolites play an important role in regulatory activities and as antagonistic agents, agrobiologicals, and pharmacological agents (Harir et al. 2018). For example, endophytic *S. hygroscopicus* TP_A045 produces metabolites such as pteridic acids A and B that show auxin-like activity and that induce root elongation in *Phaseolus vulgaris* L. (common bean) (Igarashi et al. 2002).

In addition, the genus *Streptomyces* is well known as a generator of extracellular polymeric substances (EPS) that act as siderophores or Fe^{2+} iron transporters, which can be chelated, mobilized, solubilized, and assimilated by active transport mechanisms (Singh et al. 2016). Poaceae (barley and wheat grasses) are able to effectively sequester iron by releasing phytosiderophores through their roots, and *Streptomyces* spp. can increase the availability and absorption of iron. Oats are also capable of assimilating iron by microbial siderophores (Kraemer et al. 2006), and so are the seeds of chickpea (Sathya et al. 2016). With regard to the latter crop, Gopalakrishnan et al. (2015) confirmed grain yield and plant growth enhancement by using broad-spectrum *Streptomyces* spp.

Among the isolates tested in the biological restoration of tailings landfills, *Streptomyces* sp. R05.33 and *Phyllobacterium* R01.34 have been claimed to have the greatest potential to act as PGP rhizobacteria. Zappelini et al. (2018) showed how *Streptomyces* spp. could colonize the roots of *Betula pendula* Roth (silver birch) in a red gypsum landfill in unfavorable conditions, forming spores and favoring the growth of the plant. A similar approach (birch and strains of actinomycetes as rooting agents) was also successful in the restoration of coal mine landfills (Ostash et al. 2014).

Entering fully in specific aspects of composting, Sharma et al. (2017) developed an efficient microorganism (EM) compost or bioorganic fertilizer optimized by dose of EM compost and inocula of various microorganisms (viz. *S. globisporous* C3, *Candida tropicalis* Y6, *Phanerochaete chrysosporium* VV18, and *Lactobacillus* spp.) to improve soil fertility and stimulate plant growth. The physicochemical characteristics of the EM mature compost used in ornamental crops were as follows: total C 26%; total N 1.66%; C/N ratio 15.66; humus 7.55%; available P 0.31%; pH 7.8; EC 0.38 $S \cdot m^{-1}$.

Verma et al. (2015) noted that the application of EM compost improved the lycopene content of tomato fruits, provided nutrients, and reduced the cultivation cost. The application of EM to plants would also induce higher levels of calcium compared to untreated plants (Daiss et al. 2007).

Results of the synergistic potential of compost with two *Streptomyces* strains for PGP and biocontrol against *R. solani* on pepper evidenced that this combination was highly efficient for the disease suppression (Wang et al. 2015).

In a recent contribution by Dimitrijevic et al. (2017), a superior fertilization effect of a compost obtained from mixed herbs waste was gained after inoculation with three novel mesophilic strains (viz. *S. spororaveus* CKS2, *S. microflavus* CKS6, and *S. fulvissimus* CKS 7).

13.5 *Streptomyces* as Biocontrol Agents

Traditionally, crop rotation has been an effective strategy to combat pathogens, and soil solarization has been used, for instance, to fight *F. oxysporum* (Benlioglu et al. 2005; Huang et al. 2012). Since disinfection with methyl bromide and other pesticides is currently a prohibited practice (Cebolla et al. 2000), it is necessary to find biological agents that are more efficient and safer.

Interference competition, a relevant strategy in interspecific interactions, refers to the production of secondary metabolites (e.g., enzymes, toxins, biosurfactants, antibiotics, and volatiles) that can suppress microbial opponents (Hibbing et al. 2009). In this regard, actinomycetes, particularly *Streptomyces* spp., are receiving increasing attention as biological agents (Cuesta et al. 2012) to control soil-borne pathogens (Getha and Vikineswary 2002; Gopalakrishnan et al. 2011) and to produce antibiotics. In fact, *Streptomyces* is the largest antibiotic-producing genus against clinical microorganisms and parasites (Castillo et al. 2002; Hwang et al. 2001). In addition, they produce other bioactive compounds of clinical importance such as immunosuppressants (Watve et al. 2001).

In sustainable agriculture, the potential of *Streptomyces* to produce antibiotics and other secondary metabolites can be used to control phytopathogenic fungi and bacteria (Schrey and Tarkka 2008) and even to act as nematicidals (Santos et al. 2016). Moreover, the use of *Streptomyces* as agro-antibiotics (Demain 2009) and as biocontrol agents against numerous fungal and viral pathogens is becoming increasingly popular and is reaching the commercialization stage in many countries (Trejo-Estrada et al. 1998; Macagnan et al. 2008; Gopalakrishnan et al. 2011; McGhee and Sundin 2011; Liu et al. 2014; Peng et al. 2014). Volatile antifungal compounds produced by *Streptomyces* species play an important role in biocontrol and are important because they can act as fungicides, bactericides, nematicides, herbicides, insecticides, molluscicides, etc. (Rey and Dumas 2017).

A selection of very promising *Streptomyces* spp. and their associated secondary metabolites, together with their applications, is presented in Table 13.2. The chemical structures of some of those secondary metabolites are depicted below, in

Table 13.2 Selected *Streptomyces* spp. with their associated secondary metabolites and proposed applications

Streptomyces spp.	Secondary metabolites	Applications	References
S. ambofaciens	Oxytetracycline, spiramycin, albopeptin A, albopeptin B, alpomycin	Antibacterial	Bunet et al. (2008), Pernodet et al. (1993)
S. alboflavus	Oxytetracycline, tetracycline, desertomycin A	Antibacterial	Dam et al. (2014), Ji et al. (2012), Wang et al. (2013a)
S. althioticus	Althiomycin	Antibacterial	Luo et al. (2016), Vining (2014)
S. amphotericinicus	Amphotericin	Antifungal	Cao et al. (2017)
S. anandii	Pentaene G8, gilvocarcin V, gilvocarcin M, gilvocarcin E	Antifungal	Balitz et al. (1981), Singh and Srivastava (1998)
S. atratus	Atramycin A, hydrazidomycins A, hydrazidomycins B, hydrazidomycins C, rufomycins A, rufomycins B	Antifungal	Deutsche Forschungsgemeinschaft. Senatskommission zur Beurteilung von Stoffen in der Landwirtschaft. (2001), Ueberschaar et al. (2011)
S. atrovirens	Indole-3-acetic acid	Plant growth promotion	Abd-Alla et al. (2013)
S. avermitilis	Ivermectin, abamectin	Insecticide and antihelmintic	Burg et al. (1979), Takahashi (2002)
S. bellus	Althiomycin	Antibacterial	BacDive (2019a)
S. cacaoi	Polyoxine	Antifungal	Chen et al. (2009), Funayama and Isono (2014), Goodfellow et al. (1988)
S. cellulosae	Fungichromin (pentamycin)	Antifungal	Harrison et al. (1986), Laskin and Lechevalier (1977), Li et al. (1989)
S. chattanoogensis	Natamycin	Antifungal	Du et al. (2009), Jiang et al. (2013)
S. chrestomyceticus	Lycopene, pyrrolostatin, paromomycin, aminocidin, aminosidine, neomycin E, neomycin F	Antibacterial	Kato et al. (1993), Prakash and Sharma (2014)
S. crystallinus	Hygromycin A (totomycin)	Antibacterial	Afifi et al. (2012)
S. cuspidosporus	Valinomycin	Antibacterial	Maheswari and Chandra (2000)
S. fabae	Antimicrobial activity	Antibacterial	Nguyen and Kim (2015)
S. filipinensis	pentalenolactone I, hygromycin A, filipin	Antifungal	Payero et al. (2015), Uyeda et al. (2014)

(continued)

Table 13.2 (continued)

Streptomyces spp.	Secondary metabolites	Applications	References
S. flavofungini	Bafilomycin K, aminoacylase	Antifungal	Uri and Békési (1958)
S. fradiae	Neomycin, tylosin, fosfomycin	Antibacterial	Janssen et al. (1989), Waksman and Lechevalier (1949)
S. glomeratus	Beromycin, nogalamycin	Antibacterial	Blumauerová et al. (1980)
S. griseiniger	Nigericin	Antibacterial	BacDive (2019b)
S. griseochromogenes	Blasticidin A, blasticidin B, blasticidin C, blasticidin S, pentalenene, cytomycin	Antifungal	Cutler and Cutler (1999)
S. griseoviridis	Etamycin, griseoviridin, bactobolin, prodigiosin R1, rosophilin	Antifungal	Kawasaki et al. (2009), Xie et al. (2012)
S. griseus	Produces 32 different structural types of secondary metabolites of commercial importance	Antibacterial	Graf et al. (2007), Ohnishi et al. (2008), Schatz et al. (1944)
S. halstedii	Bafilomycin B1, bafilomycin C1, deltamycin A2, deltamycin A3, magnamycin B, vicenistatin	Antifungal	Hochstein and Murai (1954)
S. heilongjiangensis	Borrelidin	Antibacterial	Liu et al. (2012)
S. hiroshimensis	Prodigiosin	Antifungal	Magae et al. (1993)
S. hygroscopicus	Geldanamycin, hygromycin B, nigericin, validamycin, cyclothiazomycin	Antifungal and antibacterial	Murakami et al. (1986)
S. kanamyceticus	Kanamycin	Antifungal and antibacterial	BacDive (2019c)
S. kasugaensis	Kasugamycin, thiolutin	Antibacterial	Hotta et al. (1996)
S. koyangensis	4-Phenyl-3-butenoic acid	Antifungal	Lee (2005), Lee et al. (2005)
S. kurssanovii	Chitinase, N-(phenylacetyl)-2-butenediamide, fumaramidmycin	Antifungal	Il'ina et al. (2000), Tikhonov et al. (1998)
S. laurentii	Thiostrepton	Antibacterial	Trejo et al. (1977)
S. lavendofoliae	Fosfazinomycin A, fosfazinomycin B, piperastatin B	Antifungal	Al-Humiany (2011), Murakami et al. (2008)
S. lavendulae	Streptothricin, lavendamycin	Antifungal	August et al. (1996), Sheldon et al. (1999)

(continued)

Table 13.2 (continued)

Streptomyces spp.	Secondary metabolites	Applications	References
S. lydicus	Actithiazic acid, natamycin, lydimycin, streptolydigin, 1-deoxygalactonojirimycin	Antifungal	Atta et al. (2015), Gómez et al. (2012), Yuan and Crawford (1995)
S. narbonensis	Narbomycin, josamycin	Antibacterial	van Balken (1997)
S. netropsis	Netropsin, distamycin A, mycoheptin	Antifungal and antibacterial	Ekzemplyarov (1977), Neilan et al. (2014)
S. omiyaensis	Chloramphenicol, pentalenolactone P	Antibacterial	Alam et al. (2004)
S. phytohabitans	Novonestmycin A, novonestmycin B	Antifungal	Bian et al. (2012), Wan et al. (2014)
S. platensis	Oxytetracycline, platensimycin, migrastatin, isomigrastatin, platencin, dorrigocin A, dorrigocin B	Antibacterial	Peterson et al. (2014), Smanski et al. (2009)
S. prasinus	Prasinomycin, validamycin, prasinon A, prasinon B	Antifungal and antibacterial	Box et al. (1973)
S. rochei	Borrelidin, butyrolactol A, butyrolactol B, uricase, streptothricin	Antifungal	Augustine et al. (2005), Irdani et al. (1996), Kanini et al. (2013)
S. roseosporus	Daptomycin	Antibacterial	Miao (2005)
S. spectabilis	Hangtaimycin, gentamicin, kanamycin, neomycin B, sisomicin, tobramycin, paromomycin, spectinabilin, spectinomycin, aminocyclitol, actinospectacin, prodigiosin, streptovaricin	Antibacterial	Kakinuma et al. (1976), Zuo et al. (2016)
S. sulfonofaciens	Pluracidomycin	Antibacterial	Miyadoh et al. (1983)
S. variegatus	Prodigiosin	Antifungal	Sveshnikova et al. (1983)
S. vitaminophilus	Pyrrolomycin	Antifungal and antibacterial	Mahan et al. (2016)
S. zaomyceticus	Zaomycin, pikromycin, glumamycin, foroxomithine	Antibacterial	Wegler (1981)

Figs. 13.2 and 13.3. Table 13.3 shows different species of *Streptomyces* and their role as biocontrol agents.

Many species of actinomycetes can inhibit various pathogenic fungi (Al-Askar et al. 2014; Hwang et al. 2001; Lim et al. 2000). Some strains of *S. humidus* (e.g., strain S5–55) have been reported to feature antagonism against *Phytophthora capsici* through direct antibiosis (sodium phenylacetate and phenylacetic acid)

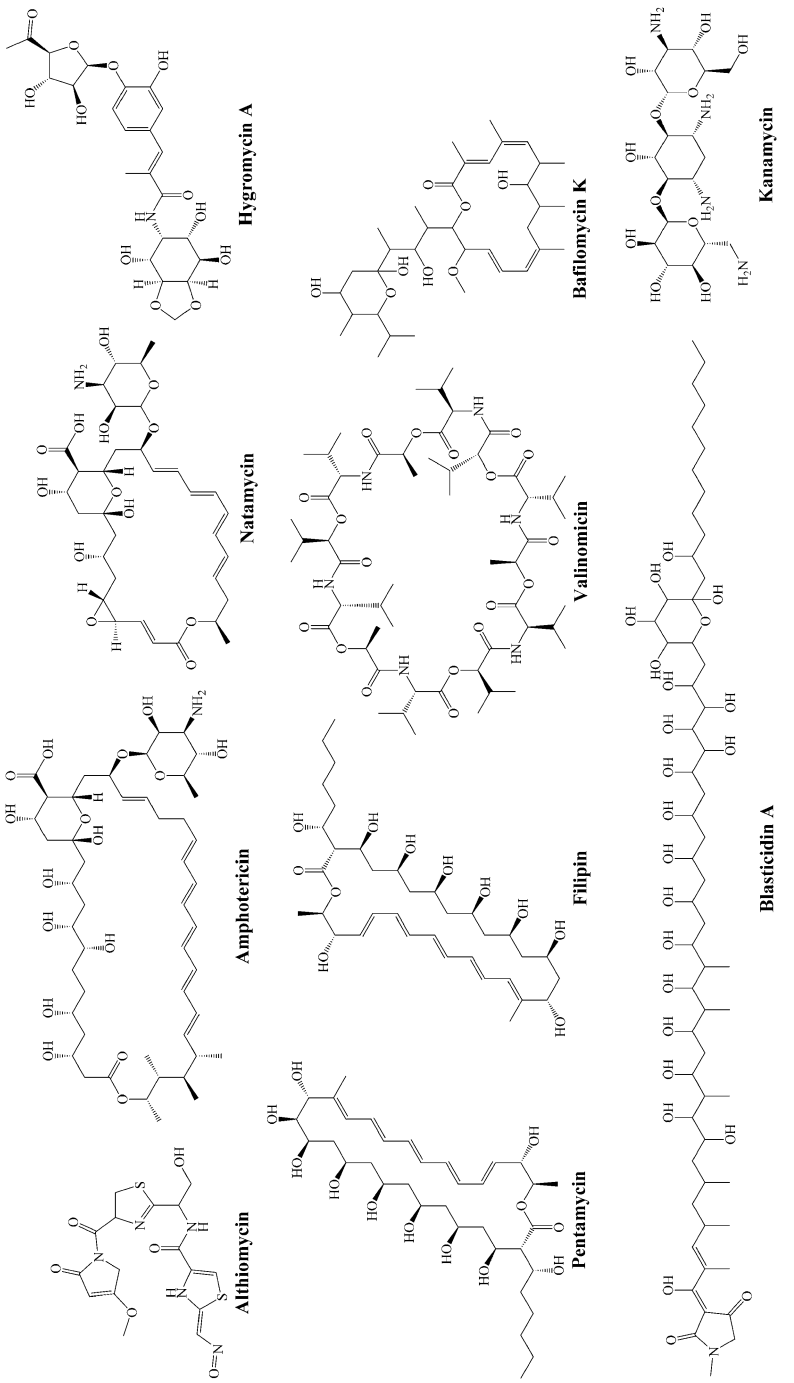

Fig. 13.2 Chemical structures of some of the secondary metabolites produced by the *Streptomyces* spp. in Table 13.2

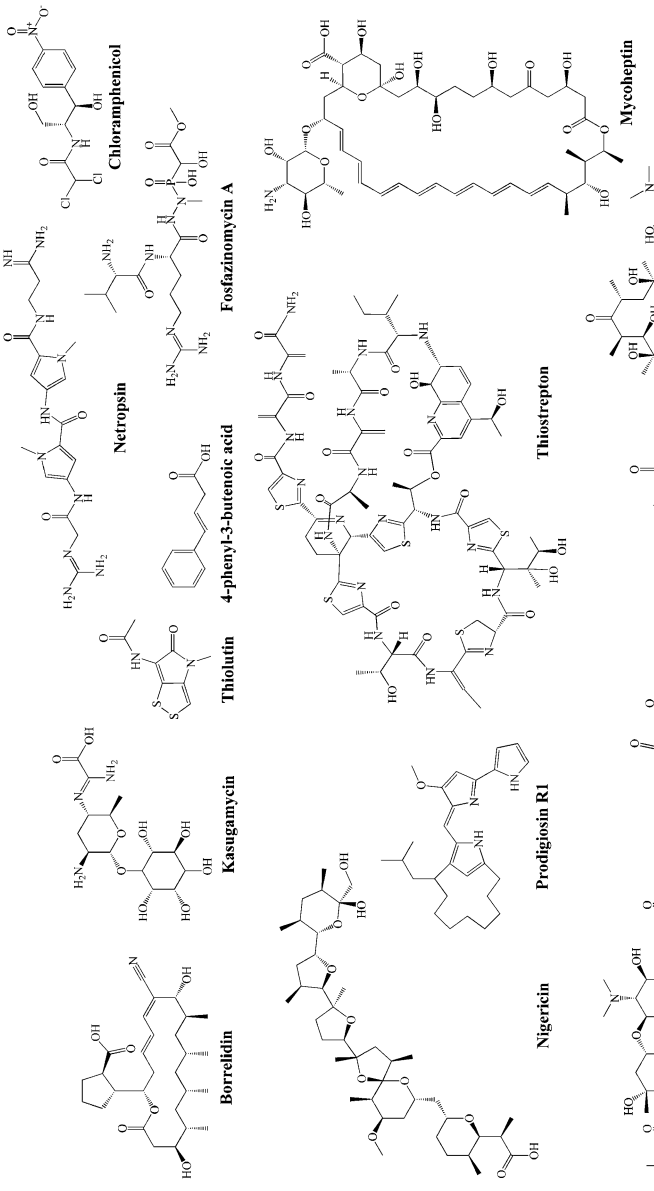

Fig. 13.3 Chemical structures of some of the secondary metabolites produced by the *Streptomyces* spp. in Table 13.2 (continued)

Table 13.3 Functions of different *Streptomyces* spp. against plant pathogens

Streptomyces spp.	Sample	Plant	Pathogen		Disease	Functions	Reference
S. humidis	Sandy soil with debris of *Diplotaxis tenuifolia*	Cucumber	*F. oxysporum* f. sp. *radicis-cucumerinum*		Root rot	Shifts in root microbiome associated with reduction of pathogen root colonization	Klein et al. (2013)
S. roseoflavus strain NKZ-259	Soil samples from the Qilian Mountains (Qinghai province, China)	Tomato and pepper	*Botrytis cinerea* *Fulvia fulva* *Curvularia lunata* *Fusarium oxysporum* *Rhizopus stolonifer* *Ustilaginoidea oryza* *Botryosphaeria ribis* *Bipolaris sorokiniana* *Bipolaris maydis*	*Botryosphaeria dothidea* *Phyllosticta ampelicida* *Valsa ceratosperma* *Colletotrichum gloeosporioides* *Alternaria alternata* *Exserohilum turcicum* *Fusarium graminearum* *Pyricularia oryzae* *Rhizoctonia cerealis*	Seedlings rot	Inhibition of mycelial growth Pathogen outbreaks were blocked after NKZ-259 fermentation broth was applied to infected detached leaves Growth promotion of tomato and pepper seedlings	Shi et al. (2018)
S. albospinus CT205	Cucumber seeds (Jiangsu, China)	Cucumber	*Fusarium oxysporum* NJAU-2 *F. oxysporum* f. sp. *cucumerinum*		Hyphal deformation Death incidence	Inhibition of the development of *F. oxysporum* NJAU-2 mycelium Induced resistance against ampicillin, cefotaxime, and amoxicillin Promoted plant growth Secreted hydrolytic enzymes that led to the leakage and	Wang et al. (2016)

					breakdown of pathogenic fungal cells	
S. pratensis LMM15	Leaves of tomatoes (Weinan, Shaanxi province, China)	Tomato	*Botrytis cinerea*	Gray mold	Inhibited hyphal growth Reduced decay incidence of gray mold on tomato fruits.	Lian et al. (2017)
S. vinaceusdrappus S5 MW2	Samples of tomato seeds	Tomato (var. Navratan)	*R. solani*	Root rot Gray mold	Inhibition of *R. solani* growth up to 65% With chitin enhanced PGP parameters	Yandigeri et al. (2015)

(Hwang et al. 2001). Nonetheless, there is still substantial interest in finding novel strains with the ability to produce safer and more effective antifungal agents.

Zhao et al. (2012) evaluated *Streptomyces* spp. for the control of *Didymella bryoniae* (responsible for gummy stem blight) and to promote growth of *Cucumis melo* L. (muskmelon). *S. hygroscopicus* (strain B04), isolated by Shen et al. (2016) from the rhizosphere soil of a healthy strawberry plant, was found to strongly inhibit the growth of phytopathogenic fungi such as *Fusarium oxysporum*. This strain can utilize many carbon sources and can produce extracellular fungal cell wall-degrading enzymes (e.g., β-1,3-glucanase, chitinase, protease, and cellulase). In a similar fashion, *S. albospinus* CT205 can inhibit the growth of *F. oxysporum* NJAU-2 (responsible for Fusarium wilt of cucumber, which leads to serious economic losses), effectively reducing the presence of this pathogen in the rhizosphere of cucumber. The biological characteristics of CT205 were measured by Wang et al. (2016), and this strain was shown to produce β-glucanase, chitinase, and a heat-resistant antagonistic substance.

In 2017, Lian et al. (2017) showed that the strain *S. pratensis* LMM15 has the ability to inhibit *Botrytis cinerea* mycelial growth and to reduce lesion expansion associated with gray mold on detached fruits and leaves. Shi et al. (2018) isolated from soil samples from Qinghai (China) a strain named NKZ-259, identified as *S. roseoflavus*, which displays high antagonistic activity against 6 fungal pathogens: *Botrytis cinerea, Curvularia lunata, Colletotrichum gloeosporioides, Fulvia fulva, Rhizoctonia cerealis*, and *Ustilaginoidea oryzae*. The use of its fermentation broth reduced tomato gray mold incidence by 66.67%, and promoted the growth of pepper and tomato seedlings, resulting in a significant increase in fresh weight, plant height, and root length. Indoleacetic acid (IAA) phytohormone production was involved.

Lytic enzymes such as chitinase and lipase that bacteria from the *Streptomyces* genus produce (Santos et al. 2016) allowed their use as biocontrol agents against nematodes (*Scutellonema bradys* in yam plants). The production of chitinase enzymes caused the destruction of the nematode cuticle, in which chitin is an important constituent (Park et al. 2002). Moreover, substances that are toxic for the phytonematodes (e.g., ammonia) were released in the decomposition process of chitin.

Finally, an important aspect to take into consideration is that antagonistic strains need to be combined with an appropriate substrate in order to improve their biological control efficacy against soil-borne diseases. Solid-state fermentation of agro-industrial residues with *Streptomyces* spp. to produce bioorganic fertilizers is currently a promising strategy for the management of agro-industrial waste. As noted above (see Shen et al. (2016)), the fermentation equipment involved is simple, economical, and suitable for multiple applications in production. In their work, solid shallow-tray fermentation of various combinations of pig manure compost, vermicompost, wheat bran, and rapeseed meal were inoculated with *S. hygroscopicus* strain B04, and the resulting bioorganic fertilizer showed a remarkable activity against fungal pathogens.

13.6 *Streptomyces* spp. in the Formation of Biofilms

Streptomyces have the capacity to form biofilms (Kim et al. 2019), which are defined as "*a bacterial community immersed in a liquid medium, with the ability to adhere to a substrate or surface and interfaces, which are embedded in an extracellular matrix produced by themselves, and which show an altered phenotype in terms of the degree of cell multiplication or the expression of their genes*" (Donlan and Costerton 2002).

In most biofilms formation, unicellular organisms come together, forming a community attached to a solid surface and covered in an exopolysaccharide matrix (Satpathy et al. 2016). The biofilm formation process consists of 5 stages: (1) preconditioning of the adhesion surface by macromolecules, either present in the bulk fluid or intentionally coated on the surface (Simões et al. 2010); (2) reversible cellular adhesion, in which attachment occurs most effortlessly on rougher and more hydrophobic surfaces, and on surfaces coated by conditioning films; (3) clonal expansion of the bacteria takes place and the production of exopolysaccharides begins, which together with proteins and nucleic acids form the matrix of the biofilm during the irreversible adhesion stage (Reisner et al. 2003); (4) maturation, in which biofilms develop into an organized structure that can be flat or mushroom-shaped (Chmielewski and Frank 2003; Klausen et al. 2003); and (5) dispersion, when the environmental conditions are unfavorable and the cells return to their planktonic form (Sauer et al. 2002).

The formation of microbial biofilm is a very complex process (Shi and Zhu 2009), which depends on the joint interaction of factors such as the environment, surface characteristics, and characteristics of the cell itself. To the best of the authors' knowledge, only a limited number of studies on biofilms of streptomycetes have been reported in the literature (de Jong et al. 2009; Khiyami et al. 2005; Kim and Kim 2004; Morales et al. 2007; Winn et al. 2014). From a biotechnology point of view, the most important were *S. setonii* 75Vi2 (ATCC 39116), which degraded microbial inhibitors in diluted corn stover and starch pyrolysis liquors (Khiyami et al. 2005), and *S. griseus*, which was cultivated as a biofilm in a tubular reactor (Winn et al. 2014).

Compost is a medium capable of providing electrochemically active biofilms for the oxidation of organic compounds and the transfer of electrons (Dulon et al. 2006). Parot et al. (2008) investigated the effect of adding acetate to garden compost to promote the development of electrochemically efficient biofilms.

In agricultural soils, the stage prior to the invasion of plant tissues occurs on the surface of roots, leaves, or seeds, through the establishment of sessile populations of phytopathogenic bacteria (Monier and Lindow 2003). The development of biofilms contributes to phytopathogenic bacteria's virulence by way of the blockage of xylem vessels (Mansfield et al. 2012). On the other hand, biofilms can participate in the biological control processes of pathogens through mutualistic relationships between rhizobacteria and plants (Chin-A-Woeng et al. 2000; Espinosa-Urgel et al. 2002). The self-aggregation characteristic of bacteria has implications for the production of inoculants for agriculture (Bogino et al. 2013) and the protection effects of biofilms would reduce the toxicity of the compounds to the cells (Burmølle et al. 2014). In

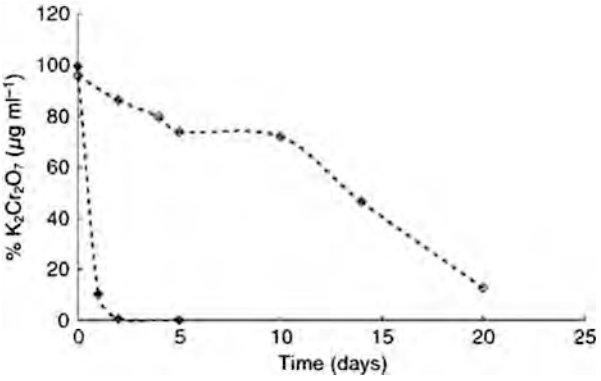

Fig. 13.4 Cr(VI) removal assay in M63 medium. Dashed lines indicate removal by cells forming biofilms on glass beads (filled diamond) or by control planktonic cells (open diamond). Reproduced with permission from Morales et al. (2007). Copyright John Wiley & Sons

particular, the biofilms formed by *Streptomyces* are capable of eliminating $K_2Cr_2O_7$ from contaminated soils. In Fig. 13.4 it can be seen that the biofilms of the strain *Streptomyces* sp. CG252 completely eliminated $K_2Cr_2O_7$ in 3 days, while the planktonic form of this same strain was only able to eliminate 80–90% and needed more than double the time. Therefore, this strain of *Streptomyces* can be a promising candidate for detoxification of sites containing this heavy metal (Morales et al. 2007).

13.7 Conclusions

Compost is a self-heated substrate formed when organic materials are broken down and recycled by successive groups of microorganisms in various composting stages. Several studies have shown that the compost microbial population is highly dominated by actinomycetes and that their presence can potentially serve as an indicator of compost maturity, provided that they participate in suppressing pathogens in the curing stage. Among the microbial biomass that colonizes composts, the versatile *Streptomyces* species produce various lytic enzymes that can break down cellulose and other insoluble organic polymers and produce nutrients that can be used by plants. Because of their capability to form spores and subsist to adverse conditions in the soil, they are also more competitive than other microbes. *Streptomyces* are active producers of antibiotics and compost colonized by these microorganisms contains strains that have plant growth-promoting abilities and that can be used as biofertilizers. Various *Streptomyces* spp. are antagonists of plant pathogens and can thus save the plant from attacks by dangerous fungi and bacteria. As such, they can be regarded as biocontrol agents in several cropping systems.The identification of efficient *Streptomyces* spp. is necessary to stimulate further research on their utility in converting agricultural wastes to organic manure for soil amendment and to attain higher crop productivity.

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ARTÍCULOS SOBRE ENFERMEDADES DE LA MADERA DE LA VID

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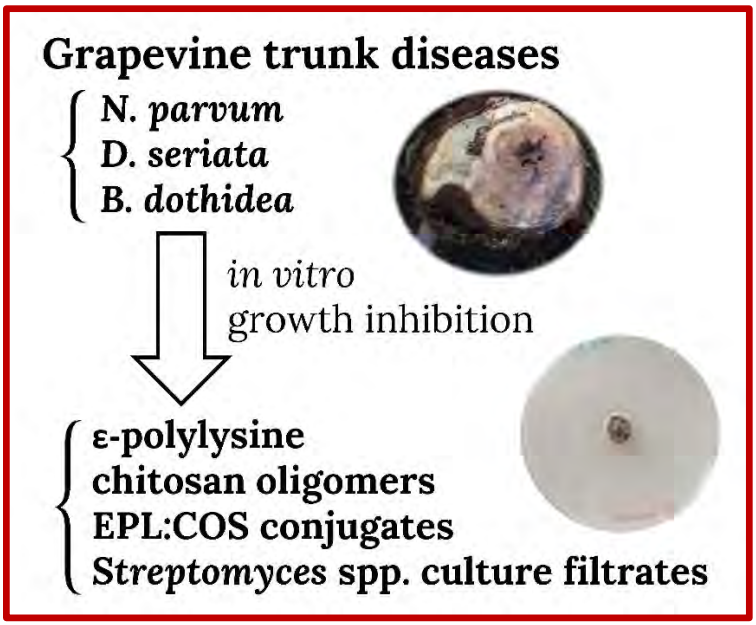

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Abstract: Grapevine trunk diseases (GTDs) are a major threat to the wine and grape industry. The aim of the study was to investigate the antifungal activity against *Neofusicoccum parvum*, *Diplodia seriata*, and *Botryosphaeria dothidea* of ε-polylysine, chitosan oligomers, their conjugates, *Streptomyces rochei* and *S. lavendofoliae* culture filtrates, and their binary mixtures with chitosan oligomers. In vitro mycelial growth inhibition tests suggest that the efficacy of these treatments, in particular those based on ε-polylysine and ε-polylysine:chitosan oligomers 1:1 *w/w* conjugate, against the three Botryosphaeriaceae species would be comparable to or higher than that of conventional synthetic fungicides. In the case of ε-polylysine, EC_{90} values as low as 227, 26.9, and 22.5 $\mu g \cdot mL^{-1}$ were obtained for *N. parvum*, *D. seriata*, and *B. dothidea*, respectively. Although the efficacy of the conjugate was slightly lower, with EC_{90} values of 507.5, 580.2, and 497.4 $\mu g \cdot mL^{-1}$, respectively, it may represent a more cost-effective option to the utilization of pure ε-polylysine. The proposed treatments may offer a viable and sustainable alternative for controlling GTDs.

Keywords: *Botryosphaeria dothidea*; conjugate complexes; *Diplodia seriata*; grapevine trunk diseases; *Neofusicoccum parvum*

1. Introduction

Grapevine trunk diseases (GTDs) have been reported in most grapevine producing regions worldwide, causing a serious decline and loss of productivity. These diseases include black dead arm, caused by *Botryosphaeria dothidea*; esca, which includes vascular symptoms and internal white rot in the trunk; eutypiosis, caused by *Eutypa lata*; Petri disease; black foot; and Phomopsis dieback, being the esca complex the most frequent and increasing syndrome in almost all European countries [1]. A recent International Organization of Vine and Wine (OIV) publication reported that incidence of GTDs was 10% in Spain, 13% in France, and between 8% and 19% in Italy, and that the losses in California were at least 260 M$ per year [2].

A thorough and up-to-date panorama of the state-of-the-art of chemicals (including synthetic organic compounds, inorganic compounds, natural compounds, and plant-defense stimulating compounds) and biocontrol agents that have been tested towards GTDs can be found in the recent review paper by Mondello et al. [2].

Unfortunately, chemical fungicides traditionally used to control aforementioned fungal crop infections, such as sodium arsenite, carbendazim, or tecobunazole, have several drawbacks in terms of toxicity and efficacy, and, in recent years, public pressure to reduce their use has increased. In fact, concerns have been raised about both their environmental impact and the potential associated health risks. In this context, the use of natural antifungals as a feasible alternative is receiving increasing attention.

Among the tested natural compounds, Nascimento et al. [3] reported the antifungal effect of chitosan on several fungal species involved in grapevine decline. Greenhouse experiments using foliar sprays of chitosan on potted grapevine plants growing in a substrate artificially infected with *Phaeomoniella chlamydospora* or *Ilyonectria liriodendri* demonstrated that chitosan significantly improved plant growth and decreased disease incidence. More recently, Cobos et al. [4] reported that chitosan oligosaccharides, garlic extract, and vanillin were able to significantly reduce infection in pruning wounds by *Diplodia seriata*. Galarneau et al. [5] also examined the potential role of antimicrobial phenolic compounds on *Neofusicoccum parvum* and *D. seriata*, two causal fungi of Botryospheria dieback.

ε-polylysine (EPL), a natural antimicrobial produced from aerobic bacterial fermentation by *Streptomyces albulus*, widely used in Japan and USA as an antimicrobial agent in food products, could also be a promising antifungal agent [6]. Although it has been reported to have a strong activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* [7], either alone or in chitosan conjugate compounds, its efficacy has not been assayed against GTDs.

In a similar fashion, even though beneficial bacteria inhabiting the rhizosphere and/or the endosphere of plants and their secondary metabolites have been put forward by some authors to reduce grapevine pathogen diseases [6], information reported in the literature is limited [8–12]. These biocontrol agents, such as *Streptomyces* spp., would affect pathogen performance by antibiosis, competition for niches and nutrients, interference with pathogen signaling, or by stimulation of host plant defenses.

The aim of the study presented herein has been to assess the in vitro antifungal activity of EPL, EPL:chitosan oligomers (EPL:COS) conjugates, and secondary metabolites from two beneficial actinobacteria (*Streptomyces rochei* and *S. lavendofoliae*) to control *N. parvum*, *D. seriata*, and *B. dothidea*, three of the most frequently isolated fungal pathogens in GTDs.

2. Results

2.1. Vibrational Analysis of the ε-polylysine: Chitosan Conjugates

The vibrational spectra of conjugates prepared with six different EPL:COS mass ratios were examined in order to confirm their secondary structure and to determine the most suitable proportion (Figure 1).

The absorption bands at 1150 cm^{-1} and 1018 cm^{-1} were assigned to asymmetric stretching of the C−O−C bridge and to the skeletal vibration of C−O stretching, respectively [13–15]. The absorption band at 895 cm^{-1} could be assigned to the β-D-configuration. There was a shift of amide/amino bands in the reaction products, indicating the progress of Maillard reaction: the absorption peaks at 1659 cm^{-1} and 1597 cm^{-1} (associated with amino groups characteristic of chitosan oligomers) disappeared, and new bands at 1665 cm^{-1} and 1565 cm^{-1} were observed. The appearance of these bands suggest that a Schiff base (C=N bond) was formed between the reducing end of chitosan and the amino groups [16]. Thus, the Fourier-Transform Infrared (FTIR) results showed that ε-polylysine had actually attached to chitosan.

An interesting feature was that the absorbance of the bands associated with Schiff base formation were stronger in the 1:1 EPL:COS conjugate than in the spectra of conjugates prepared with other EPL:COS ratios. Thus, the Schiff base for the 1:1 conjugate seems to feature the desired balance of components to undergo the Amadori rearrangement with formation of ketosamines, but avoiding their subsequent decomposition observed in more COS-rich conjugates. This result was in good agreement with the findings of Liang et al. [7] for EPL:chitosan, who concluded that the conjugate with EPL and chitosan ratio of 1:1 exhibited the strongest antibacterial and antifungal activity. Consequently, the 1:1 EPL:COS conjugate was chosen for the mycelial growth inhibition tests in this study.

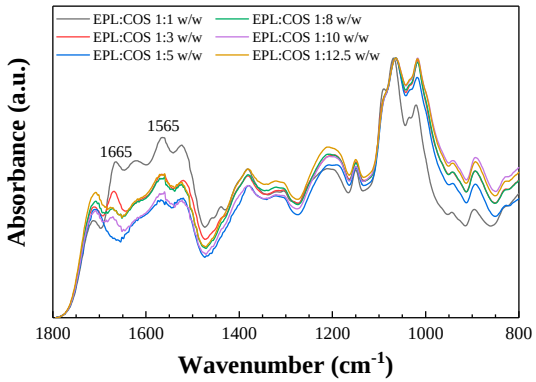

Figure 1. Comparison of the attenuated total reflection (ATR)-Fourier-Transform Infrared (FTIR) spectra of ε-polylysine:chitosan oligomers conjugates prepared with different ε-polylysine:chitosan oligomers mass ratios. Only the fingerprint region is shown.

2.2. Mycelial Growth Inhibition Tests

The in vitro radial growth inhibition attained by each of the treatments against *N. parvum* is depicted in Figure 2, showing only for one replicate per treatment and dose. Those attained against *D. seriata* and *B. dothidea* are depicted in Figures S1 and S2, respectively. The values across the three replicates for the three Botryosphaeriaceae species are summarized in Figure 3.

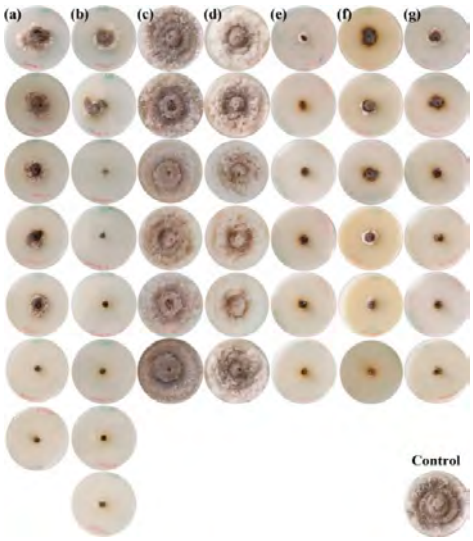

Figure 2. *N. parvum* mycelial growth inhibition assays for: (**a**) chitosan oligomers; (**b**) ε-polylysine; (**c**) *S. rochei* secondary metabolites; (**d**) *S. lavendofoliae* secondary metabolites; (**e**) ε-polylysine:chitosan (1:1 *w/w*) conjugates; (**f**) *S. rochei* secondary metabolites + chitosan oligomers (1:1 *w/w*); and (**g**) *S. lavendofoliae* secondary metabolites + chitosan oligomers (1:1 *w/w*). The concentration of the treatments decreases from top to bottom (doses for each treatment are indicated in Table 3). The petri dish in the bottom right corner shows the PDA control. Only one replicate per each treatment and dose is shown.

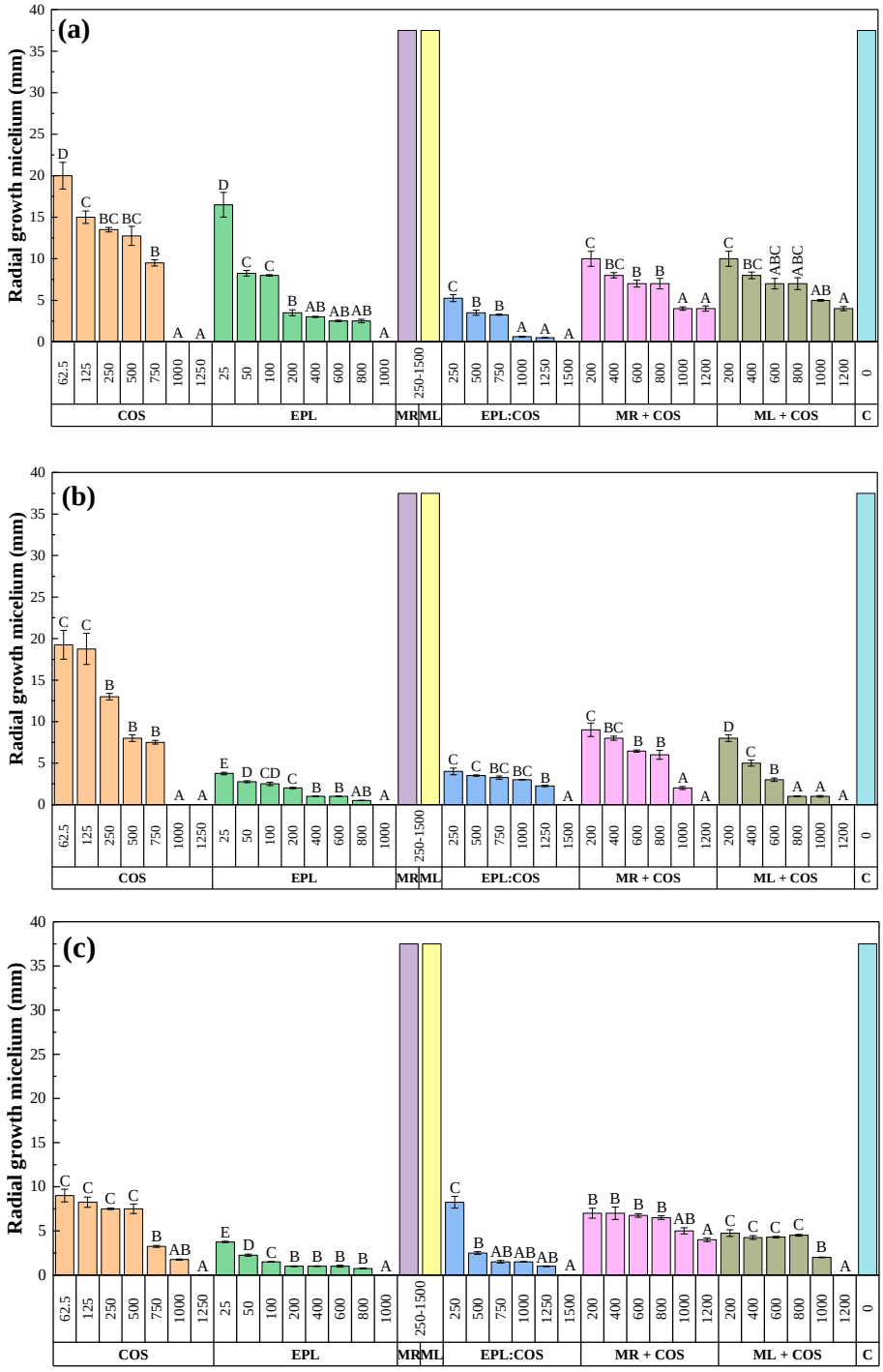

Figure 3. Radial growth values of (**a**) *N. parvum*; (**b**) *D. seriata*; and (**c**) *B. dothidea* in the presence of the different treatments under study at different concentrations (in μg·mL^{-1}). COS, EPL, MR, ML and C stand for chitosan oligomers, ε-polylysine, *S. rochei* secondary metabolites, *S. lavendofoliae* secondary metabolites and control, respectively. For MR and ML only one column is shown, since no inhibition was detected at any concentration in the 250–1500 μg·mL^{-1} range. Concentrations labelled with the same uppercase letters are not significantly different at $p < 0.05$ by Tukey's test. All values are presented as the average of three repetitions. Error bars represent the standard deviation across three replicates.

The increase in the treatment doses resulted in a reduction in the radial growth of the mycelium in all cases, with statistically significant differences amongst the various concentrations (Figure 3),

except for the *S. rochei* and *S. lavendofoliae* secondary metabolites-only based treatments (MR and ML, respectively), for which no inhibition was observed.

Doses in the 1000–1500 $\mu g \cdot mL^{-1}$ range were required to attain full inhibition of the three Botryosphaeriaceae species for the COS, EPL, and EPL:COS conjugate treatments. As regards the activity of MR+COS and ML+COS treatments, differences were observed as a function of the fungal pathogen species. Full inhibition of *D. seriata* was attained for both treatments at a dose of 1200 $\mu g \cdot mL^{-1}$, whereas it was only observed for ML+COS in the case of *B. dothidea*. MR+COS treatment led to 89% inhibition at the same dose for this latter pathogen. In the case of *N. parvum*, the highest doses of MR+COS and ML+COS led to 83% and 89% inhibition, respectively.

The sensitivity tests results may also be expressed in terms of effective concentrations EC_{50} and EC_{90}, that is, the concentrations that reduce mycelial growth by 50% and 90%, respectively (Table 1). Goodness-of-fit analyses revealed good r^2 and low sum of standard errors, showing that parameter fits of sigmoid curves to the dose-response data were significant. In view of the obtained theoretical values, the activity of the treatments—in general terms—would follow the sequence EPL > EPL:COS > ML+COS > COS > MR+COS.

Table 1. Effective concentrations that inhibited mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively).

Pathogen	Concentration ($\mu g \cdot mL^{-1}$)	Treatment				
		COS	**EPL**	**EPL:COS**	**MR + COS**	**ML + COS**
N. parvum	EC_{50}	60.7	16.0	11.2	67.2	46.7
	EC_{90}	1270.0	227.0	507.5	2074.2	1101.7
D. seriata	EC_{50}	94.3	0.3	11.6	45.1	30.7
	EC_{90}	1120.7	26.9	580.2	906.9	498.2
B. dothidea	EC_{50}	1.8	0.4	4.2	15.8	10.7
	EC_{90}	689.5	22.5	497.4	1019.0	490.3

3. Discussion

3.1. Efficacy of the Treatments

In relation to the efficacy of the composites, although the review paper by Mondello et al. [2] provides a qualitative comparison of different treatment against GTDs, specific inhibition rates with their associated concentrations or effective concentrations were not provided. A survey of such values against the three Botryosphaeriaceae species under study is summarized in Table 2 for comparison purposes.

It may be observed that the EC_{50} values for the treatments presented herein (Table 1), in particular those of EPL and EPL:COS conjugate, were comparable to or better than those of popular synthetic organic compounds used to control GTDs, and only slightly lower than the excellent activities reported for AgNPs.

The results presented for COS were in excellent agreement with those reported by Nascimento et al. [3] and Cobos et al. [4]. However, with regard to this latter study, it should be noted that while the use of polyphenols, such as vanillin or those found in garlic extract, may be suitable against *D. seriata* and other Botryosphaeriaceae strains [17], it may not be advisable against *N. parvum*. Galarneau et al. [5] recently found that *N. parvum* was either uninhibited or promoted by phenolic compounds such as gallic acid, epicatechin, rutin, or piceid. In fact, the authors explained that the ability of *N. parvum* to tolerate these phenolics or utilize them as carbon sources would contribute to its greater virulence compared to *D. seriata*.

Table 2. Concentration values and associated inhibition rates, or EC_{50} values, reported in the literature for other active compounds against the three Botryosphaeriaceae species under study.

Fungicide	Fungal Species	Concentration ($\mu g \cdot mL^{-1}$)	Inhibition rate (%)	EC_{50}($\mu g \cdot mL^{-1}$)	Ref.
Tebuconazole	*N. parvum* *D. seriata*			90 150	[18]
Pyraclostrobin	*N. parvum* *D. seriata*			100 250	[18]
Carbendazim, tebuconazole, iprodione, fludioxonil, fluazinam, flusilazole, penconazole, procymidone, myclobutanil, pyraclostrobin	*N. parvum* *D. seriata* *B. dothidea*			360–440 * 530–620 * 450 *	[19]
Carbendazim Tebuconazole Iprodione	*N. parvum*			40 130 750	[20]
Tecobunazole	*D. seriata*			300	[21]
Fe NPs (FeNPs + neem leaf extract)	*D. seriata* *B. dothidea*	100 (FeNPs / FeNPs+neem 1:1)	79/80.3 83/82.5		[22]
AgNPs AgNPs	*N. parvum*	40 30	84 81		[23] [24]
Lemon essential oil (limonene, neral, β-pinene, and γ-terpinene) in DMSO	*B. dothidea*	2500	48.1		[25]
Chitosan oligosaccharin (mol. wt. <3 kDa)	*Botryosphaeria* sp.			1.56	[3]
Chitosan oligosaccharides Vanillin Garlic extract	*D. seriata*	1000 1000 40000	100 89.8 75.3		[4]

* Data pooled across fungicides to provide mean EC_{50} values for isolate sensitivity in the original study.

The *Streptomyces* spp. secondary metabolites-based treatments showed an unexpected lack of activity when used alone. In fact, the percentage of inhibition of radial growth (PIRG) values, shown in Tables S1–S3, were negative, i.e., the growth of the pathogens was promoted. This was not a case of hormetic response, provided that increasing the concentration did not result in inhibition. The observed mycelial growth promotion may be tentatively ascribed to the presence of molasses and yeast extract in the culture filtrates, together with a poor absorption and bioavailability of the active ingredients in the water-based culture filtrates, resulting from their insolubility or very poor solubility in water.

In relation to one of the active compounds present in the culture filtrates under test, lankacidin, Harada et al. [26] stated that lankacidin-group antibiotics are scarcely soluble in water and that the parts that dissolved are rapidly decomposed to compounds with no antimicrobial activity. To overcome this problem, they prepared inclusion compounds with cyclodextrins. In this study, this solubility problem was solved by forming polyelectrolyte complexes (PECs) with a polycationic polymer, i.e., chitosan oligomers. These chitosan-based PECs have been reported to exhibit favorable physicochemical properties and to preserve chitosan's biocompatible characteristics [27], which has made this approach very popular in the drug delivery fields [28,29]. In fact, Zhang et al. [30] previously reported that chitosan behaves as an efficient carrier to deliver streptomycin.

3.2. Mechanism of Action

Concerning the mechanism of action (MOA) of the proposed treatments, although the antimicrobial activity of EPL is well documented, its MOA has only been vaguely described. Hyldgaard et al. [31] hypothesized that EPL destabilizes membranes in a carpet-like mechanism by interacting with negatively charged phospholipid head groups, which displace divalent cations and enforce a negative curvature folding on membranes that leads to formation of vesicles/micelles. According to Ye et al. [32], the antimicrobial mechanism of EPL may be attributed not only to disturbances on membrane integrity, but also to oxidative stress by ROS, and to its effects on various gene expressions.

It is worth noting that the fungicidal activity would likely benefit from the substitution of lysine with arginine residues, provided that previous works have demonstrated the superior cell permeability by

arginine polymers over lysine-containing ones [33,34]. Mechanistic evidences indicate that arginine can enhance the activity of both translocating and membrane permeabilizing peptides [35,36]. This would be a potential direction for future studies.

Regarding the inhibition mode of chitosan oligomers, Ing et al. [37] proposed several MOAs. The interaction of chitosan's positive charge with negatively charged phospholipid components would result in an increased permeability and in leakage of cellular contents. Its chelating action would deprive fungi of trace elements essential for their normal growth. Moreover, its binding to fungal DNA would inhibit mRNA synthesis and affect proteins and enzymes production.

Consequently, the activity of EPL:COS conjugates, as noted by Liang et al. [7], should be referred to an enhanced disruption of their cell membranes, leading to damages of structure, function, and permeability, leakage of intracellular components and the ultimate lysis of the cell.

3.3. Applicability to GTDs in vivo

As regards the applicability of the proposed treatments to GTDs in vivo, although it was not covered in this preliminary study, several systems may be envisaged [38]. To reduce symptoms in the field, once the wood is already infected, an approach to be explored would be to apply the products to the soil (injector pole) or to the trunk (trunk injections), mimicking the mechanism activated by winter spraying of sodium arsenite [39]. However, it would be expensive and time-consuming if applied on a large scale [40], and would only be cost-effective when applied in high-value vineyards [41,42].

The proposed antifungal agents may also be administrated by foliar pulverization with minor changes to the formulations (e.g., adding a surfactant as Tween-80). This would be the most practical approach considering the experience of winegrowers. According to Roblin et al. [43], the compounds sprayed on the leaf blades would be able to migrate to the fungal target in the trunk or to trigger the plant defense reaction in distal parts of the plant. In fact, successful use of foliar sprays of chitosan on grapevine plants artificially infested with *Phaeomoniella chlamydospora* or *Neonectria liriodendri* have been reported in the literature [3]. However, this application method has the major drawback that the treatments may be easily washed off by rainfall [44]. If this approach was to be chosen, sprays after the period of vintage should be useful since, at this period, the phloem sap begins to be directed in a descending flow towards the roots, assuring the transport of the compounds towards the fungi [43].

Alternatively, as a preventive measure, the active ingredients may also be used to protect pruning wounds to avoid grapevine infection and to limit fungal expansion in the plant, either as painted pastes or as liquid formulations. This application method was evaluated against *D. seriata* and *P. chlamydospora* in field trials by Cobos et al. [4], using chitosan oligosaccharides, vanillin, and garlic extract, and resulted in a significant decrease in plant mortality and in the infection rate. Nonetheless, to improve the adherence of the treatments, thickener agents would need to be added to the formulations: e.g., starches, vegetable gums, pectin, or clays such as halloysite.

3.4. Significance of the Reported Findings

Although follow-up studies involving in vivo assays and field tests would be necessary to draw firm conclusions on the effectiveness of the application of the proposed treatments, the fact that they reached higher mycelial growth inhibition than that of commercial fungicides makes them promising candidates for the effective control of botryosphaeriaceous diseases.

It is also worth noting that the three fungal species tested in the present study are not only pathogens of grapevine, but also of other commercially important woody plants. For instance, *D. seriata* and *B. dothidea* are phytopathogens of apple [22], *N. parvum* causes dieback in avocado [45], *B. dothidea* causes branch dieback of olive [46], and the three of them are associated with branch cankers on almond trees [47]. Consequently, the results of this study may also find application in other pathosystems, resulting in an even higher ecological and economic impact.

4. Materials and Methods

4.1. Reagents, Bacteria and Fungi

High molecular weight chitosan (CAS 9012-76-4; 310000-375000 Da) was purchased from Hangzhou Simit Chemical Technology Co., Ltd. (Hangzhou, China). ε-polylysine (CAS 25104-18-1), phosphate buffer (for microbiology, APHA, pH 7.2), ethyl acetate (CAS 141-78-6; ≥99.5%), and citric acid (CAS 77-92-9; ≥99.5%) were supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Neutrase® 0.8L enzyme was supplied by Novozymes (Bagsvaerd, Denmark). Potato dextrose agar (PDA), yeast extract, and Bacto™ Peptone were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Starch casein agar (SCA), Mueller Hinton agar, and malt extract agar (MEA) came from Oxoid Ltd. (Hampshire, UK). Molasses were supplied by ACOR, Sociedad Cooperativa General Agropecuaria (Castilla y León, España).

The three fungal isolates under study, viz. *Diplodia seriata* (ITACYL_F079), *Neofusicoccum parvum* (ITACYL_F111), and *Botryosphaeria dothidea* (ITACYL_F141), were supplied by ITACYL, Instituto Tecnológico Agrario de Castilla y León (Castilla y León, España).

The two *Streptomyces* spp. strains from which secondary metabolites were produced, *Streptomyces lavendofoliae* (DSM 40217) and *Streptomyces rochei* (DSM 41729) were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig, Germany).

4.2. Equipment

A probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) was used for solutions sonication.

To incubate the flasks, controlling the temperature and the stirring speed, an ECOLAN 60 (Labolan; Esparza de Galar, Navarra, Spain) orbital stirrer incubator was used.

Functional groups were identified by Fourier-Transform Infrared spectroscopy with a Nicolet iS50 (Thermo Scientific, Waltham, MA, USA) apparatus equipped with a diamond attenuated total reflection (ATR) module. The spectra were collected in the 400–4000 cm^{-1} region with a 1 cm^{-1} spectral resolution; 64 scans were co-added and the resulting interferogram was averaged. The ATR-FTIR spectra were corrected using the advanced ATR correction algorithm [47] available in OMNIC™ software suite.

4.3. Preparation of Chitosan Oligomers

Chitosan oligomers were obtained according to the enzymatic procedure described by Santos-Moriano et al. [48], with slight modifications. 20 g of high molecular weight chitosan were dissolved in 1000 mL of Milli-Q water by adding citric acid under constant stirring at 60 °C. Once dissolved, Neutrase® 0.8 L (1.67 $g{\cdot}L^{-1}$) was added in order to degrade the polymer chains. The mixture was sonicated for 3 min in cycles of 1 min with sonication and 1 min without sonication to keep the temperature in the 30–60 °C range [14]. At the end of the process, a solution with a pH in the four to six interval with oligomers of molecular weight < 2000 Da was obtained.

4.4. ε-polylysine Treatment

For the preparation of the ε-polylysine treatment, 2 g of EPL were dissolved in 1000 mL of Milli-Q water. The mixture was sonicated for 3 min in cycles of 1 min with sonication and 1 min without sonication so that the temperature remained in the 30–60 °C range.

4.5. Synthesis of ε-polylysine: Chitosan Oligomers Conjugates

Conjugated complexes of ε-polylysine and chitosan oligomers were prepared at different mass ratios, namely 1:1, 1:3, 1:5 1:8, 1:10, and 1:12.5 *w/w*, respectively. The appropriate amounts of each component were dissolved in Milli-Q water using sonication (5 cycles of 5 min/cycle, taking care

not to exceed 60 °C). The resulting solutions were lyophilized, and then heated at 60 °C under 60% relative humidity for 24 h. This synthesis procedure was analogous to other procedures described in the literature for the preparation of EPL:COS conjugates through Maillard reaction [7,49,50]. Only the conjugate with the highest expected activity was assayed in the mycelial growth inhibition tests.

4.6. Secondary Metabolites Production from Streptomyces spp. Strains

Two strains of the genus *Streptomyces*, viz. *Streptomyces lavendofoliae* DSM 40217 and *Streptomyces rochei* DSM 41729 were seeded on starch casein agar medium plates at 28 °C for 10 days. The plates were stored at 4 °C. For long-term storage, lyophilizates from both strains were used.

In order to obtain the secondary metabolites, the method described by Sadigh-Eteghad et al. [51] was followed. Once the fermentation was complete, each final solution of the cultures of both strains was treated with 50 mL of phosphate buffer (pH 6.4) and was sonicated for 5 min. The solutions were then filtered through sterile muslin cloth twice. These solutions (culture filtrates) were used for the mycelial growth inhibition tests.

In order to determine the concentration of bioactive compounds in aforementioned solutions (and the doses used in the inhibition tests), the filtrates were centrifuged, and the supernatant was extracted with 100 mL of ethyl acetate. The solvent with the crude bioactive compounds was concentrated under reduced pressure and then lyophilized. The culture filtrates had a concentration of approx. 2000 $\mu g \cdot mL^{-1}$ (1958 $\mu g \cdot mL^{-1}$ for *S. lavendofoliae* secondary metabolites and 1877 $\mu g \cdot mL^{-1}$ for *S. rochei* secondary metabolites), in agreement with Pazhanimurugan et al. [52]. The bioactive compounds in the secondary metabolites of *S. lavendofoliae* and *S. rochei* are summarized in Table S4.

4.7. Synthesis of Chitosan Oligomers-secondary Metabolites Inclusion Compounds

Secondary metabolites, either from *S. lavendofoliae* or from *S. rochei*, and chitosan oligomers mixtures were prepared by mixing in 1:1 (*w*/*w*) ratio of their respective solutions (2000 $\mu g \cdot mL^{-1}$ of bioactive compounds + 2000 $\mu g \cdot mL^{-1}$ COS), followed by sonication. The resulting solutions (ML+COS and MR+COS) were assayed at different concentrations in the inhibition tests.

4.8. In vitro Mycelial Growth Inhibition Tests

The biological activity of the treatments under study was determined by the agar dilution method: aliquots of the original solutions of the various treatments, obtained by dilution of the respective "mother" solutions, were incorporated into the PDA medium to obtain the final concentrations indicated in Table 3. It should be clarified that the tested concentrations were not the same all treatments due to difficulties associated with the estimation of the molecular weights of the polymeric reagents from their viscosities. Petri dishes containing only PDA culture medium (20 mL) were used as the control.

The mycelial discs of pathogen (5 mm in diameter) were removed from the margins of 7-day-old PDA cultures and transferred to the petri dishes (in triplicate). Plates were incubated at 25 °C. The measurements of fungal growth for *D. seriata* and *N. parvum* were taken two, four and five days after inoculation. In contrast, for *B. dothidea*, measurements were carried out two, four and six days after inoculation, provided that mycelial growth was slower for this later fungus in the control plates.

The inhibition of mycelial growth, or the efficacy of the compound analyzed, for each treatment and concentration, was calculated by the formula:

$$\text{Percentage inhibition of radial mycelium growth (\%)} = ((R_1 - R_2)/R_1) \times 100 \quad (1)$$

where R_1 and R_2 correspond to the average radial growth of the fungal mycelium in the control medium (pure PDA) and in the fungicide-amended medium, respectively.

The results were also expressed as the effective concentrations that reduced mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively), which were determined by the regression of the radial growth inhibition values (%) against the $\log_{10}$ values of the concentrations of antifungal compounds

using PROBIT in IBM SPSS Statistics v.25 software. This regression procedure fits the dose-response curve to a sigmoid and calculates the values, with 95% CI, of the dose variable that correspond to a series of probabilities.

Table 3. Concentrations assayed for each of the treatments in the mycelial growth inhibition tests. COS, PL, MR and ML stand for chitosan oligomers, ε-polylysine, *S. rochei* secondary metabolites, and *S. lavendofoliae* secondary metabolites, respectively.

Treatment	Concentrations Assayed in the Mycelial Growth Inhibition Tests (μg·mL^{-1})
COS	62.5, 125, 250, 500, 750, 1000, 1250, 1500
EPL	25, 50, 100, 200, 400, 600, 800, 1000
MR	250, 500, 750, 1000, 1250, 1500
ML	250, 500, 750, 1000, 1250, 1500
EPL:COS	250, 500, 750, 1000, 1250, 1500
MR+COS	200, 400, 600, 800, 1000, 1200
ML+COS	200, 400, 600, 800, 1000, 1200

4.9. Statistical Analyses

Data were subjected to analysis of variance (ANOVA) in IBM SPSS Statistics v.25 software. Tukey's HSD test at 0.05 probability level ($p < 0.05$) was used for the *post hoc* comparison of means.

5. Conclusions

The efficacy of ε-polylysine, chitosan oligomers, ε-polylysine:chitosan oligomers conjugates, two *Streptomyces* spp. secondary metabolites, and the combinations of the latter two with chitosan oligomers were examined in vitro against *N. parvum*, *D. seriata* and *B. dothidea*. On the basis of vibrational spectroscopy data, a 1:1 *w/w* mass ratio was chosen for the EPL:COS conjugate, for which an optimum Schiff base was formed. From the mycelial growth inhibition tests it was found that, in spite of the remarkable contents in bioactive compounds in the culture filtrates, the secondary metabolites of *S. rochei* and *S. lavendofoliae* did not inhibit any of the GTD-related fungi, probably due to hydrophobicity reasons. In contrast, upon formation of polyelectrolyte complexes with chitosan oligomers, inhibitions above 80% were attained. In view of the calculated effective concentration values, the antifungal activity of the treatments would follow the sequence EPL > EPL:COS > ML+COS > COS > MR+COS. EC_{50} values below 100 μg·mL^{-1} were obtained for all the assayed treatments, suggesting that they could be a viable alternative to conventional synthetic fungicides. In particular, ε-polylysine and ε-polylysine:chitosan oligomers may be put forward as the most promising options, due to the high efficacy of the former and the trade-off between efficacy and cost associated with the latter. In the current context in which the use of synthetic chemical pesticides is more and more restricted, this work constitutes a necessary step for developing efficient treatments that take into account the importance of environmental protection within the scope of sustainable development.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6382/8/3/99/s1, Figure S1: *D. seriata* mycelial growth inhibition assays, Figure S2: *B. dothidea* mycelial growth inhibition assays, Table S1: Radial growth of mycelium (RG) and percentage of inhibition of radial growth (PIRG) of the different treatments against *N. parvum* two, four and five days after inoculation, Table S2: Radial growth of mycelium (RG) and percentage of inhibition of radial growth (PIRG) of the different treatments against *D. seriata* two, four and five days after inoculation, Table S3: Radial growth of mycelium (RG) and percentage of inhibition of radial growth (PIRG) of the different treatments against *B. dothidea* two, four and six days after inoculation, Table S4: Bioactive secondary metabolites produced by *S. lavendofoliae* and *S. rochei*.

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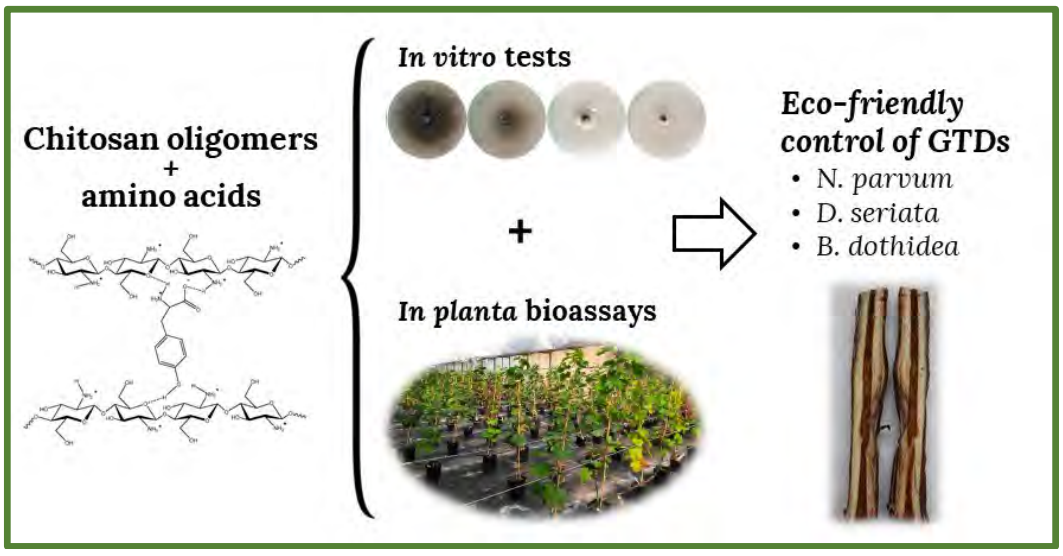

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On the Applicability of Chitosan Oligomers-Amino Acid Conjugate Complexes as Eco-Friendly Fungicides against Grapevine Trunk Pathogens

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Abstract: In a context in which the incidence and severity of grapevine fungal diseases is increasing as a result of both climate change and modern management culture practices, reducing the excessive use of phytosanitary products in viticulture represents a major challenge. Specifically, grapevine trunk diseases (GTDs), caused by several complexes of wood decay or xylem-inhabiting fungi, pose a major challenge to vineyard sustainability. In this study, the efficacy of chitosan oligomers (COS)–amino acid conjugate complexes against three fungal species belonging to the *Botryosphaeriaceae* family (*Neofusicoccum parvum*, *Diplodia seriata*, and *Botryosphaeria dothidea*) was investigated both in vitro and in planta. In vitro tests led to EC_{50} and EC_{90} effective concentrations in the 254.6−448.5 and 672.1−1498.5 $\mu g \cdot mL^{-1}$ range, respectively, depending on the amino acid involved in the conjugate complex (viz. cysteine, glycine, proline or tyrosine) and on the pathogen assayed. A synergistic effect between COS and the amino acids was observed against *D. seriata* and *B. dothidea* (synergy factors of up to 2.5 and 2.8, respectively, according to Wadley's method). The formulations based on COS and on the conjugate complex that showed the best inhibition rates, COS−tyrosine, were further investigated in a greenhouse trial on grafted vines of two varieties ("Tempranillo" on 775P and "Garnacha" on 110R rootstock), artificially inoculated with the mentioned three *Botryosphaeriaceae* species. The in planta bioassay revealed that the chosen formulations induced a significant decrease in disease severity against *N. parvum* and *B. dothidea*. In summary, the reported conjugate complexes may be promising enough to be worthy of additional examination in larger field trials.

Keywords: *Botryosphaeriaceae*; chitosan; fungicide; GTD; IPM; tyrosine; *Vitis vinifera*

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1. Introduction

The so-called grapevine trunk diseases (GTDs) represent one of the greatest threats to vineyards in the last 20–25 years, as a consequence of changes in the management and intensification of the crop, the increase in the production of propagating plant material, the banning of chemicals or the existence of a climate change scenario. The International Organization of Vine and Wine (OIV) has estimated that the incidence rate of GTDs is approximately 10, 13 and 13.5% of Spanish, French and Italian vineyards, respectively [1]. At a global level, economic losses caused by GTDs exceed US$1.5 billion/year [2].

Among these, ascomycetous taxa belonging to the family *Botryosphaeriaceae* are responsible for large losses due to their incidence, especially in young grapevine plants coming

from nurseries. Pathogenicity studies have shown that grapevine-associated species belonging to the genera *Botryosphaeria*, *Lasiodiplodia* and *Neofusicoccum* are among the fastest colonizing wood fungi and are therefore considered the most virulent cause of wood diseases [3]. External symptoms produced by this pathogenic complex include death of the cordons, canes, shoots and buds, stunting, bud necrosis, bleached canes, reduced bunch set and bunch rots, while internal symptoms like brown wood streaking and wedge-shaped discolorations are very frequent [4,5]. Together with these disease symptoms, these and other related GTD fungi are known to produce toxic metabolites [6], some of them well characterized by chemical methods, whose toxicity has been proven on different organs and tissues of several *Vitis vinifera* L. cultivars [7].

A comprehensive overview of the current state-of-the-art concerning chemicals (including inorganic, synthetic organic, natural, and elicitor compounds), biocontrol agents (BCAs) or preventive and post-infection management practices that have been examined against GTDs may be found in the review papers by Vincenzo, et al. [8], Gramaje, Urbez-Torres and Sosnowski [3] and Mondello, Songy, Battiston, Pinto, Coppin, Trotel-Aziz, Clement, Mugnai and Fontaine [1]. However, it is necessary to clarify that at present there are no last-generation chemical methods or alternative treatments with proven efficacy [9], which explains why preventive cultural measures are generally used [10].

In order to comply with the European legislation currently in force (Article 14 in European Directive 2009/128/EC), the implementation of integrated pest management (IPM) methods has become a priority objective in plant disease control worldwide. The efforts oriented towards the selection and/or development of rootstocks and varieties with certain levels of tolerance against different trunk mycoses have not been successful to date [11–16], and the use of strategies involving endophytic microorganisms as microbial antagonists (BCAs) obtains a certain degree of protection, but no single BCA application has been able to control GTDs at similar rates to those shown by chemical fungicides, which are now banned [17]. Hence, other alternative/complementary strategies have to be explored and improved, such as the application of substances of natural origin that are safe, effective and sustainable from an environmental point of view [18].

As regards this latter option, polysaccharide-amino acid conjugates are drawing much attention due to their biocompatibility, design flexibility, adjustable degradability, and similarity—in terms of structure—to natural glycoproteins [19].

It is worth noting that plant host defense peptides (HDPs) or antimicrobial peptides (AMPs), generally cysteine-rich (nodule-specific cysteine-rich peptides, NCRs), are considered one of the main barriers developed by plants to fight infective agents [20–22], and are now being studied as antimicrobial agents against drug-resistant bacteria and other biomedical applications [23,24]. Amongst the different types of HDPs, the Snakin class is particularly interesting, as it encompasses the principal cysteine-rich peptides and given that the Snakin/GASA gene family has been identified in the grapevine [25]. As noted by Álvarez, et al. [26], cysteine is a keystone metabolite in the immune response pathways of plants, functioning as a precursor for many defense compounds (for example, phytoalexins, thionins, glucosinolates, etc.), and is associated with high resistance rates to both bio- and necrotrophic phytopathogens. In a recent study by Roblin, et al. [27], it was reported that cysteine may be able to control fungal diseases either by acting directly on fungal development and/or functioning as an early signal that elicits the plant's host reaction. In relation to GTDs, the same group also chose cysteine as a one of the chemicals in their experimental model aimed at the elaboration of preventive and/or curative treatments of esca syndrome [28].

Regarding polysaccharides, chitosan, a well-known compound with proven control properties, has been assayed against GTDs in different formulations: e.g., chitosan oligomers can protect pruning wounds inoculated with *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams and *Diplodia seriata* de Not. in field trials [29]; high molecular weight chitosan reduced mycelial growth of *Botryosphaeria* sp., *Phomopsis* sp., *Eutypa lata* (Pers.) Tul. & C. Tul., *Neonectria liriodendri* Halleen, Rego & Crous, 2006, *P. chlamydospora*

and *Fomitiporia* sp. [30]; oleoyl-chitosan nanoparticles reduced the mycelium growth of *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not. [31]; chitosan oligomers/propolis/silver nanoparticles composites have been tested against *D. seriata* [32]; and ε-polylysine:chitosan oligomers conjugates showed antifungal activity against *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Diplodia seriata*, and *B. dothidea* [33].

In connection with polysaccharide-amino acid conjugates, several examples for medical applications have been recently reported [34–36], but applications in the field of agronomy are still at a very early stage of development. To the best of the authors' knowledge, there is only one recent study on chitosan oligomers–amino acid conjugates against *Fusarium culmorum* (Wm.G. Sm.) Sacc., in spelt (*Triticum spelta* L.) by some of the co-authors of this work [37].

The aim of this study was to assess both the in vitro and in vivo antifungal efficacy of chitosan oligomers and amino acid conjugate complexes to control three of the most prevalent fungal pathogens associated with GTDs, especially in young plants: *N. parvum*, *D. seriata*, and *B. dothidea*.

2. Materials and Methods

2.1. Fungal Isolates

The three fungal isolates under study, viz. *N. parvum* (ITACYL_F111), *D. seriata* (ITACYL_F079) and *B. dothidea* (ITACYL_F141), were all isolated from diseased grapevine plants from D.O. Ribera de Duero and supplied as lyophilized vials (later reconstituted and refreshed as PDA subcultures) by the Agricultural Technological Institute of Castilla and Leon (ITACYL, Valladolid, Spain) [38].

2.2. Reagents and Preparation of Chitosan Oligomers and Bioactive Formulations

Chitosan (CAS 9012-76-4; high MW: 310,000–375,000 Da) was supplied by Hangzhou Simit Chem. & Tech. Co. (Hangzhou, China). The four amino acids (cysteine, CAS 52-90-4; glycine, CAS 56-40-6; proline, CAS 147-75-3; and tyrosine, CAS 60-8-4) were purchased from Panreac (Barcelona, Spain). Citric acid (CAS 77-92-9), sodium alginate (CAS 9005-38-3) and calcium carbonate (CAS 471-34-1) were purchased from Sigma-Aldrich Química (Madrid, Spain). Neutrase^{TM} 0.8 L enzyme was supplied by Novozymes A/S (Bagsværd, Denmark). Potato dextrose agar (PDA) was purchased from Becton Dickinson (Bergen County, NJ, USA).

Chitosan oligomers (COS) were prepared according to the procedure previously reported in [33]. Cysteine (Cys), glycine (Gly), proline (Pro) and tyrosine (Tyr) solutions were obtained by dissolution of the amino acids (with 99% purity) in sterile double distilled water at an initial concentration of 3000 $\mu g \cdot mL^{-1}$. The COS–amino acid conjugate complexes were obtained by mixing of the respective solutions in a 1:1 (v/v) ratio. The mixture was then sonicated for 15 min in five 3-min periods (so that the temperature did not exceed 60 °C) using a probe-type ultrasonicator (model UIP1000hdT; Hielscher Ultrasonics, Teltow, Germany).

2.3. In Vitro Tests of Mycelial Growth Inhibition

The fungicidal potential of the different compounds was determined employing an agar dilution method [39]; briefly, aliquots of stock solutions were incorporated onto the PDA medium to obtain the usual concentrations defined in the EUCAST standard antifungal susceptibility testing procedures [40]. Then, mycelial plugs (⌀ = 5 mm) of each pathogen coming from the margin of 7-day-old PDA cultures were transferred to plates incorporating the above mentioned concentrations for each compound (3 plates per treatment/concentration, with 2 replicates) and incubated 7 days at 25 °C in the dark. Control plates consisted of PDA medium without any amendment.

Mycelial growth rates were determined by calculating the average diameter of 2 perpendicular colony axes for each replicate. Growth inhibition of each treatment and concentration was calculated at the end of the incubating period according to the formula:

$$((d_c - d_t)/d_c) \times 100, \quad (1)$$

where d_c represents the average diameter of the fungal colony of the control and d_t is the average diameter of the treated fungal colony.

Results were also expressed as both EC_{50} and 90% effective concentrations, estimated by means of PROBIT analysis in IBM SPSS Statistics v.25 (IBM; Armonk, NY, USA) software.

Synergy factors were determined according to Wadley's method to quantify the level of interaction [41].

2.4. Greenhouse Bioassays in Grafted Plants

Together with the experiments of fungal pathogens growth inhibition in vitro, bioassays with the mentioned natural products and formulations were performed in grapevine plants in order to scale the protective capabilities of these compounds against three *Botryosphaeriaceae* species responsible of GTDs on young grapevine plants. Thus, plant material consisted of 48 plants each of varieties "Tempranillo" (CL. 32 clone) (2-years old) and "Garnacha" (VCR3 clone) (one year old) grafted on 775P and 110R rootstocks, respectively. Plants were planted on 3.5 L plastic pots with a mixed substrate of peat and autoclaved natural soil (75:25), incorporating slow release fertilizer when needed. Plants were maintained in the greenhouse with drip irrigation and anti-weed ground cover for six months (June-December) (Figure 1a). One week after placing them in the greenhouse, grafted plants were inoculated with three pathogens and either COS or COS−Tyr treatments. Five repetitions were arranged for each pathogen/control product and grapevine plant combination (cultivar/rootstock), together with 4 repetitions per pathogen and variety as positive control plus 3 repetitions of negative controls (inoculating only the bioactive product) for each treatment (Table S1).

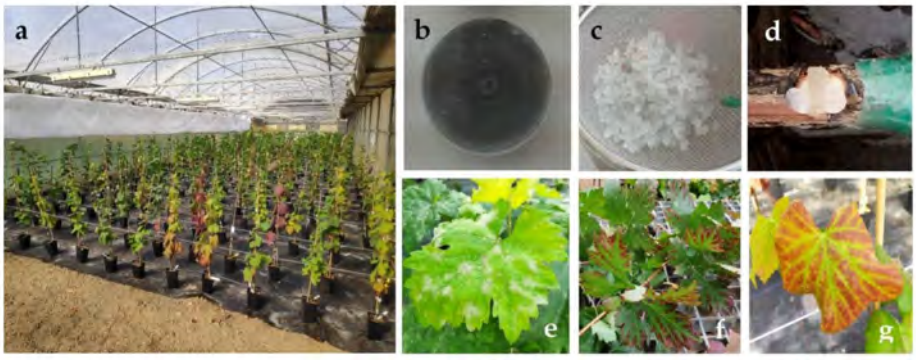

Figure 1. Bioassays in the greenhouse with grafted grapevine plants. (**a**): Bioassay overview; (**b**): fresh culture of *Neofussicoccum parvum* on PDA plate; (**c**): calcium alginate beads including control product; (**d**): inoculation method; (**e**): leaves infected with powdery mildew; (**f**,**g**): presence of foliar symptoms in grapevine plants.

For the fungal inocula, pure cultures of *N. parvum*, *D. seriata* and *B. dothidea* were maintained as fresh colonies in 9 cm Ø Petri dishes with PDA medium at 25 °C in the dark (Figure 1b). When necessary, the strains were subcultured in the aforementioned medium to keep them fresh and viable before use. Inoculations of both pathogens and control products were carried out on the trunk of the living plants at two sites per individual (separated at least 5 cm among them) below the grafting point and not reaching the root crown. In the case of fungal strains, agar plugs from fresh PDA cultures of each fungus in question were used as fungal inoculum. In the defined points of each grapevine plant, slits (with a scalpel) of approx. 3 mm in diameter and 0.5 cm deep were done. After this,

0.5 cm diameter agar plugs were inoculated and placed in such a way that the mycelium was in contact with the incision of the stem. Two calcium alginate beads (Figure 1c) including the different control products assayed were placed at both sides of the agar plug (Figure 1d). For this, beads were prepared as follows; each biological compound was added to a 3% sodium alginate solution in a 2:8 ratio (20 mL treatment/80 mL sodium alginate). Then, the solution incorporating each treatment was dispensed drop by drop onto a 3% calcium carbonate solution to spherify (polymerize) the beads containing the mentioned treatments. Finally, both discs and beads were covered with cotton soaked in sterile double distilled water and sealed with Parafilm^{TM} tape. During the culturing period, application of copper to control powdery mildew (Figure 1e) was performed in mid-July, together with a first sprouting (followed by periodic sprouting). In addition, releasing of *Amblyseius* (*Typhlodromips*) *swirskii* Athias-Henriot for biological control of whitefly, thrips and spider mite, *Encarsia formosa* Gahan/*Eretmocerus eremicus* Rose & Zolnerowich for whitefly and *Aphelinus abdominalis* Dalman for aphids at the end of July (Biobest Group NV, Almería, Spain) were also performed.

Potted grapevine plants were examined weekly during the whole assay period by taking photographs (Figure 1f,g) in cases where different foliar symptoms including intern-ervial necroses) were observed. Six months after inoculation, plants were removed and two sections of the inoculated stems between the grafting point and the root crown were prepared, opened longitudinally and the length of the vascular necroses (tracheomycosis) caused by the different pathogens was evaluated. For this, the length of the vascular necroses was measured longitudinally on upper and lower directions from the inoculation point for both halves of the longitudinal cut, and the measures of these were statistically analyzed and compared depending on the type of pathogen and product formulation employed. All the data were compared with controls.

At the beginning of the assay, some of the grapevine plants did not sprout or died in the first month after transplantation. These were removed and examined to verify the presence of pre-existing root rot in the plant material related to this circumstance, and analyzed in the laboratory to isolate the possible responsible fungal species. The rest of the plants removed and measured at the end of the assay were finally processed to re-isolate the different pathogenic taxa previously inoculated. Thus, in order to fulfill Koch's postulates, wood fragments approximately 0.5 cm long surrounding the different vascular necroses (1–2 cm around the wounds) were washed, surface sterilized, placed in PDA plates amended with streptomycin sulphate (to avoid bacterial contamination) and incubated at 26 °C in the dark in a culture chamber for 2–3 days.

2.5. Statistical Analyses

Differences in the in vitro mycelial growth inhibition results were assessed by analysis of variance (ANOVA) followed by post hoc comparison of means through Tukey's test at $p < 0.05$ (provided that the homogeneity and homoscedasticity requirements were satisfied, according to the Shapiro–Wilk and Levene tests [42]). In the case of in planta results, the Johnson transformation [43,44] was first used to transform the data to follow a normal distribution, and then descriptive statistics, ANOVA and Tukey's tests of the necrosis lengths were performed. The SPSS Statistics v.25 software was used (IBM; Armonk, NY, USA).

3. Results

Below are shown the results of the assays carried out to test the antifungal capacity of a series of conjugates based on chitosan polymers and certain amino acids, for the control, both in vitro and in plant, of some taxa of the *Botrysphaeriaceae* family involved in the so-called wood diseases in young grapevine plants.

3.1. Mycelial Growth Inhibition Tests

The results of the growth inhibition tests are presented in Figure 2. The performances of the amino acid-only treatments were much lower than those of the treatments based on

COS, either alone or in combination with them (Figures S1–S3). Concerning the dosage of the compounds assayed, higher inhibition was obtained upon increase of the concentration for all treatments.

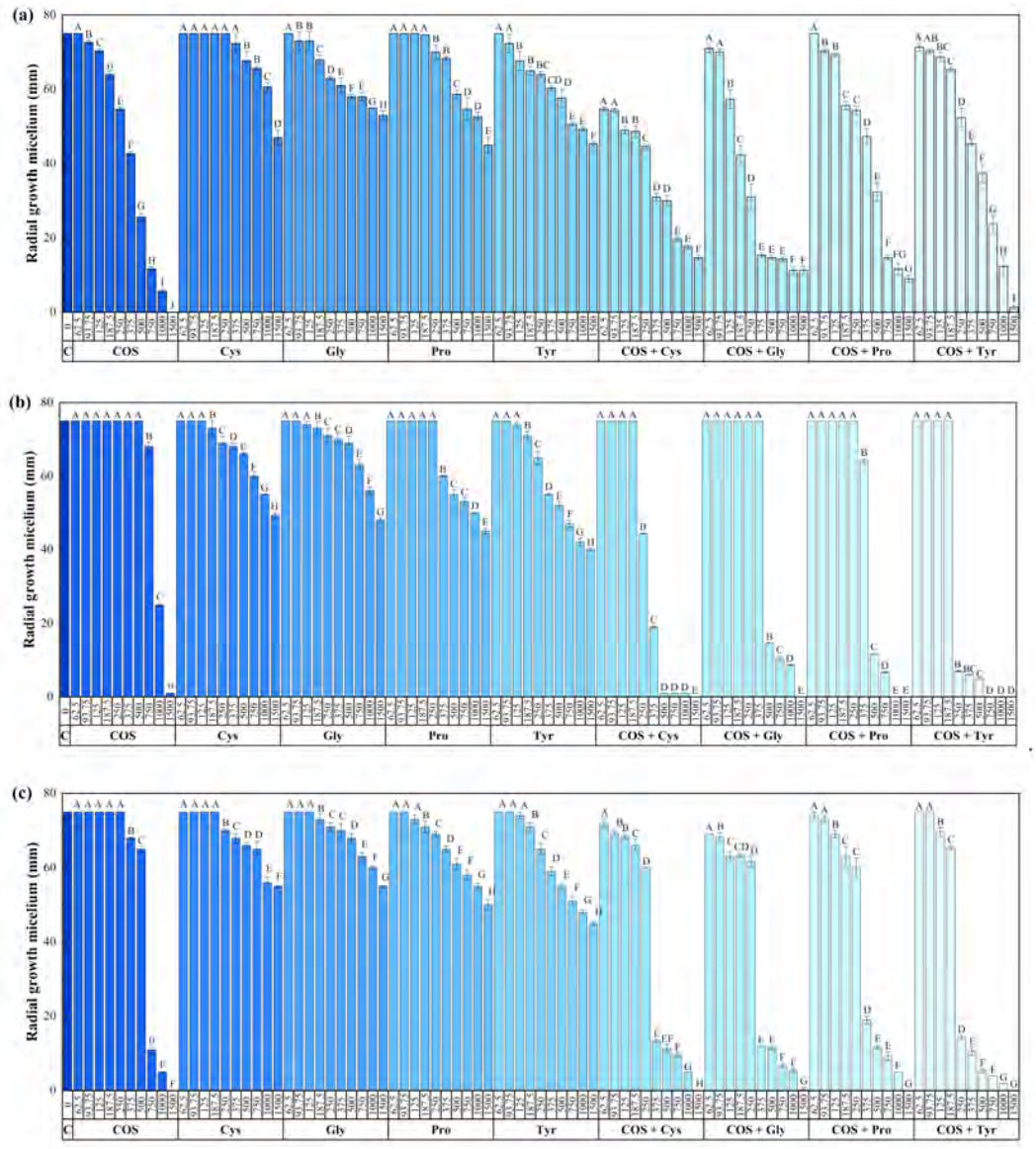

Figure 2. Colony growth values of (**a**) *N. parvum*, (**b**) *D. seriata* and (**c**) *B. dothidea* strains when cultured in PDA plates containing several control products, i.e., chitosan oligomers (COS), cysteine (Cys), glycine (Gly), proline (Pro), tyrosine (Tyr), and the respective COS–amino acid (1:1 v/v) conjugate compounds. The same letters above concentrations mean that they are not significantly different at $p < 0.05$. Error bars represent standard deviations.

The effective in vitro concentrations are summarized in Table 1 for comparison purposes (effective concentrations for amino acids alone are not presented, provided that full inhibition was not attained even at the highest assayed concentration, so a reliable fitting could not be obtained). In the case of *N. parvum*, a synergistic effect was only observed for COS-Cys and COS-Tyr in the EC_{50} values. Conversely, for *D. seriata* and *B. dothidea*, a synergistic effect was observed for all the COS−amino acid conjugate complexes, particularly evident for COS-Tyr, with estimated synergy factors (SF) of 2.03 and 2.29 in the EC_{50} values and SF of 2.48 and 2.84 in the EC_{90} values, respectively.

Table 1. EC_{50} and EC_{90} effective concentrations, expressed in $\mu g \cdot mL^{-1}$.

Pathogen	Effective Concentration	COS	COS–Cys	COS–Gly	COS–Pro	COS–Tyr
N. parvum	EC_{50}	320.9	208.8	417.8	402.9	258.9
	EC_{90}	967.4	1347.0	1498.5	1439.0	1021.4
D. seriata	EC_{50}	448.1	297.8	448.5	398.7	254.6
	EC_{90}	1360.6	774.6	1286.7	1086.5	672.1
B. dothidea	EC_{50}	425.8	306.2	291.1	316.0	255.1
	EC_{90}	1339.2	897.9	887.9	907.4	707.7

COS = chitosan oligomers; Cys = cysteine; Gly = glycine; Pro = proline; Tyr = tyrosine.

3.2. In Vivo Tests

After removing, cutting and measuring vascular necroses present in the different treated grafted plants, it was primarily observed that no statistically significant differences were obtained among neither between plant combination (cultivar/rootstock) nor between upper and lower wounds (Figure S4). In fact, in this latter case, the Pearson correlation coefficient was 0.738.

Upon comparison of necrosis lengths in the negative controls (i.e., plants whose wounds were only treated with the bioactive product, with no pathogen inoculation) and positive controls (i.e., plants inoculated only with pathogens), significant differences between pathogens in terms of their aggressiveness were only observed for the lower wound (Table S2). The most aggressive fungus was *N. parvum*, while *D. seriata* showed an intermediate virulence, and *B. dothidea* induced the least necrosis. This can be ascribed to both differences in the wood decay enzymatic activities and in the ability of these fungi to metabolize major grapevine phytoalexins [45,46].

When the effect of the treatments on the infection rates of the three pathogens was studied, significant differences were found between the treated plants and the positive control in the case of *N. parvum* and *B. dothidea* (Table 2). On the other hand, the synergistic behavior between COS and tyrosine observed in vitro (particularly evident for *B. dothidea*) was not reflected in statistically significant differences (compared with single COS treatment) in the plant bioassay at the greenhouse scale.

Table 2. Analysis of variance (ANOVA) of the lengths of vascular necroses for *N. parvum* (left), *B. dothidea* (center) and *D. seriata* (right).

N. parvum	Upper Wound	Lower Wound	*B. dothidea*	Upper Wound	Lower Wound	*D. seriata*	Upper Wound	Lower Wound
Positive control	0.848 a	0.895 a	Positive control	0.529 a	0.397 a	Positive control	0.609 a	0.486 a
COS	0.258 b	0.351 b	COS-Tyr	−0.121 b	−0.196 b	COS	0.145 b	0.412 a
COS-Tyr	0.257 b	0.217 b	COS	−0.136 b	−0.236 b	COS + tyr	0.332 ab	0.279 a
Negative control	−1.444 c	−1.210 c	Negative control	−1.444 c	−1.210 c	Negative control	−1.444 c	−1.210 b
Pr > F	<0.0001	<0.0001	Pr > F	<0.0001	<0.0001	Pr > F	<0.0001	<0.0001
Significant	Yes	Yes	Significant	Yes	Yes	Significant	Yes	Yes

Treatments/controls labelled with the same letters are not significantly different at $p < 0.05$.

Finally, in the case of *D. seriata*, significant differences were only observed in the upper wounds, with a better performance of the treatment based solely on COS, an unexpected result on the basis of the effective concentration values reported in Table 1. In the lower wounds, no significant differences were seen, but the COS−Tyr treatment seemed to show a better performance than that based solely on COS, in line with the results of the in vitro tests.

Plants prematurely removed from the bioassay displayed basal rots (due to *Rhizoctonia solani* J.G. Kühn and *Neonectria* spp.) already present in the starting material. Moreover, the rest of the plants of the assay were submitted to Koch' postulates, isolating the previously inoculated pathogens in most (80%) of them.

As previously mentioned, together with the vascular necrosis, during the whole assay period it was observed that many of the grapevine plants exhibited certain foliar symptoms (Figure 1f,g), probably due to a long-dispersal action of phytotoxins produced by the inoculated pathogens. The production of such type of secondary metabolites by these and other GTD-related fungi is well known [7,47–49]. Among these, low molecular weight lipophilic phytotoxins (for example, naphthalenone pentaketides, melleins and polyphenols) produced by the different *Bot* taxa could be responsible for the observed symptoms (i.e., moderate to severe withering and necrotic spots). When analyzing such symptoms, no correlations were observed among either the plants inoculated exclusively with the pathogens and the controls without any fungus or the treated plants, probably due to the basal phytosanitary status of the propagation material, which could also influence the appearance of these foliar symptoms, regardless the treatment assayed.

4. Discussion

4.1. Comparison of the Efficacy of the Treatments

Regarding chitosan only-based treatments, chitosan oligosaccharides (molecular weight < 3000 Da) at a concentration of 1000 $\mu g \cdot mL^{-1}$ were reported to completely inhibit the mycelial growth of *D. seriata* and *B. dothidea* when performing in vitro assays [29]. These values are of the same order of magnitude as the EC_{90} values presented herein, so differences may be attributed to the isolate-dependency of the susceptibility profile.

For the same strains of *N. parvum* and *D. seriata*, EC_{90} values of 1270 and 1120.7 $\mu g \cdot mL^{-1}$ were attained for COS with molecular weight < 2000 Da in [33]. In this case, differences may be tentatively ascribed to slight differences in the molecular weight, polymerization degree or deacetylation degree of COS, which are known to influence its efficacy against phytopathogenic fungi [50,51].

In relation to non-in vitro bioassays with chitosan, Cobos, Mateos, Alvarez-Perez, Olego, Sevillano, Gonzalez-Garcia, Garzon-Jimeno and Coque [29] reported that 96.8% growth inhibition of *D. seriata* was attained in autoclaved vine shoots using chitosan oligosaccharides, although at a much higher concentration (25 $mg \cdot mL^{-1}$). In artificially inoculated plants, the same authors found a significant reduction in the incidence of *D. seriata* when the pruning wounds were treated with chitosan oligosaccharides and other natural compounds, decreasing lipid peroxidation levels and guaiacol peroxidase (GPX) activity (a recognized stress marker). Albeit for different GTD pathogens, Nascimento, Rego and Oliveira [30]—in greenhouse experiments carried on potted grapevine plants (cultivar "Castelão") growing in a substrate artificially infested with *Phaeomoniella chlamydospora* or *Neonectria liriodendri*—observed that foliar sprays of chitosan oligosaccharin (<3 kDa) only reduced the disease incidence of *P. chlamydospora*, but had an effect against *N. liriodendri* similar to that of some selected fungicides (tebuconazole, cyprodinil + fludioxonil and carbendazim + flusilazole).

Concerning aminoacids, cysteine has been reported to have an inhibitory effect on the in vitro mycelial growth of *P. chlamydospora* and *Phaeoacremonium minimum*; at a concentration of 10 mM (that is, 1216 $\mu g \cdot mL^{-1}$), an inhibition of 77% for *P. chlamydospora* and 58% for *P. minimum* was attained. The respective EC_{100} values were 15 and 20 mM (1824 and 2432 $\mu g \cdot mL^{-1}$) [28]. At a 10 mM concentration, it exhibited a strong inhibition (79–100%) against various strains of *E. lata*, while lower efficacies were observed against other fungal species associated with other grapevine diseases (*P. chlamydospora* and *Phaeoacremonium aleophilum*, *Botryosphaeria parva* and *B. obtusa*, that were inhibited by 63%, 40%, 54% and 40%, respectively) [52].

Regarding analogous polysaccharide-peptide based formulations, little information is available in the literature. The EC_{90} values attained with a COS−ε-polylysine conjugate

(507.5, 580.2 and 497.4 μg·mL^{-1} for *N. parvum*, *D. seriata* and *B. dothidea*, respectively [33]) were better than those attained in this work for the COS−Tyr conjugate (1021.4, 672.1 and 707.7 μg·mL^{-1}, respectively), but—from an economic perspective—the latter formulation would be much more viable (given that the price of ε-polylysine is much higher than that of tyrosine: 245 €/100 mg vs. 58 €/100 g). An additional advantage of the COS–Tyr formulation could be its versatility as a crop protection product: EC_{50} and EC_{90} values against *Fusarium culmorum* (320 and 1107 μg·mL^{-1}, respectively) were of the same order of magnitude as those reported herein [37].

If the EC_{50} values for COS and the COS−amino acid conjugate complexes are compared with those reported for chemical fungicides used in GTDs control, it may be observed that the efficacies would be comparable: for example, Pitt, et al. [53] found values in the 360–440, 530–620 and 450 μg·mL^{-1} range for *N. parvum*, *D. seriata* and *B. dothidea*, respectively, taking data pooled across fungicides (viz. carbendazim, fluazinam, fludioxonil, flusilazole, iprodione, myclobutanil, penconazole, procymidone, pyraclostrobin and tebuconazole) to estimate average EC_{50} values for isolate sensitivity. Nonetheless, if one considers the excellent EC_{50} values reported by Olmo, et al. [54] against *N. parvum* and *D. seriata* for tebuconazole (90 and 150 μg·mL^{-1}, respectively) and pyraclostrobin (100 and 250 μg·mL^{-1}, respectively), it becomes apparent that there is still room for improvement in the efficacy of the natural composites.

4.2. Mechanism of Action

A panorama of the molecular mechanisms behind chitosan interactions with plants and fungi has been recently presented in the review paper by Lopez-Moya, et al. [55]. With regard to its role as an antimicrobial agent, it is well established that it can permeabilize fungal plasmatic membranes (triggering intracellular production of ROS and cell death), arrest germination and growth by deprivation of nutrients (which leads to cell wall architecture modification), alter gene expression (e.g., affecting oxidoreductase activity, respiration and transport gene ontology functions), etc.

Regarding the precise function of amino acids in the response of plants to pathogens, it is not well established: on one hand, they are required for growth and metabolism in microorganisms, and on the other hand, careful optimization of composition and concentration can produce antimicrobial effects [56]. Besides this, changes in the contents of amino acids appear to be a common characteristic of plant response to GTDs. For instance, in a recent study of the wood metabolomic responses of wild (*Vitis vinifera* subsp. *sylvestris*) and cultivated grapevine (*V. v.* subsp. *vinifera*) to infection with *N. parvum*, Labois, et al. [57] found that the metabolic response of the former to the infection featured a faster and more intense alteration in primary metabolites in comparison to the latter, accompanied by a higher induction of various resveratrol oligomer contents. Infection by *N. parvum* caused an increase in alanine, β-alanine and glycine, and a decrease in aspartic acid, asparagine and serine.

To the best of the authors' knowledge, no data on the role of tyrosine on GTDs has been published to date. Nonetheless, cysteine has been reported to be involved in signaling, plant resistance and antifungal development [27]. Like other amino acids, cysteine can be transported along the vascular tissues of vines over long distances, and it can induce dramatic alterations in the structural organization of the mycelium (nucleus, mitochondria, vacuoles and cell wall), causing the death of the hyphae [52]. Octave, Amborabé, Luini, Ferreira, Fleurat-Lessard and Roblin [52] hypothesized that the action of cysteine may be based on its ability to interfere with a certain metabolic pathway and also by triggering the secretion of ergosterol, which presents properties of an elicitor.

As regards the mode of action of the conjugate complex, it may be the result of an enhanced additive fungicidal effect per se, and/or via a concurrent action on diverse fungal metabolic sites. In a previous work [37], we also hypothesized that conjugation of COS and Tyr increases the cationic surface charge of COS, enhancing the linkage (through

electrostatic interactions) to the negatively charged site-specific binding receptors on the fungal membrane.

4.3. Significance of the Reported Findings

The three fungal species tested in the present study belong to the *Botryosphaeriaceae* family, a group of polyphagous ascomycetous taxa associated not only with grapevine diseases, but also pathogenic on a vast range of woody plants, specially forestry species [58], stone and pome fruits (i.e., almond, peach, apple, apricot, etc.) [59], and even on woody crops of recent implantation and extension in Spain such as pistachio [60,61]. Furthermore, *D. seriata* and *B. dothidea* have been cited as phytopathogens on apple [62], while *N. parvum* causes avocado dieback [63], *B. dothidea* causes Botryosphaeria blights and cankers on olive trees [64], and as mentioned, the three of them are commonly related to branch cankers on almond trees [54]. Therefore, the findings obtained in the present study may also be applied and extended to other basic Mediterranean crops that usually share these types of pathogens. In fact, many authors have verified that many of the pathogens of crops such as olive, stone fruit or grapevine, share plant hosts during some phase of their life cycle and are isolated repeatedly from adjacent crops [65], resulting in an even higher ecological and economic impact. Thus, any type of research in the control of global and aggressive pathogens such as *B. dothidea* [66] or *N. parvum* [67] is relevant, since the incidence and economic importance of the losses caused by both fungi has been increasing in recent years, especially in grapevine crop due to damage to young plants coming from the nursery. Both taxa tend to have a prolonged latent or endophytic phase [68], which makes their detection very difficult, especially in quarantine inspection surveys, since their symptoms occasionally appear in situations of stress of the plant host. Furthermore, in the nurseries that produce young grafted vine plants in Spain, *N. parvum* is considered one of the main mycoses associated with propagation material [69], being ultimately responsible for the uprooting of thousands of hectares of grapevine plants in the first years after their plantation.

4.4. Limitations of the Study and Further Research

A clear limiting factor in the *in planta* bioassays was the choice of calcium alginate as a dispersion medium to protect the pruning wounds, given that it limited the amount of the bioactive solution that could be incorporated to the matrix to approximately 20% (otherwise gelation was not attained). Considering that the initial concentration of the bioactive solutions was 3000 $\mu g \cdot mL^{-1}$, the formulations were tested at a concentration of *ca*. 600 $\mu g \cdot mL^{-1}$, that is, at values closer in many cases to the EC_{50} value than to the EC_{90} one. This would explain why, even though significant differences were observed, a higher degree of protection was not attained. The use of other thickener agents (e.g., pectin, vegetable gums, starches, or halloysite, which are cheaper than alginate) should be assayed in future studies. Alternatively, more advanced delivery methods, such as the use of lignin nanocarriers (analogous to those recently reported by Wurm's group [70,71] for azoxystrobin, pyraclostrobin, tebuconazole, and boscalid delivery) could overcome the aforementioned limitation.

Since the results obtained here refer only to the reduction of vascular necrosis in artificially inoculated grapevine plants, complementary tests will be required in future multiyear assays to correlate these levels of protection with the intensity and incidence of foliar symptoms, harvest yield, etc., to have a more complete view of the effect of this type of alternative substances.

Another particularly interesting aspect to be considered in follow-up studies would be the inclusion of synthetic chemicals as additional treatments, provided that this would allow direct comparisons with the natural products both in terms of efficacy and cost-effectiveness.

5. Conclusions

Conjugate complexes of chitosan oligomers (with MW < 2 kDa) and amino acids, inspired in plant HDPs, were assayed for their control effects against three *Botryosphaeriaceae* fungi responsible for some of the GTDs. In vitro growth inhibition tests revealed a synergistic effect between COS and the amino acids against two of the pathogens, viz. *D. seriata* and *B. dothidea*, which was not present (or was very weak) in the case of *N. parvum*. The lowest EC_{50} and EC_{90} effective concentrations, comparable to those reported for conventional synthetic fungicides used in the control of these mycoses, were obtained for the COS−tyrosine conjugate complex. Hence, this formulation and the one based on COS alone were further assayed for wound protection applications in a greenhouse bioassay conducted on potted grapevines of two varieties ("Tempranillo" on 775P and "Garnacha" on 110R rootstock), which were artificially inoculated with the mentioned pathogenic species. A significant decrease in vascular necrosis severity was observed for *N. parvum* and *B. dothidea*, while the efficacy against *D. seriata* was only statistically significant for the upper wounds. Taking into consideration that the incidence and economic importance of the losses caused by the former two fungi has been increasing in recent years, and that they affect many other woody plants (not only grapevine), the reported formulations may pose a promising alternative to synthetic chemical pesticides for the protection of trunk diseases of woody crops.

6. Patents

The work reported in this manuscript is related to Spanish patents P201931118 and P201831106.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/2/324/s1, Figures S1–S3: Sensitivity tests for *N. parvum, D. seriata* and *B. dothidea*; Figure S4: Box-plot of the lengths of the vascular necroses in the upper and lower wounds; Table S1: Repetitions for each of the plant/treatment combinations in the greenhouse bioassay; Table S2: ANOVA of lengths of the vascular necroses in the positive and negative controls for the three fungi under study.

Author Contributions: Conceptualization, E.P.-L., J.M.-G. and V.G.-G.; methodology, J.M.-G., J.C.-G. and V.G.-G.; validation, J.C.-G., V.G.-G. and P.M.-R.; formal analysis, J.C.-G., V.G.-G. and P.M.-R.; investigation, L.B.-D., N.L.-L., V.G.-G., J.C.-G., J.M.-G. and P.M.-R.; resources, J.M.-G. and P.M.-R.; data curation, J.C.-G.; writing—original draft preparation, L.B.-D., N.L.-L., V.G.-G., J.C.-G., J.M.-G. and P.M.-R.; writing—review and editing, V.G.-G. and P.M.-R.; visualization, L.B.-D. and N.L.-L.; supervision, V.G.-G. and P.M.-R.; project administration, V.G.-G., J.M.-G. and P.M.-R.; funding acquisition, J.M.-G. and P.M.-R. All authors have read and agreed to the published version of the manuscript.

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Physicochemical Characterization of *Crithmum maritimum* L. and *Daucus carota* subsp. *gummifer* (Syme) Hook.fil. and Their Antimicrobial Activity against Apple Tree and Grapevine Phytopathogens

Eva Sánchez-Hernández; Laura Buzón-Durán; Celia Andrés-Juan; Belén Lorenzo-Vidal; Jesús Martín-Gil; Pablo Martín-Ramos

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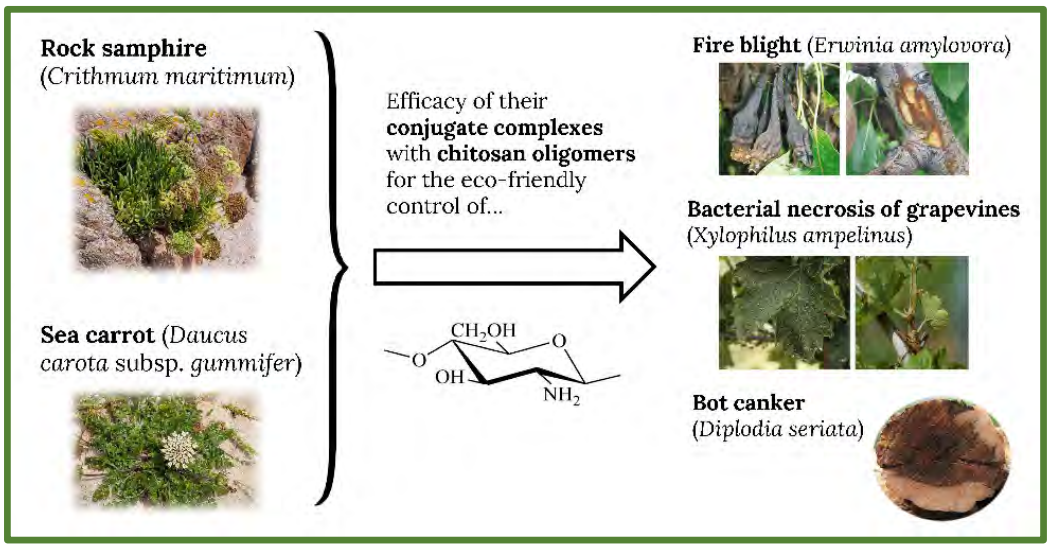

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Physicochemical Characterization of *Crithmum maritimum* L. and *Daucus carota* subsp. *gummifer* (Syme) Hook.fil. and Their Antimicrobial Activity against Apple Tree and Grapevine Phytopathogens

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Abstract: *Crithmum maritimum* and *Daucus carota* subsp. *gummifer* are two species of the Apiaceae family that share multiple characteristics: both are halophitic, live on cliffs in the same geographic habitats, and are edible. While *C. maritimum* is rich in essential oils and flavonoids, *D. carota* is rich in terpenes and a gum producer. In the work presented herein, the biomass of these two wild plants and the bioactive compounds present in their extracts have been studied by elemental and thermal analysis, infrared spectroscopy, and gas chromatography-mass spectroscopy. To explore their bioactivities, both their hydroalcoholic extracts and their major constituents (apiole in *C. maritimum* and geranyl acetate in *D. carota*), either alone or in combination with chitosan oligomers, were assayed in vitro against bacterial and fungal pathogens that affect apple trees (*Malus domestica*) and grapevine (*Vitis vinifera*). Remarkable inhibition was observed against *Erwinia amylovora*, the causal agent of fire blight in apple; *Xylophilus ampelinus* [syn. *Erwinia vitivora*], the causal agent of bacterial blight of grapevine; and *Diplodia seriata*, a virulent pathogen of grapevines that also causes canker, leaf spot and fruit rot of apple. In view of their effectiveness against these three phytopathogens, a potential application of these two medicinal plants in organic farming may be envisaged.

Keywords: antibacterial; antifungal; apiole; chitosan; *Diplodia seriata*; *Erwinia amylovora*; geranyl acetate; Viticulture; *Xylophilus ampelinus*

1. Introduction

Crithmum maritimum L., the sole species of *Crithmum* genus, is a perennial wild plant that is found on cliffs in southern and western coasts of the British Isles, on western and Mediterranean coasts of Europe, in North Africa and the Canary Islands. It is known as *samphire*, *rock samphire*, *sea fennel*, and, in Asturias (Spain), as *cenoyo de mar*. It belongs to the Apiaceae family and is an oleaginous halophyte. It has fleshy, divided aromatic leaves, which have a hot and spicy taste (Figure 1). A detailed morphological description, together with a discussion of its eco-physiological responses to salt stress, may be found in the review paper by Atia, et al. [1].

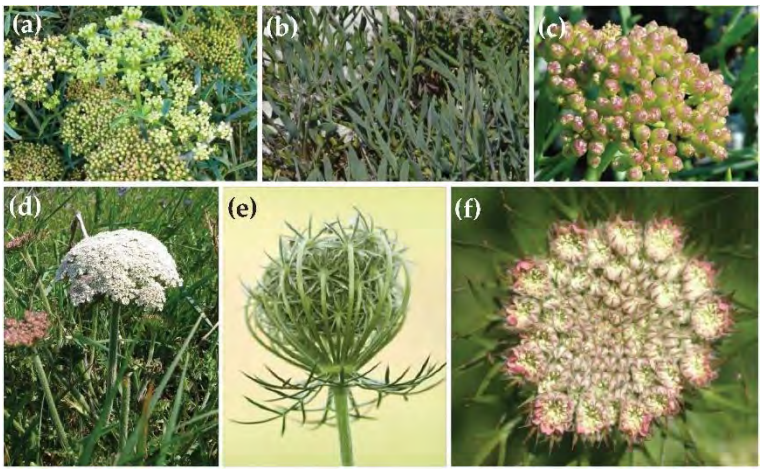

Figure 1. Morphology of *Crithmum maritimum* L. (**a**–**c**) and *Daucus carota* subsp. *gummifer* Hook.fil. (**d**–**f**).

Daucus carota subsp. *gummifer* (Syme) Hook.fil. is also a member of the Apiaceae, and is a herb of maritime cliffs, dunes, and grasslands. It is mostly found on the northern coast of Spain, although it may also be found on southern and western coasts of Britain. The common names of this *D. carota* subspecies include *sea carrot*, *wild carrot*, *bird's nest*, *bishop's lace*, and *Queen Anne's lace*. In Spanish language, it is named *zanahoria de acantilado* (tr. cliff carrot). It is hairy, with a stiff, solid stem (Figure 1). The leaves are tripinnate, finely divided, lacy, triangular in shape. Its flowers—small and white, clustered in flat, dense umbels—are sometimes battered and fried. The root is edible while young, but it quickly becomes too woody to consume. The leaves are also edible in little quantities. It contains small amounts of toxicant cyanogenic glycosides [2].

These two medicinal plants have been reported to produce interesting secondary metabolites [3]. Spectrometric analyses of the contents of flavonoids, tannins, and total polyphenols in the aerial parts of rock samphire collected on the Adriatic coast of Croatia in different growth stages were reported by Males, et al. [4], with the highest contents of above components in the samples collected before flowering. Phenolic acids, such as caffeic, chlorogenic, ferulic, p-hydroxybenzoic, p-coumaric vanillic, protocatechuic, and syringic acids were identified by Bartnik, et al. [5]. According to Pavela, et al. [6], the essential oils (EO) of *C. maritimum* show notable variability in chemical composition, being dominated by dillapiole and γ-terpinene (French EO), limonene and γ-terpinene (central Italy EO), and thymol methyl ether and γ-terpinene (Sicilian EO).

In turn, *D. carota* subs. *gummifer* has been reported to contain high contents of monoterpenes (83.9%), the major compounds being geranyl acetate [7] and pinenes. The daucane sesquiterpene, carotol, has also been found in relatively high amounts (11%) [8].

With regard to the potential applications of these bioactive compounds, the antimicrobial activity of the EO of *C. maritimum* has been assayed against common food-borne bacteria, finding significant inhibition against *Escherichia coli*, *Candida albicans*, *Listeria innucia*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* [9,10]. Its antimicrobial activity against a panel of microorganisms, including clinical isolates and food-borne pathogens, has also been studied [11]. The EO obtained from *D. carota* (albeit not for subsp. *gummifer*) has been assayed against *S. aureus*, *E. coli*, *P. aeruginosa*, *Enterobacter aerogenes*, *B. subtilis*, *Campylobacter jejuni*, *Microsporum canis*, and *C. albicans* by Rossi, et al. [12], Ozcelik, et al. [13], and Pavoni, et al. [14]. Only Valente, et al. [8] and Nawel, et al. [15] explored the EO from *D. carota* subs. *gummifer* as a natural source of antifungals against clinical strains of bacteria, yeast, and filamentous fungi.

Nonetheless, to the best of the authors' knowledge, the efficacy of these wild plants extracts has barely been explored against pathogens affecting crop species: *C. maritimum* EO has only been tested against *Erwinia carotovora* (which causes beet vascular necrosis, blackleg of potato and other vegetables, and slime flux on various tree species) by

Ruberto, et al. [16], and against *Mycogone perniciosa* (which causes severe crop losses in common mushroom cultivation) by Glamoclija, et al. [17]. In this work, their application to the control of apple tree (*Malus domestica* Borkh.) and grapevine (*Vitis vinifera* L.) pathogens, in particular against two bacteria, namely *Erwinia amylovora* (Burrill) and *Xylophilus ampelinus* (Panagopoulos, 1969) Willems et al., 1987 [syn. *Erwinia vitivora*], and a fungus, viz. *Diplodia seriata* De Not., is evaluated.

Erwinia amylovora is the causal agent of fire blight, a major global threat to commercial apple and pear production [18]. It is cataloged as a quarantine organism in the European Union, and it has been included in the top 10 plant pathogenic bacteria [19]. A panorama of this pathogen's biology, epidemiology, and control may be found in the recent review by Zhao, et al. [20]. *X. ampelinus* (syn. *Xanthomonas ampelina* and *Erwinia vitivora* [21]), the causal agent of bacterial necrosis of grapevines (known as "maladie d'Oléron" in France and "mal nero" in Italy), severely affects grape crops, resulting in harvest losses as high as 70% of typical yield [22]. The European and Mediterranean Plant Protection Organization (EPPO) categorizes *X. ampelinus* as a quarantine A2 organism, and it is also a quarantine pest for the North American Plant Protection Organization (NAPPO) and the Interafrican Phytosanitary Council (IAPSC). Regarding *D. seriata*, it is a member of the Botryosphaeriaceae family, which are known to be pathogens, endophytes, and saprophytes on a wide range of woody hosts. *D. seriata* is a primary and virulent pathogen of grapevines [23,24], but it also causes frog-eye leaf spot, black rot and canker of apples [25–27].

Taking into consideration that EU regulation (Article 14 in Directive 2009/128/EC, Council Regulation (EC) 834/2007, Commission Regulation (EC) 889/2008, Regulation (EU) 2019/1009, etc.) promotes the use of formulations based on natural products for Integrated Pest Management (IPM), valorization of these two halophytes from the Asturian coast (Spain) as antimicrobial agents for crop protection is proposed. To explore this possibility, a physicochemical characterization of *C. maritimum* and *D. carota* subsp. *gummifer* is first presented, followed by in vitro studies of the efficacy of their hydromethanolic extracts against the above-referred phytopathogens.

2. Material and Methods

2.1. Plant Material and Chemicals

C. maritimum and *D. carota* subsp. *gummifer* samples were collected in the cliffs near the beach of San Antolín (Naves, Llanes, Asturias, Spain—43°26′32.3″ N 4°51′59.6″ W) in early August, in full flowering. Plant parts from different specimens (n = 10 for each species) were thoroughly mixed to obtain separate composite samples for roots, leaves, stems, and flowers.

Chitosan (CAS 9012-76-4; high MW: 310,000–375,000 Da) was supplied by Hangzhou Simit Chem. & Tech. Co. (Hangzhou, China). Neutrase^{TM} 0.8 L enzyme was supplied by Novozymes A/S (Bagsværd, Denmark). Chitosan oligomers (COS) were prepared according to the procedure previously reported in [28].

Apiole (1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene, CAS 523-80-8) was purchased from Cymit Química SL (Barcelona, Spain). Geranyl acetate (trans-3,7-dimethyl-2,6-octadien-1-yl acetate, CAS 105-87-3), methanol (UHPLC, suitable for mass spectrometry, CAS 67-56-1), TSA (tryptic soy agar, CAS 91079-40-2) and TSB (tryptic soy broth, CAS 8013-01-2) were acquired from Sigma-Aldrich Química (Madrid, Spain). PDA (potato dextrose agar) was supplied by Becton Dickinson (Bergen County, NJ, USA).

2.2. Bacterial and Fungal Isolates

The two bacterial isolates, *Erwinia amylovora* (Burrill) and *Xylophilus ampelinus* (Panagopoulos, 1969) Willems et al., 1987 were supplied by the Spanish Type Culture Collection (CECT), with NCPPB 595 and CCUG 21976 strain designations, respectively. The fungal isolate under study, *D. seriata* (code ITACYL_F098, isolate Y-084-01-01a) was isolated from 'Tempranillo' diseased grapevine plants from protected designation of origin (PDO) Toro (Spain) and supplied as lyophilized vials (later reconstituted and refreshed as

PDA subcultures) by the Agricultural Technological Institute of Castilla and Leon (ITACYL, Valladolid, Spain) [29].

2.3. Preparation of Plant Extracts

C. maritimum and *D. carota* subsp. *gummifer* flowering aerial parts were mixed (1:20, *w*/*v*) with a methanol/water solution (1:1 *v*/*v*) and heated in a water bath at 50 °C for 30 min, followed by sonication for 5 min in pulse mode with a 1 min stop for each 2.5 min, using a probe-type ultrasonicator model UIP1000hdT (Hielscher Ultrasonics, Teltow, Germany). The solution was then centrifuged at 9000 rpm for 15 min and the supernatant was filtered through Whatman No. 1 paper. Aliquots were lyophilized for CHNS analyses.

2.4. Plant Biomass and Extracts Physicochemical Characterization

Elemental analyses were carried out with a LECO (St. Joseph, MI, USA) CHNS-932 apparatus (model No. 601-800-500).

Thermal gravimetric (TGA) and differential scanning calorimetry (DSC) analyses were carried out by means of a simultaneous TG-DSC2 (Mettler Toledo; Columbus, OH, USA), in N_2:O_2 (4:1), with a flow heating rate of 20 °C·min^{-1}.

The infrared vibrational spectra were registered using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 Fourier-transform infrared spectrometer, equipped with an in-built diamond attenuated total reflection (ATR) system. The spectra were collected with a 1 cm^{-1} spectral resolution over the 400–4000 cm^{-1} range, taking the interferograms that resulted from co-adding 64 scans.

The colorimetric quantification of total polyphenol content (TPC) and total flavonoid content (TFC) was conducted according to the procedures described in [30], using an Agilent (Santa Clara, CA, USA) UV-Vis Cary 100 spectrometer. Contents were expressed in GAE (gallic acid equivalents) and CE (catechin equivalents), respectively. Total carotenoids in *D. carota* subsp. *gummifer* were also determined spectrophotometrically, following the methodology described by Garcia Camacho, et al. [31].

The hydroalcoholic plant extracts were studied by gas chromatography-mass spectrometry (GC-MS) at the Research Support Services (STI) at Universidad de Alicante (Alicante, Spain), using a gas chromatograph model 7890A coupled to a quadrupole mass spectrometer model 5975C (both from Agilent Technologies). The chromatographic conditions were: injection volume = 1 µL; injector temperature = 280 °C, in splitless mode; initial oven temperature = 60 °C, 2 min, followed by ramp of 10 °C/min up to a final temperature of 300 °C, 15 min. The chromatographic column used for the separation of the compounds was an Agilent Technologies HP-5MS UI of 30 m length, 0.250 mm diameter, and 0.25 µm film. The mass spectrometer conditions were: temperature of the electron impact source of the mass spectrometer = 230 °C and of the quadrupole = 150 °C; ionization energy = 70 eV. NIST11 library was used for compound identification.

2.5. In Vitro Antimicrobial Activity Assessment

The antibacterial activity was assessed according to CLSI standard M07-11 [32], using the agar dilution method to determine the minimum inhibitory concentration (MIC). In short, an isolated colony of *E. amylovora* in TSB liquid medium was incubated at 30 °C for 18 h. Serial dilutions were then conducted, starting from a 10^8 CFU·mL^{-1} concentration, to obtain a final inoculum of ~10^4 CFU·mL^{-1}. Bacterial suspensions were then delivered to the surface of PDA plates, to which the bioactive products had previously been added at concentrations ranging from 62.5 to 1500 µg·mL^{-1}. Plates were incubated at 30 °C for 24 h. In the case of *X. ampelinus*, the same procedure was followed, albeit at 26 °C. Readings were taken after 24 h. MICs were determined visually in the agar dilutions as the lowest concentrations of the bioactive products at which no bacterial growth was visible. All experiments were run in triplicate, with three 3 plates per treatment/concentration.

The antifungal activity of the different treatments was determined using the agar dilution method according to EUCAST standard antifungal susceptibility testing proce-

dures [33], by incorporating aliquots of stock solutions onto the PDA medium to obtain concentrations in the 62.5–1500 $\mu g \cdot mL^{-1}$ range. Mycelial plugs (∅ = 5 mm), from the margin of 1-week-old PDA cultures of *D. seriata*, were transferred to plates incorporating the above-mentioned concentrations for each treatment (3 plates per treatment/concentration, with 2 replicates). Plates were incubated at 25 °C in the dark for a week. PDA medium without any amendment was used as the control. Mycelial growth inhibition was estimated according to the formula: $((d_c - d_t)/d_c) \times 100$, where d_c and d_t represent the average diameters of the fungal colony of the control and of the treated fungal colony, respectively. Effective concentrations (EC_{50} and EC_{90}) were estimated using PROBIT analysis in IBM SPSS Statistics v.25 (IBM; Armonk, NY, USA) software.

The level of interaction, i.e., synergy factors, were determined according to Wadley's method [34].

2.6. Statistical Analysis

The results of the inhibition of mycelial growth of *D. seriata* as affected by the different concentrations of the treatments were statistically analyzed using one-way analysis of variance (ANOVA), followed by *post hoc* comparison of means through Tukey's test at $p < 0.05$. IBM SPSS Statistics v.25 software was used.

3. Results and Discussion

3.1. Plant Biomass Characterization

3.1.1. Elemental Analysis of Plant Fractions

The C, H, N, and S percentages of *C. maritimum* components were in the 36.6−40.0%, 6.2–6.3%, 0.7–1.6%, and 0.05–0.1% range, respectively, and those of *D. carota* subsp. *gummifer* in the 39.7−42.8%, 6.3−6.4%, 0.6–2.5%, and 0.0–0.3% range, respectively (Table S1). The distribution of N content showed maximum values in the flowering aerial parts, resulting in C/N ratio values noticeably lower than those found for stems and roots. Regarding the elemental analysis of the gels that resulted from the concentration by vacuum evaporation of the hydroalcoholic extracts of the flowering aerial parts of *C. maritimum* and *D. carota* subsp. *gummifer*, presented in Table S2, slightly higher C/N ratios than those reported in Table S1 were observed.

3.1.2. Thermal Characterization of Flowering Aerial Parts

The DSC curve of the flowering aerial parts of *C. maritimum* (Figure S1) showed exothermic peaks at 290, 330, and 416 °C, in good correspondence with the exothermal effects associated with xylan and lignin [35]. From the TG curve, the ash content was 2.8%. In the case of the umbel of *D. carota* subsp. *gummifer* (Figure S2), exothermal effects occurred at 323, 402, and 444 °C, and the ash content was 2%.

3.1.3. Vibrational Characterization

The FTIR spectra of the various fractions of *C. maritimum* (Table S3) showed the specific bands characteristic of oleaginous plants. In particular, the lipid acyl chains absorb at 2916 and 2848 cm^{-1}, and at 1516 and 1320 cm^{-1}, while at 1732 cm^{-1} the ester carbonyl IR response could be observed. The intensity of these bands was in agreement with the high concentrations of oils that this halophyte can store [36].

The spectra from *D. carota* subsp. *gummifer* (Table S3) featured three specific bands of carotenes at ~1514, ~1147, and ~1009 cm^{-1}. The intensity of the bands at 2360 and 2158 cm^{-1} (attributed to CN stretching) pointed to the presence of appreciable amounts of cyanogen glycosides and anthocyanin. Moreover, the intensity of the amide bands also suggested a significant amount of protein. A notable amount of pectin esters may be inferred from the presence of bands at 2918, 1598, and ~808 cm^{-1}, justifying the ability of this plant to produce gum. With regard to the spectrum from the concentrated gel obtained by evaporation of the hydromethanolic extract of *D. carota* subsp. *gummifer* (Figure S3), the peaks at 2916, 2849, 1732, 1369, 1237, 1144, 1095, and 1015 cm^{-1} were found to be in good

correlation (shifts below 20 cm^{-1}) with those of geranyl acetate (2926, 2858, 1742, 1377, 1233, 1163, 1108, and 1024 cm^{-1}).

3.1.4. On the Usefulness of the Above Physicochemical Techniques

Valuable information may be retrieved from the elemental analysis data: C/N ratios can shed light on the relative presence of carbohydrates and lipids vs. amines, amides, nitriles, and nitro compounds. Hence, the aerial parts, in which the lowest C/N ratios were registered, are to be used if one would like to obtain a high content of bioactive heterocyclic compounds in the hydroalcoholic extracts.

Infrared spectral fingerprinting is useful to identify and/or fingerprint pectins, proteins, aromatic phenolics, cellulose, hemicellulose, etc. without—in most cases—the need for any physical separation [37]. The Apiaceae dicotyledonous herbs *C. maritimum* and *D. carota* are spectroscopically very different from the Gramineae due their higher degree of esterification, which can be crudely assessed by the ratio of the areas of the ester band (at around 1730 cm^{-1}) to the polysaccharides band (at 1170–970 cm^{-1}). Nevertheless, the spectra of *C. maritimum* exhibited five specific bands of cellulose (1472 cm^{-1}, 1320 cm^{-1}, 1104 cm^{-1}, 1074 cm^{-1}, 1034 cm^{-1}), and presence of xylan and lignin could also be inferred from the TG-DTG data for *C. maritimum*. This would support the hypothesis of Abideen, et al. [38], who put forward that the lignocellulosic biomass of this plant could be a potential source of biomass for bioethanol production.

On the other hand, given that the fatty acid methyl ester composition of the oils from *C. maritimun* and *D. carota* is comparable to those of other oil crops used for biodiesel production [39], and taking into consideration that their ash contents are not high, their valorization for this application, proposed by Sotiroudis, et al. [36], certainly deserves further attention.

Notwithstanding the above considerations on the utility of thermal and vibrational techniques for plant characterization and applications, they suffer from limitations to identify specific phytochemicals, making it necessary to make use of other more elucidative techniques, such as GC-MS (see below).

3.2. Extracts Characterization

3.2.1. Phenolic Contents

Extracts from *C. maritimum* from the Cantabrian Sea coast showed total phenolic contents (4.6–8.3 mg GAE·g^{-1} dw) and total flavonoid contents (3.0–5.6 mg CE·g^{-1} dw) similar for those reported for Mediterranean origins, such as Tunisia [30,40] (4.1–7.9 mg GAE·g^{-1} dw and 2.9–6.1 mg CE·g^{-1} dw) or the Adriatic coast in Croatia [4] (4.7–9.5 mg GAE·g^{-1} dw and >3.7 mg CE·g^{-1} dw).

For *D. carota* subsp. *gummifer*, the total phenolic content (5.0 mg GAE·g^{-1}) was lower than those found by Ksouri, et al. [41] for *D. carota* L. spp. *carota* extracts (between 7.1 and 13.8 mg GAE·g^{-1}). With regard to the amount of carotenoid components in the umbel extract, by our terpene analyses, it was 81 mg β-car/100 g dw, slightly lower than that reported for *D. carota* leaves (83.5 mg β-car/100 g dw) [42].

3.2.2. Active Components by GC-MS Analysis

GC-MS of *C. maritimum* hydromethanolic extracts (Table 1, Figure S4) allowed the identification of 1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene (apiole) [m/z = 77, 106, 121, 149, 177, 207, 222], methylthymol [m/z = 91, 119, 149, 164], and 1,2-dimethyl-3-phenylcyclopropene [m/z =129] as major components. Apiole and methylthymol are in correspondence with dill-apiole (an isomer of apiole) and thymol methyl ether, two of the major components of *C. maritimum* essential oil from Kélibia and Monestir reported by Jallali, et al. [30]. Dill-apiole was also referred by Ngom, et al. [43], Houta, et al. [9], and Ben Mustapha, et al. [44]; and thymol methyl eter by Alves-Silva, et al. [45] and Nabet, et al. [10]. The main difference between the composition of our extracts and those reported by some authors [9,10,46] is the absence of γ-terpinene and sabinene in the extracts presented herein. The presence of the polyacetylene falcarinol [m/z = 41, 55, 77, 91, 115, 129, 145,

159, 173] was in correspondence with that of falcarindiol reported by Meot-Duros, et al. [47] and Ngom, et al. [43]. 1,2-dimethyl-3-phenylcyclopropene constitutes a class of mini-tag probes that participate in fast biorthogonal ligations reactions with 1,2,3,4-tetrazines and photoclickable tetrazoles [48].

Table 1. Compounds identified in *C. maritimum* hydromethanolic extract by GC-MS.

Peak	R_t (min)	Area (%)	Tentative Assignments
11	9.842	2.78	benzene, 2-methoxy-4-methyl-1-(1-methylethyl)- (also named methylthymol); 3-methoxy-p-cymene (also named 2-isopropyl-5-methylanisole or tymol methyl ether)
15	11.005	0.88	2-methoxy-4-vinylphenol (or 4-vinylguaiacol); 1-(2-hydroxy-5-methylphenyl)ethanone; 3-methoxyacetophenone
21	14.068	0.80	1,2,3-trimethoxy-5-allylbenzene (or elemicin)
22	15.163	54.58	1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene (or apiole)
33	18.143	0.92	ethyl 2-(3-hydroxyphenyl)acetate methanol, cyclohexylphenyl-1-(4-hydroxyphenyl)-2-(3-hydroxyphenyl)ethane
49	19.170	3.78	falcarinol; propenoic acid, 3-(cycloheptatrien-7-yl-, methyl ester N,N-dimethyl-1H-inden-2-amine
50	20.499	2.79	1-methyl-4-nitrosobenzene; bicyclo[4.2.0]octa-1,3,5-trien-7-ol
51	20.777	23.83	1,2-dimethyl-3-phenylcyclopropene; α-methyl-2-naphthalenemethanol dimethyl; 1,2-diethenyl tricyclo[3.1.0.0(2,4)]hexane-3,6-dicarboxylate

Major constituents of the hydromethanolic extract of *D. carota* subsp. *gummifer* (Table 2, Figure S5) were: (Z)-3,7-dimethyl-2,6-octadien-1-ol, acetate (or geranyl acetate) [m/z = 41, 69, 80, 93, 107, 121, 136, and 154]), any of the three following: 1,2-dicyclohexyl-1,1-propanedicarbonitrile; 1,6-dibromohexane or 3-methylbut-2-enoic acid, 3,5-dimethylphenyl ester; anhydro-4,6-dimethyl-3-[p-chlorophenyl]-7-hydroxy-1,2,4-triazolo[1,5-a]pyrimidinium-5-one and/or bromocyclohexane; and γ-sitosterol. For comparison purposes, Gil Pinilla, et al. [7] reported the presence of geranyl acetate, linalool, sabinene, terpinen-4-ol, geraniol, α-pinene, and β-pinene in the EO of *D. carota* subsp. *gummifer* from Santander, Cantabria, Spain. The main constituents of *D. carota* subsp. *maritimus* and *D. carota* "Nantes" EOs (from Turkey) reported by Majdoub, et al. [49] and Keser, et al. [50] were geranyl acetate, β-bisabolene, γ-bisabolene, terpinolene, elemicin, myristicin, 5-caffeoylquinic acid, 5-feruoylquinic, and dicaffeic acid. In our study, instead of carotol sesquiterpene, reported by Valente, et al. [8], cariophyllene [m/z = 41, 55, 69, 79, 91, 119, 133, 147, 161, 175, and 189], cariophyllene oxide and farnesene sesquiterpenes were found. Caryophyllene oxide was also reported as a major compound of the hydrosol extract from aerial parts of *Daucus carota* subsp. *sativus* by Tabet Zatla, et al. [51]. Bisabolene was also registered, although as *trans*-Z-α-bisabolene epoxide (R_t = 17.105) and in small amounts. For a thorough comparison of the main components of *D. carota* from different origins, the interested reader is referred to Bendiabdellah, et al. [52].

The possibility of exploitation of the two studied plants for agricultural chemicals industry applications is supported by above GC–MS results: apiole and dill-apiole, major constituents of *C. maritimum*, have been shown to be a good insecticide when they were isolated from the roots of *Anethum graveolus* L. [53], whereas geranyl acetate, the major component of *D. carota*, has antifungal and anti-inflammatory properties, referred in the studies by Gonçalves, et al. [54] and by Khayyat and Sameeh [55]. Thymol, a phytochemical from *C. maritimun*, interferes with the formation and viability of hyphae and induces morphological alterations in the envelope (i.e., the plasma membrane and the mannoproteins, enzymes, beta-glucans, and chitin of the wall) of *C. albicans*, and it also exhibits anti-inflammatory effects by reducing the production and gene expression of the pro-inflammatory mediators [56]. Falcarinol has also been identified as an important

antifungal compound, inhibiting spore germination of various fungi in concentrations ranging from 20 to 200 μg·mL^{-1} [57].

Table 2. Compounds identified in *D. carota* subsp. *gummifer* hydromethanolic extract by GC-MS.

Peak	R_t (min)	Area (%)	Tentative Assignments
6	6.219	1.12	1,6-anhydro-2,4-dideoxy-β-D-ribo-hexopyranose; propanoic acid, 2,2-dimethyl-, hexyl ester; 2-methylbutanal
20	11.925	22.73–39.68	(Z)-3,7-dimethyl-2,6-octadien-1-ol, acetate (or geranyl acetate)
22	12.519	2.70	caryophyllene; bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]
26	13.254	1.87	2,6-dimethyl-3,5,7-octatriene-2-ol; geranyl acetate, 2,3-epoxy-
28	13.756	1.49	(E,Z)-α-farnesene; 6-epi-shyobunol; epiglobulol
34	14.569	1.30	caryophyllene oxide; cyclohexaneethanol, 2-methylene-
40	15.528	1.35	1,2,3,5-cyclohexanetetrol, (1α,2β,3α,5β)-; 4-methyl-5-propyl-nonane; trichloroacetic acid, 4-methylpentyl ester
55	19.418	2.61	4-hydroxy-4-(4,6-dimethylcyclohex-3-enyl)butan-2-one; 3-buten-2-one, 4-(3-hydroxy-6,6-dimethyl-2-methylenecyclohexyl)-; 7,8-epoxy-α-ionone
59	19.920	0.65	spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-; biciclo[4,1,0]heptan-3-ol,3,7,7-trimethyl-, [1S-1α,3α,6α]-
62	20.163	1.23	3-carene; tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-
63	20.431	1.16	5-ethyl-2,4-dimethyl-2-heptene; hexan-3-yl (E)-2-methylbut-2-enoate
84	23.201	1.33	hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
86	23.566	2.42	anhydro-4,6-dimethyl-3-[p-chlorophenyl]-7-hydroxy-1,2,4-triazolo[1,5-a]pyrimidinium-5-one
91	24.593	2.38	(9Z,12Z)-1,3-Dihydroxypropan-2-yl octadeca-9,12-dienoate (or β-monolinolein)
97	25.299	5.50	1,2-dicyclohexyl-1,1-propanedicarbonitrile; 1,6-dibromohexane; 3-methylbut-2-enoic acid, 3,5-dimethylphenyl ester
99	25.480	4.92	3-ethyl-2-butenoic acid, phenyl ester; bromocyclohexane
103	25.947	1.71	3-methyl-but-2-enoic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester
107	30.192	2.52–6.95	γ-sitosterol

3.3. In Vitro Antimicrobial Activity

3.3.1. Antibacterial Activity

The inhibition of both *C. maritimum* and *D. carota* extracts against *Erwinia amylovora* and *Xylophilus ampelinus* were similar (Table 3), although it was slightly higher against *X. ampelinus* in the case of *C. maritimum*. As regards the activities of the two main active principles, viz. pure apiole (an essential oil) and pure geranyl acetate (a monoterpene), the obtained results were comparable to or lower than those of the plant extracts. Another was the case for the conjugate complexes, in which a synergistic behavior was observed among COS and the phytochemicals in all cases. The best results against *E. amylovora* were attained with the COS-*C. maritimum* complex (MIC = 187.5 μg·mL^{-1}), while against *X. ampelinus* the lowest MIC (125 μg·mL^{-1}) corresponded to the COS-geranyl acetate complex, followed by the COS-*C. maritimum* complex (MIC = 250 μg·mL^{-1}).

The above results of antibacterial effect of chitosan-phytochemical conjugates against *Erwinia* spp. were in accordance with the previous reports [58,59], in which the chitosan-phytochemical conjugates exhibited higher antimicrobial activity than that of unmodified chitosan. For instance, Kim, et al. [60] reported that the MICs of chitosan-phytochemical

conjugates ranged from 32 to 512 μg·mL^{-1} against foodborne pathogens, while the MICs of the unmodified chitosan were in the 128−1024 μg·mL^{-1} range.

Table 3. Antibacterial activity of chitosan oligomers (COS), *C. maritimum* and *D. carota* subsp. *gummifer* extracts, pure apiole and geranyl acetate, and their corresponding conjugate complexes (COS−*C. maritimum*, COS−*D. carota*, COS−apiole and COS−geranyl acetate) against the two phytopathogenic bacteria under study at different concentrations (expressed in μg·mL^{-1}).

Pathogen	Compound	62.5	93.7	125	187.5	250	375	500	750	1000	1500
E. amylovora	COS	+	+	+	+	+	+	+	+	+	-
	C. maritimum	+	+	+	+	+	+	+	+	+	-
	D. carota	+	+	+	+	+	+	+	+	+	-
	Apiole	+	+	+	+	+	+	+	+	+	-
	Geranyl acetate	+	+	+	+	+	+	+	+	+	+
	COS-apiole	+	+	+	+	+	+	+	-	-	-
	COS-geranyl acetate	+	+	+	+	+	+	+	+	-	-
	COS-*C. maritimum*	+	+	+	+	-	-	-	-	-	-
	COS-*D. carota*	+	+	+	+	+	+	-	-	-	-
X. ampelinus	COS	+	+	+	+	+	+	+	+	+	-
	C. maritimum	+	+	+	+	+	+	+	-	-	-
	D. carota	+	+	+	+	+	+	+	+	-	-
	Apiole	+	+	+	+	+	+	+	+	+	-
	Geranyl acetate	+	+	+	+	+	+	+	+	+	-
	COS-apiole	+	+	+	+	+	+	+	-	-	-
	COS-geranyl acetate	+	+	+	-	-	-	-	-	-	-
	COS-*C. maritimum*	+	+	+	+	+	-	-	-	-	-
	COS-*D. carota*	+	+	+	+	+	+	-	-	-	-

"+" and "-" indicate presence and absence of bacterial growth, respectively.

In line with Kim, et al. [60], it may be speculated that the mechanism of action behind this enhanced behavior operates via multiple mechanisms: positively charged chitosan can interact with the negatively charged bacterial cell surface, which leads to a weakening of the cell wall, either by cell wall damage alone or accompanied by cell lysis. Conjugation with phytochemicals may increase the osmotic pressure-induced disruption and shrinkage of the bacterial membrane because of a reduction in the permeability of the membrane to intracellular components, and the conjugates may also form a barrier on the bacterial surface and prevent the entry of nutrients. It may also be hypothesized that conjugation with phytochemicals increases the affinity of chitosan for the bacterial cell envelope because of an enhanced lipophilicity (conferred—in the case of apiol—by the allyl side chain bonded to the aromatic ring; and, in the case of geranyl acetate, by the presence of two double bonds in the unsaturated chain). In any case, it should be taken into consideration that further research is required to support aforementioned hypotheses.

3.3.2. Antifungal Activity

Diplodia seriata mycelial growth inhibition results are presented in Figure 2 and Figrue S6. The preconized antifungal activity of *D. carota* [8], based on its relatively high content of terpenes, was not observed in our assays. That of *C. maritimum* was also low, with EC_{50} and EC_{90} values of 832 and 2933 μg·mL^{-1}, respectively. Even when its main component, apiole (whose antifungal effect has been referred to the presence of two methoxyl groups in positions 2, 3 of their benzene ring, optimum to gain a correct balance of hydrophilicity-lipophilicity [61]), was assayed as a pure substance, the results were moderate, with EC_{50} and EC_{90} values of 333 and 822 μg·mL^{-1}, respectively.

These results are in line with the low activity of *D. carota* EO against certain *Candida* spp. and *Aspergillus* spp. reported by Valente, et al. [8], and with the lack of activity of apiole against *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporium* reported by Meepagala, et al. [62].

Another was the case for geranyl acetate, the main component of *D. carota*: when it was assayed as a pure substance, it led to EC_{50} and EC_{90} values as low as 147 and 172 μg·mL^{-1}, respectively.

Regarding the activity of the conjugate complexes with COS, an enhancement in the antifungal activity was registered in all cases. The lowest EC_{50} and EC_{90} values were obtained for COS-geranyl acetate (68 and 113 μg·mL^{-1}, respectively) and for COS-*C. maritimum* extract (75 and 331 μg·mL^{-1}, respectively), for which a synergy factor above 5 was obtained (Table 4).

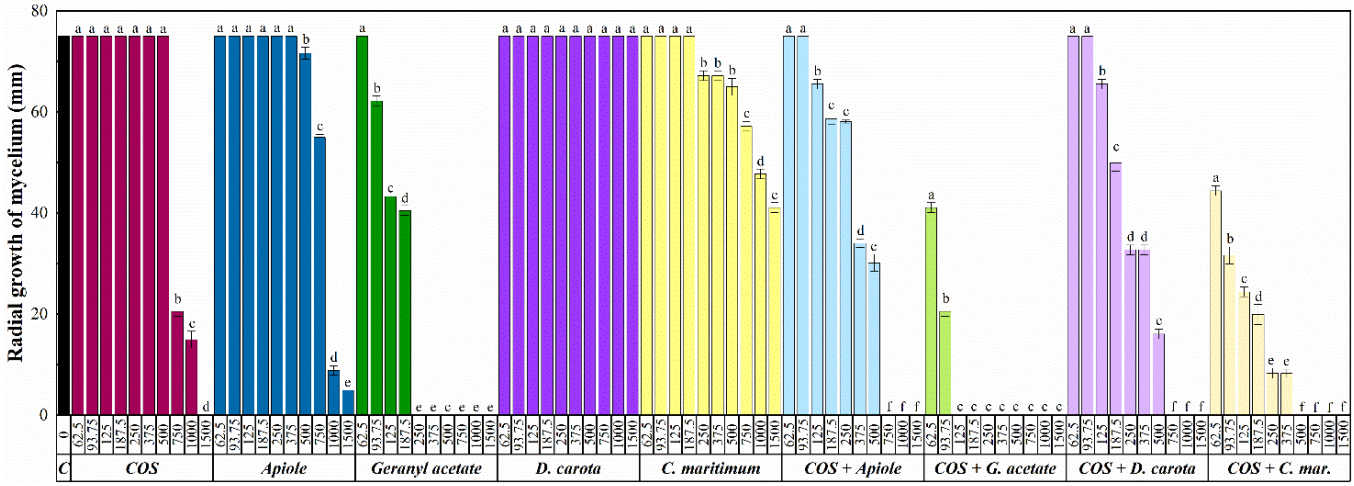

Figure 2. Radial growth of the mycelium for *D. seriata* in in vitro tests conducted in PDA medium with different concentrations (62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000, and 1500 μg·mL^{-1}) of chitosan oligomers (COS), *C. maritimum* extract and pure apiole, *D. carota* extract and pure geranyl acetate, and their respective conjugate complexes. The same letters above concentrations mean that they are not significantly different at $p < 0.05$. Error bars represent standard deviations.

Table 4. EC_{50} and EC_{90} effective concentrations for the different treatments, expressed in μg·mL^{-1}, and synergy factors estimated according to Wadley's method.

Effective Concentration	COS	Apiole	Geranyl Acetate	*D. carota*	*C. maritimum*	COS-Apiol	COS-Geranyl Acetate	COS-*D. carota*	COS-*C. maritimum*
EC_{50}	744	807	147	–	832	333	68	269	75
EC_{90}	1180	1353	272	–	2933	822	113	633	331
SF						1.53	3.91	–	5.08

SF = synergy factor.

The molecular mechanisms behind chitosan interactions with fungi have been recently discussed in a review paper by Lopez-Moya, et al. [63]. Nonetheless, the information available on the mechanism of synergistic action of COS-phytochemical conjugates is not well-established yet. It has been hypothesized that it may be the result of an enhanced additive fungicidal effect *per se*, and/or via a concurrent action on diverse fungal metabolic sites. The conjugation with phytochemicals may increase the cationic surface charge of COS, enhancing the linkage (through electrostatic interactions) to the negatively charged site-specific binding receptors on the fungal membrane [28,64–66].

3.3.3. Comparison with Efficacies Reported in the Literature

Results from studies on the antimicrobial activity of the specific bioactive substances under study (*C. maritimum* and *D. carota* extracts, apiol, and geranyl acetate) against diverse foodborne and clinical bacteria and fungi are summarized in Table 5. The reported MICs and IC_{50} values are generally lower than those reported herein (in this work, the lowest MIC values were 125 and 187.5 μg·mL^{-1} against *X. ampelinus* and *E. amylovora*, respectively, and the lowest EC_{50} and EC_{90} values against *D. seriata* were 68 and 113 μg·mL^{-1}, respec-

tively), but it is worth noting that there are certain pathogens for which no inhibition could be attained, and that there is a large variability in the reported values depending on the bioactive product (and its provenance) and even as a function of the strain/isolate for the same pathogen. A comparison with the values reported for other phytopathogens was not possible, given that no inhibition could be attained using a hexane extract of *C. maritimum* leaves against *Erwinia carotovara* subsp. *carotovora*, and the minimum inhibitory quantity (MIQ = 1 µL/disc) reported using *C. maritimum* roots essential oil against *Mycogone perniciosa* was not expressed in standard units.

A comparison can instead be made with the efficacy of other natural products reported in the literature against the actual phytopathogens under study. To the best of the authors' knowledge, no assays with plant-derived products have been conducted against *X. ampelinus*, but *E. amylovora* has the subject of several studies, summarized in Table 6. In this work, the lowest MIC value against *E. amylovora* was 187.5 $\mu g \cdot mL^{-1}$, better than those attained with the extracts from Damask rose and golden wreath wattle flowers, *Conocarpus lancifolius* leaves and different phenolic extracts from clove, oregano, artichoke, or walnut shells. Nonetheless, lower MICs have been reported for the resinous exudates from *Adesmia boronioides* and alkaloids from African rue seeds.

Table 5. Antibacterial and antifungal activities of *C. maritimum* and *D. carota* extracts, apiol, and geranyl acetate reported in the literature.

Phytochemical	Product Type	Microorganisms	Effectiveness	Ref
Apiole	EO from rhizomes of *Athamanta turbith* 33–49% apiole	Bacteria:	MIC ($mg \cdot mL^{-1}$)	[67]
		E. coli ATCC 25922	43.3	
		P. aeruginosa ATCC 27853	>86.6	
		S. aureus ATCC 25923	43.3	
		S. epidermidis ATCC 12228	86.6	
		M. luteus ATCC 10240	43.3	
		K. pneumoniae NCIMB 9111	>86.6	
		Fungi:		
		C. albicans ATCC 10259	>86.6	
	EO from aerial parts of *Piper holtonii* 57% apiole	Fungi:	IC_{50} ($\mu g \cdot mL^{-1}$)	[61]
		Colletotrichum acutatum	<50	
		Botryodiplodia theobromae	36.16	
Geranyl acetate	EO of lemongrass varieties 0.5–1% geranyl ac.	Bacteria:	MIC ($\mu g \cdot mL^{-1}$)	[55]
		P. aeruginosa	4.5–9	
		S. aureus	4.5–18	
	EO from aerial parts of *Thapsia minor*: 83% geranyl acetate	Fungi:	MIC ($\mu L \cdot mL^{-1}$)	[54]
		C. albicans ATCC 10231	>20	
		C. tropicalis ATCC 13803	>20	
		C. krusei H9	10–20	
		C. guillermondii MAT23	1.25	
		C. parapsilosis ATCC 90018	2.5–5	
		T. rubrum CECT 2794	0.32	
		M. gypseum CECT 2905	0.64	
		M. canis FF1	0.32–0.64	
		C. neoformans CECT1078	0.32	
		E. floccosum FF9	0.16	
		A. flavus F44	>20	
		A. niger ATCC16404	>20	
		A. fumigatus ATCC 46645	10–20	

Table 5. *Cont.*

Phytochemical	Product Type	Microorganisms	Effectiveness	Ref
D. carota subsp. *gummifer*	EO of aerial parts, 37% geranyl acetate	Fungi: *C. albicans* ATCC 10231 *C. tropicalis* ATCC 13803 *C. krusei* H9 *C. guillermondii* MAT 23 *C. parapsilosis* ATCC 90018 *T. rubrum* CECT 2794 *M. gypseum* CECT 2908 *M. canis* FF1 *E. floccosum* FF9 *A. flavus* F44 *A. niger* ATCC 16404 *A. fumigatus* ATCC 46645	MIC ($\mu L \cdot mL^{-1}$) >20 10 >20 1.25 >20 0.32 0.64 0.64 0.32 >20 10 2.5	[8]
	EO of aerial parts 52–77% geranyl ac.	Bacteria: *E. coli* ATCC 25922 *P. aeruginosa* ATCC 27853 *S. aureus* ATCC 25923 *B. cereus* ATCC 9634 *E. faecalis* ATCC 29212 *K. pneumoniae* ATCC 10031	MIC ($mg \cdot mL^{-1}$) >6.0 >6.0 5.1 3.8 4.3 >6.0	[15]
D. carota subsp. *hispidus*	EO of aerial parts	Bacteria: *E. coli* ATCC 35218 *S. aureus* ATCC 25923 *E. faecalis* ATCC 29212	MIC ($mg \cdot mL^{-1}$) 1.25 2.5 1.25	[68]
C. maritimum	Plant extract and EO of aerial parts	Bacteria: *E. coli* ATCC 10536 *P. aeruginosa* ATCC 9027 *S. aureus* ATCC 6538 *B. cereus* ATCC 11778	IC_{50} = 0.47 $mg \cdot mL^{-1}$ (Kélibia) and 3.3 $mg \cdot mL^{-1}$ (Monastir)	[30]
	Hydromethanolic extract of aerial parts	Fungi: *E. coli* ATCC 25922 *C. albicans* ATCC 10231	MIC ($\mu g \cdot mL^{-1}$) 0.11 0.11	[10]
	Hexane extract of leaves	Bacteria: *E. coli* BCC 3.08.001 and ATCC 4157 *B. cereus* BCC 3.05.002 *M. luteus* ATCC 10240 *E. carotovora* BCC 3.08.031 Fungi: *C. albicans* BCC 3.08.036.	MIC ($\mu g \cdot mL^{-1}$) - 50 50 - -	[47]
	Volatile oils of leaves	Fungi: *C. albicans* ATCC 10231 *C. guillermondii* MAT23 *C. neoformans* CECT 1078 *E. floccosum* FF9 *T. rubrum* CECT 2794 *M. gypseum* CECT 2908 *M. canis* FF1	MIC ($\mu g \cdot mL^{-1}$) 2.5–5 0.32–2.5 0.32–0.64 0.08–0.32 0.08–0.32 0.08–1.25 0.08–0.64	[69]
	Essential oil of roots	*M. perniciosa*	MIQ = 1 µL/disc	[17]

In relation to the antifungal activity against *D. seriata*, the lowest EC_{50} and EC_{90} values for the products assayed herein were 68 and 113 $\mu g \cdot mL^{-1}$, respectively. These were substantially lower than those attained with other natural compounds. For instance, a concentration of 1000 $\mu g \cdot mL^{-1}$ was required to completely inhibit the mycelial growth of *D. seriata* for chitosan oligosaccharides (molecular weight < 3000 Da) [80]; and only

96.8% growth inhibition was reached for chitosan at 25 mg·mL^{-1} [80]. Growth inhibition percentages of 20.6, 90.5, 47.7, 68.2, and 77.8% were reported by Cobos, et al. [80] for *Evernia prunastri* lichen extract (4%), garlic extract (10%), lemon peel extract (10%), propolis (10 mg·mL^{-1}), and vanillin (5 mg·mL^{-1}), respectively. If COS-conjugate complexes are considered instead, the EC_{90} values attained with a COS–ε-polylysine conjugate (580 µg·mL^{-1}) [28], and a COS–tyrosine conjugate (672 µg·mL^{-1}) [65] were substantially higher than those obtained for COS-geranyl acetate and COS-*C. maritimum* extract, and comparable to those of COS-apiol and COS-*D. carota* subsp. *gummifer* extract.

Table 6. Natural products assayed against *Erwinia amylovora*.

Phytochemical	Effective Dose	Ref.
EO of *Rosa damascena* flowers	MCB = 1386.5 µg·mL^{-1}	[70]
Water extract (7.4% *w*/*w*) of *Acacia saligna* flowers	MIC = 300 µg·mL^{-1}	[71]
Alkaloids extract from *Conocarpus lancifolius* leaves	MIC > 200 µg·mL^{-1}	[72]
Phenolic extracts from: *Syzygium aromaticum* *Origanum vulgare* *Cynara cardunculus* var. *scolymus* stem *Juglans regia* shells	MIC (mg·mL^{-1}) 10.2 91% inhibition at 41.0 48% inhibition at 41.0 No inhibition	[73]
Exudate from *Adesmia boronioides* (8.5% resin/fresh plant)	MIC = 64 µg·mL^{-1}	[74]
Alkaloids extr. from *Peganum harmala* seeds	MIC = 50 µg·mL^{-1}	[75]
Extracts from *Coccoloba uvifera* leaves: Aqueous Acetone Ethanol	Diam. inhib. zone (mm) at 2500 µg·mL^{-1} 8 ± 1 10 ± 1 14	[76]
EO from: *Cinnamomum zeylanicum* *Laurus nobilis* *Thymus vulgaris* *Syzygium aromaticum* *Pinus* spp. *Cymbogon citratus* *Mentha spicata* *Melaleuca alternifolia*	Diam. inhib. zone (mm), concentr. N/A 31.2 22 20.6 18 17 13 13 12	[77]
EO from aerial parts of flowering: *Thymus vulgaris* *Satureja hortensis*	Diam. inhib. zone (mm), concentr. N/A 25 25	[78]
EOs extr. by steam or hydrodistillation from: *Melissa officinalis* flowers/leaves *Mentha arvensis* aerial part *Nepeta cataria* flowering tops *Origanum compactum* aerial part *Origanum vulgare* aerial part *Thymus vulgaris* aerial part	Diam. inhib. zone (cm), concentr. N/A 6.17–8.7 7.67–12.7 12.1–24.00 21.33–29.3 14.50–25.5 14.33–37.0	[79]

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; EO = essential oil; N/A = not available.

4. Conclusions

The hydromethanolic extract of the aerial parts of *C. maritimum* was found to be rich in apiole (55%) and that of *D. carota* in geranyl acetate (40%). In the in vitro assays, a strong

synergistic behavior was observed upon conjugation of the bioactive constituents of plant extracts with chitosan oligosaccharides, with synergy factors in the 3.9–5.1 range. For the COS-*C. maritimum* complex, MIC values of 187.5 and 250 $\mu g \cdot mL^{-1}$ were obtained against *E. amylovora* and *X. ampelinus*, respectively; and EC_{50} and EC_{90} values of 75 and 331 $\mu g \cdot mL^{-1}$ were found against *D. seriata*. For COS-*D. carota* extract, a MIC value of 375 $\mu g \cdot mL^{-1}$ was observed against the two bacterial phytopathogens; and an EC_{90} of 633 $\mu g \cdot mL^{-1}$ was attained against *D. seriata*. Taking into consideration that the conjugate complexes of both halophyte extracts showed a better performance than other natural compounds reported in the literature against *E. amylovora* and *D. seriata*, they may be put forward as promising antimicrobial treatments, either in organic agriculture or as a substitute for treatments based on chemical synthesis fungicides in conventional management.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050886/s1, Table S1: Elemental (CHNSO) composition (wt.%) of *C. maritimum* and *D. carota* fractions; Table S2: Elemental composition (wt.%) of *C. maritimum* and *D. carota* flowering aerial parts concentrate hydromethanolic extracts; Table S3: Main bands in the ATR-FTIR spectra of various *C. maritimum* and *D. carota* subsp. *gummifer* fractions and their assignments; Table S4: GC/MS analysis of *C. maritimum* hydromethanolic extract; Table S5: GC/MS analysis of *D. carota* subsp. *gummifer* hydromethanolic extract; Figure S1: TG, DSC and DTG curves for *C. maritimum*; Figure S2: TG, DSC and DTG curves for *D. carota* subsp. *gummifer*; Figure S3: ATR-FTIR spectrum of *D. carota* subsp. *gummifer* hydromethanolic extract; Figure S4: GC-MS spectrum of *C. maritimum* hydromethanolic extract; Figure S5: GC-MS spectrum of *D. carota* subsp. *gummifer* hydromethanolic extract; Figure S6: Sensitivity test for *D. seriata*.

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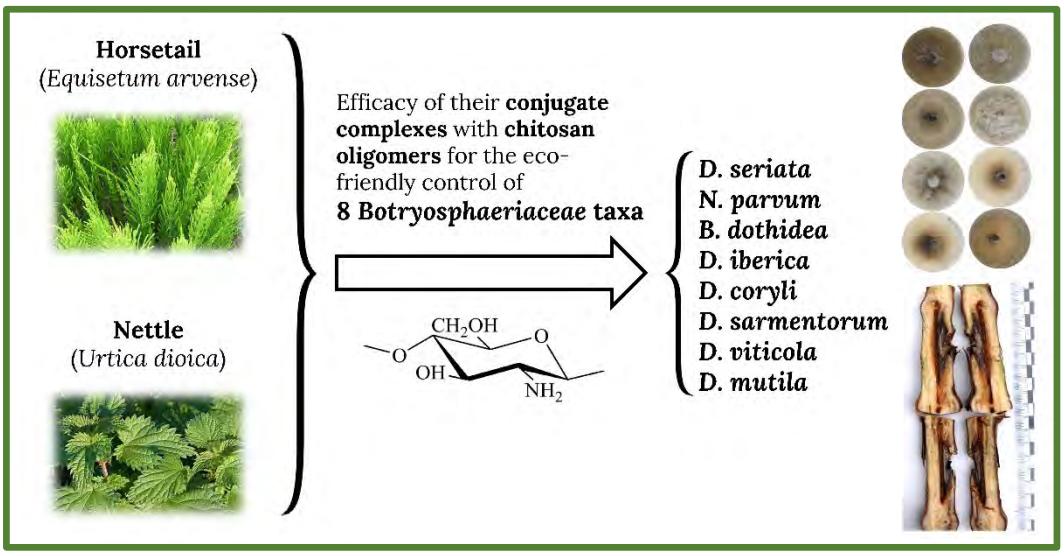

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Assessment of Conjugate Complexes of Chitosan and *Urtica dioica* or *Equisetum arvense* Extracts for the Control of Grapevine Trunk Pathogens

Natalia Langa-Lomba [1,2], Laura Buzón-Durán [3], Pablo Martín-Ramos [1], José Casanova-Gascón [1,*], Jesús Martín-Gil [3], Eva Sánchez-Hernández [3] and Vicente González-García [2]

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Abstract: In the work presented herein, we analyze the efficacy of three basic substances that comply with European Regulation (EC) No 1107/2009, namely chitosan, horsetail (*Equisetum arvense* L.) and nettle (*Urtica dioica* L.), for the control of grapevine trunk diseases (GTDs) in organic farming. The *E. arvense* and *U. dioica* aqueous extracts, prepared according to SANCO/12386/2013 and SANTE/11809/2016, have been studied by gas chromatography–mass spectrometry (GC-MS), identifying their main active constituents. The three basic substances, either alone or in combination (forming conjugate complexes), have been tested in vitro against eight *Botryosphaeriaceae* species, and in vivo, in grafted plants artificially inoculated with *Neofusicoccum parvum* and *Diplodia seriata*. A clear synergistic behavior between chitosan and the two plant extracts has been observed in the mycelial growth inhibition tests (resulting in EC_{90} values as low as 208 $\mu g \cdot mL^{-1}$ for some of the isolates), and statistically significant differences have been found in terms of vascular necroses lengths between treated and non-treated plants, providing further evidence of aforementioned synergism in the case of *D. seriata*. The reported data supports the possibility of extending the applications of these three basic substances in Viticulture beyond the treatment of mildew.

Keywords: basic substances; *Botryosphaeriaceae*; chitosan; fungicide; GTDs; horsetail; nettle; *Vitis vinifera*

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1. Introduction

Phytofungicides are receiving increasing attention as an alternative to synthetic fungicides for the management of many fungal plant diseases [1,2], due to their advantages in terms of safety, easy biodegradability, environmental friendliness and low toxicity.

In the European Union, some of the active substances allowed in organic production (viz. bio-sourced and traditional botanical extracts, light supports/aids and plant defense enhancers), have been approved as 'basic substances' under the EU plant protection products regulation (Article 23 of (EC) No 1107/2009) [3]. These basic substances are listed in Part C of the Annex to Regulation 540/2011, and include *Equisetum arvense* L., chitosan hydrochloride, *Urtica* spp., *Salix* spp. cortex, mustard seeds powder and *Allium cepa* L. bulb extract, among others.

Chitosan exhibits antimicrobial properties, but also functions as an elicitor, stimulating natural defense mechanisms [4]. The accepted and potential mechanisms of action behind its antimicrobial properties are thoroughly discussed in the review paper by Ma, et al. [5].

According to SANCO/12388/2013, it can be used in water solution for application on various crops, including 'fruit berries and small fruit'.

Horsetail (*E. arvense*) was the first approved basic substance, in 2014. A complex mixture of biologically-active carbohydrates [6], flavonoids [7] and antioxidants [8] can be obtained from its dried aerial parts. Silicic, tartaric, protocatechuic and caffeic acids, as well as apigenin, kaempferol and isoquercitrin have been found in its extracts [9–11]. Horsetail can be used in accordance with SANCO/12386/2013. In the particular case of *Vitis vinifera* L., discussed in this work, Appendix II includes its use as a fungicide for the control of downy (*Plasmopara viticola* (Berk. & M.A.Curtis) Berl. & De Toni) and powdery (*Erysiphe necator* (Schwein.) Burrill) mildews by foliar application, but extensions of its use against other fungal diseases on vegetable crops and horticulture are being analyzed [12].

More recently, Commission Implementing Regulation (EU) 2017/419 approved *Urtica* spp. as a basic substance. Its biological activity has been referred to its content in acetic, chlorogenic and formic acids, rutin, lecithin and L-prunasin [13]. Review report SANTE/11809/2016 contemplates its use in grapevine to control downy mildew by foliar spraying.

Regarding the applicability of these three basic substances as natural antifungal products for crop-protection, that of chitosan is well-established, as discussed in a recent review by Mukhtar Ahmed, et al. [14]. There are also studies on the antimicrobial properties of extracts from *E. arvense* [15,16] and other *Equisetum* spp. [10,17–19], and *Urtica* spp. extracts [20–24].

Nonetheless, to the best of the authors' knowledge, while chitosan has been tested against grapevine trunk diseases (GTDs) in various studies [25–27], the other two basic substances have not (the application of *E. arvense* extracts against fungal pathogens in relation to grapevine has been limited to assays against *P. viticola* [28] and ochratoxigenic moulds [29]). Taking into consideration that an enhanced antifungal activity generally results from the formation of conjugate complexes between chitosan and other substances of natural origin [30–32], and that the legal framework would place no obstacles to a combined use of already approved basic substances, this possibility deserves to be explored, since it can be instrumental in controlling GTDs, which are among the main challenges facing modern Viticulture [33].

Even though some of GTDs-associated problems have been described for at least a century, from the 1990s there has been a notable advance in unraveling the etiology and epidemiology of a series of complex syndromes first collectively known as grapevine esca [34]. Despite the numerous advances made in the generation of knowledge about this type of pathologies, in the last 25-30 years the incidence and economic losses in the sector due to these mycoses have not stopped increasing [35]. At present, it is commonly accepted that there are several factors that are influencing the advance of this type of phytopathological problems in the vineyard, highlighting above all the changes in cultural practices, the prohibition of certain fungicidal substances and the high demand for propagation material. Concerning current approaches employed to prevent and control these pathologies, Mondello, et al. [33] summarized, in an extensive revision, the different trials and strategies assayed in the last 25 years to find and make available to the market different GTD control strategies, based on a wide-range of organic and inorganic compounds, both synthetic and natural, and on biocontrol agents (BCAs). Some of these approaches have included natural compounds, just in the same way as the ones assayed in the present work.

The aim of the study presented herein has been to explore the effectiveness of aforementioned three basic substances and their conjugate complexes against certain GTDs, with a view to providing scientific evidence to support their extension to other applications in Viticulture beyond the treatment of diseases that affect the green organs.

2. Materials and Methods

2.1. Fungal Isolates

The eight fungal isolates employed in the study represented some of the main *Botryosphaeriaceae* taxa associated with the so-called Botryosphaeria dieback/Black Dead Arm disease in Spain (Table 1) and were supplied as lyophilized vials (later reconstituted and refreshed as PDA subcultures) by the Agricultural Technological Institute of Castilla and Leon (ITACYL, Valladolid, Spain).

Table 1. Fungal isolates used in the study.

Code	Isolate	Binomial Nomenclature	Geographical Origin	Host/Date
ITACYL_F098	Y-084-01-01a	*Diplodia seriata* De Not.	Spain (DO Toro)	Grapevine (Tempranillo) 2004
ITACYL_F111	Y-091-03-01c	*Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L.Phillips	Spain (Navarra, nursery)	Grapevine (Verdejo) 2006
ITACYL_F141	Y-127-02-01	*Botryosphaeria dothidea* (Moug.) Ces. & De Not.	Spain (Galicia)	Grapevine 2005
ITACYL_F066	T-046-05-3B	*Dothiorella iberica* A.J.L.Phillips, J.Luque & A.Alves	Spain	Grapevine (Tempranillo) 2009
ITACYL_F187	Y-291-24-01	*Diplodia coryli* Fuckel	Spain (Gordoncillo, León)	Grapevine (Prieto Picudo) 2010
ITACYL_F081	Y-051-04-03a	*Dothiorella sarmentorum* (Fr.) A.J.L.Phillips, A.Alves & J.Luque	Spain (DO Tierra de León)	Grapevine (Prieto Picudo) 2004
ITACYL_F118	Y-103-08-01	*Dothiorella viticola* A.J.L.Phillips & J.Luque	Spain (Extremadura)	Grapevine 2004
ITACYL_F080	Y-050-05-01c	*Diplodia mutila* (Fr.) Mont.	Spain (DO Ribera de Duero)	Grapevine 2004

2.2. Reagents and Preparation of Chitosan Oligomers and Bioactive Formulations

Chitosan (CAS 9012-76-4; high MW: 310,000–375,000 Da) was supplied by Hangzhou Simit Chem. & Tech. Co. (Hangzhou, China). Citric acid (CAS 77-92-9), sodium alginate (CAS 9005-38-3) and calcium carbonate (CAS 471-34-1) were purchased from Sigma-Aldrich Química (Madrid, Spain). Neutrase^{TM} 0.8 L enzyme was supplied by Novozymes A/S (Bagsværd, Denmark). Potato dextrose agar (PDA) was purchased from Becton Dickinson (Bergen County, NJ, USA). For the preparation of the *E. arvense* and *U. dioica* extracts, European Pharmacopoeia certified dry plants were purchased from El Antiguo Herbolario (Alicante, Spain).

Chitosan oligomers (COS) were prepared according to the procedure previously reported in [31]. The obtaining of the *E. arvense* and *U. dioica* extracts was conducted according to Appendix I in SANCO/12386/2013 and SANTE/11809/2016, respectively. In short, horsetail extract was obtained by water decoction: 200 g of dry plant were macerated in 10 L of water for 30 min (soaking) and then boiled for 45 min. After cooling down, the decoction was filtrated and further diluted 10-fold with water, to obtain a final concentration of 2000 µg/mL. In the case of nettle extract, dry nettle leaves (15 g/L) were macerated 3 to 4 days at 20 °C, followed by filtering and dilution of the filtrate to obtain a final concentration of 2000 µg/mL.

The COS–nettle extract and COS-horsetail extract complexes were obtained by mixing of the respective solutions in a 1:1 (*v*/*v*) ratio. The mixture was then sonicated for 15 min in five 3-min pulses (so that the temperature did not exceed 60 °C) using a probe-type ultrasonicator (model UIP1000hdT; Hielscher Ultrasonics, Teltow, Germany; 1000 W, 20 kHz).

Infrared spectroscopy was used to confirm the formation of the conjugate complexes (see supporting information).

2.3. Horsetail and Nettle Extracts Characterization

Taking into consideration that materials of plant origin are usually characterized by a high variability of phytochemical composition, resulting from both genetic and environmental variability (due to the influence of weather and soil fertility on the content of active substances), and that extraction procedures also influence the content of bioactive compounds, the aqueous plant extracts were characterized by gas chromatography-mass spectrometry (GC-MS) at the Research Support Services (STI) at Universidad de Alicante (Alicante, Spain). A gas chromatograph model 7890A coupled to a quadrupole mass spectrometer model 5975C (both from Agilent Technologies, Santa Clara, CA, USA) was used. The chromatographic conditions were: injection volume = 1 µL; injector temperature = 280 °C, in splitless mode; initial oven temperature = 60 °C, 2 min, followed by ramp of 10 °C/min up to a final temperature of 300 °C, 15 min. The chromatographic column used for the separation of the compounds was an Agilent Technologies HP-5MS UI of 30 m length, 0.250 mm diameter and 0.25 µm film. The mass spectrometer conditions were: temperature of the electron impact source of the mass spectrometer = 230 °C and of the quadrupole = 150 °C; ionization energy = 70 eV. NIST11 library was used for compound identification.

2.4. In Vitro Tests of Mycelial Growth Inhibition

The fungicidal potential of the different compounds was determined employing an agar dilution method [36]; briefly, aliquots of stock solutions of each product combination were incorporated onto the PDA medium to concentrations in the 62.5−1500 $\mu g \cdot mL^{-1}$ range. Then, mycelial plugs (5 mm in diam.) of each pathogen coming from the margin of 7-day-old PDA cultures were transferred to plates incorporating the above-mentioned concentrations for each compound (3 plates per treatment/concentration, with 2 replicates) and incubated for 6 days (in the case of *N. parvum* and *D. seriata*) or 7 days (for the other six fungi) at 25 °C in the dark, which was the amount of time needed for the isolates to the reach the Petri dish border in the control plates (which consisted of PDA medium without any amendment). Thus, a total of 2400 plates were scored and analyzed as a result of having tested five treatments (COS, *E. arvense* extract, *U. dioica* extract, COS-*E. arvense* conjugate complex and COS-*U. dioica* conjugate complex) at 10 concentrations per treatment against eight fungal pathogens.

Mycelial growth rates were determined by calculating the average diameter of 2 perpendicular colony axes for each replicate. Growth inhibition of each treatment and concentration was calculated and compared with controls at the end of the incubating period according to the formula:

$$((d_c - d_t))/d_c \times 100 \quad (1)$$

where d_c and d_t represent the average diameters of the fungal colony of the control and the treated fungal colony, respectively.

Results were also expressed as both EC_{50} and EC_{90} effective concentrations, estimated by means of PROBIT analysis in R statistical software [37].

2.5. Greenhouse Bioassays in Grafted Plants

Together with the experiments of mycelial growth inhibition in vitro, bioassays with the mentioned basic substances and their conjugate complexes (which comply with EU regulation) were performed in living young grapevine plants in order to scale the protective capabilities of these compounds against two *Botryosphaeriaceae* species responsible for grapevine trunk diseases (GTDs). *Neofusicoccum parvum* and *D. seriata* were then selected on the basis of their prevalence/frequency of isolation in Spain and adjacent areas [38], especially in young grapevine plants coming from nurseries [39]. In summary, potted plants were artificially infected with the two mentioned pathogens, treated simultane-

ously with the different conjugate complexes and, finally, protection effects were analyzed from the comparison of the vascular lesions produced after the different treatments tested with the different controls. Briefly, plant material consisted of 47 plants each of cultivars "Tempranillo" (CL. 32 clone) (2-years old) and "Garnacha" (VCR3 clone) (one year old) grafted on 775P and 110R rootstocks, respectively. Plants were grown on 3.5 L plastic pots containing a mixed substrate of moss peat and sterilized natural soil (75:25), incorporating slow release fertilizer when needed. Plants were maintained in the greenhouse with drip irrigation and anti-weed ground cover for six months (June–December 2020). One week after placing them in the greenhouse, grapevine plants were inoculated with the mentioned two *Botryosphaeriaceae* taxa together with either COS, COS-nettle or COS-horsetail treatments simultaneously. Five repetitions were arranged for each pathogen/control product and plant combination (cultivar/rootstock), together with 4 positive controls per pathogen and cultivar plus 3 negative controls (incorporating only the bioactive product) for each treatment (Table S1). Inoculations of both pathogens and control products were carried out directly on the trunk of the living plants at two sites per plant stand (separated a minimum of 5 cm among them) below the grafting point and not reaching the root crown. For the pathogens, agar plugs coming from 5-days-old fresh PDA cultures of each species were used as fungal inoculum. In the mentioned two inoculation points of each grapevine plant, slits (made up with a scalpel) of approx. 3 mm in diameter and 0.5 cm deep were done. After this, 0.5 cm diameter agar plugs were inoculated and placed in such a way that the mycelium was in contact with vascular tissue in the stem. Calcium alginate beads served as dispersal matrix, including the different control products and conjugates assayed, and beads were placed at both sides of the agar plug. For this, beads were prepared as follows; each control product was added to a 3% sodium alginate solution in a 2:8 ratio (20 mL compound/80 mL sodium alginate). Then, this solution was dispensed drop by drop onto a 3% calcium carbonate solution to spherify (polymerize) in beads of 0.4–0.6 cm diameter containing the different control treatments. Finally, both agar plugs and beads were covered with cotton soaked in sterile bi-distilled water and sealed with Parafilm^{TM} tape. During the assay period, application of copper (cuprous oxide 75%, Cobre Nordox^{TM} 75 WG) to control downy mildew outbreaks was performed in mid-July, together with a first sprouting (followed by periodic sprouting). Grapevine plants were visually examined weekly during the whole assay period for the presence foliar symptoms including both inter-nerval and nerval necroses. After six months in the greenhouse, plants were removed and two sections of the inoculated stems between the grafting point and the root crown were prepared, sectioned longitudinally and the length of the vascular necroses (tracheomycosis) caused by the different pathogens evaluated. Thus, the length of the vascular necroses was measured longitudinally on upper and lower directions from the inoculation point for both halves of the longitudinal cut, and the average measures of these statistically analyzed and compared depending on the type of pathogen and product formulation employed. All the data were compared with controls. Finally, grapevine plants removed and measured at the end of the assay were also processed to re-isolate the different pathogenic taxa previously inoculated. Thus, in order to fulfill Koch's postulates, 0.5 cm long wood chips exhibiting vascular necroses (1–2 cm around the wounds) were washed, surface sterilized, placed in PDA plates amended with streptomycin sulphate (to prevent bacterial contamination) and incubated at 26 °C in the dark in a culture chamber for 2–3 days.

2.6. Statistical Analyses

Data obtained in the in vitro mycelial growth inhibition tests were assessed by analysis of variance (ANOVA) followed by post hoc comparison of means through Tukey's test at $p < 0.05$ (provided that the homogeneity and homoscedasticity requirements were satisfied, according to the Shapiro–Wilk and Levene tests). In the case of greenhouse assay results, since the normality and homoscedasticity requirements were not met, the

Kruskal-Wallis non-parametric test was used instead, with Conover-Iman test for post hoc multiple pairwise comparisons. R statistical software was used [37].

3. Results

3.1. Horsetail and Nettle Extracts

The spectra of the aqueous extracts for the two plant species are presented in Figure 1.

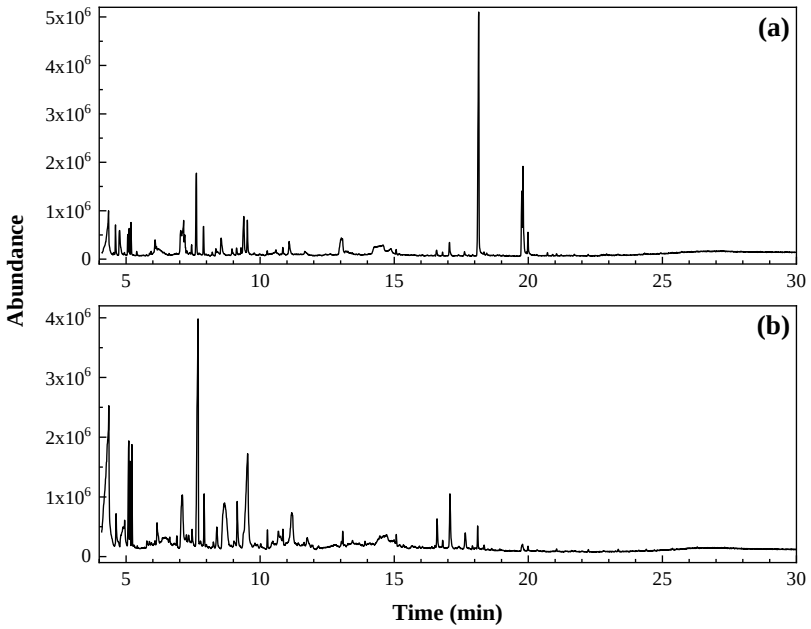

Figure 1. GC/MS spectra of (**a**) *E. arvense* and (**b**) *U. dioica* aqueous extracts.

The main constituents of *E. arvense* aqueous extract were: *n*-hexadecanoic acid or palmitic acid (18.3%), 2-furanmethanol or α-furylcarbinol (9.1%), oleic acid (5.9%), cyclopropyl carbinol (5.0%), 1,6-anhydro-β-D-glucopyranose or levoglucosan (4.1%), 4-oxo-pentanoic acid or levulinic acid (3.9%), 1-bromo-7-(tetrahydro-2-pyranyloxy)heptane (3.8%), (Z,Z)-9,12-octadecadienoic acid or cis-linoleic acid (3.7%), 3-deoxy-d-mannoic lactone (3.6%), dihydroxyacetone (2.8%), 2-ethyl-5-methyl-tetrahydrofuran (2.7%), 5-hydroxymethylfurfural (2.3%) and dihydro-4-hydroxy-2(3H)-furanone (2.2%).

Regarding *U. dioica* aqueous extract, the main phytoconstituents were found to be: 2-furanmethanol (16.7%), N-methyl-1,3-propanediamine (10.1%), thiazole (8.9%), dihydro-4-hydroxy-2(3H)-furanone (6.4%), tetrahydro-2H-pyran-2-methanol (4.1%), 4,5-dihydro-2-methyl-1H-imidazole (2.9%), (S)-2-hydroxy-2-methyl-butanedioic acid or L-citramalic acid (2.3%), 3-deoxy-d-mannoic lactone (2.2%), 2-hydroxy-2-cyclopenten-1-one (2.0%) and N-[2-(4-morpholinyl)ethyl]-α-oxo-1H-indole-3-acetamide (2.0%).

A more detailed analysis of their chemical composition is presented in Tables S2 and S3, together with a comparison with other phytochemical analyses reported in the literature for the extracts from these two plants.

3.2. In Vitro Efficacy

Fungal growth tests for *E. arvense* and *U. dioica* extracts-alone (data not shown) led to very low inhibition percentages (below 25% in all cases). More promising results of growth inhibition were observed in tests employing treatments based on COS, either alone or in combination with the plant aqueous extracts (Figure 2, Figures S3–S5). In these tests, it was observed that, concerning the amount of bioactive compound, the higher the dosage assayed, the higher the growth inhibition obtained for all treatments, with significant differences among concentrations. Together with this, a synergistic effect was observed when conjugate complexes were employed instead of the stand-alone basic substances: while full inhibition was observed for all pathogens for COS at a concentration of

1500 μg·mL^{-1} (except for *D. mutila*, for which full inhibition was reached at 1000 μg·mL^{-1}), complete fungal growth inhibition was attained at noticeably lower concentrations (ranging from 250 to 1500 μg·mL^{-1}, depending on the treatment and isolate) for the COS-plant extracts conjugate complexes. Moreover, the results showed that pathogens such as *D. viticola* or *D. mutila* were much more sensitive to the action of the conjugate complexes than the rest of the species tested: for these two taxa, noticeable reductions in mycelial growth were detected at doses of around 250 μg·mL^{-1}. On the other hand, differences were also observed in the ability to control fungal growth between the two types of conjugates, at least for some of the pathogens: species such as *D. iberica* or *D. coryli* were found to be more sensitive to the action of the COS-*E. arvense* conjugate than to the COS-*U. dioica* treatment, both in terms of the level of reduction of growth rates at the same concentrations and in terms of the lethal dose.

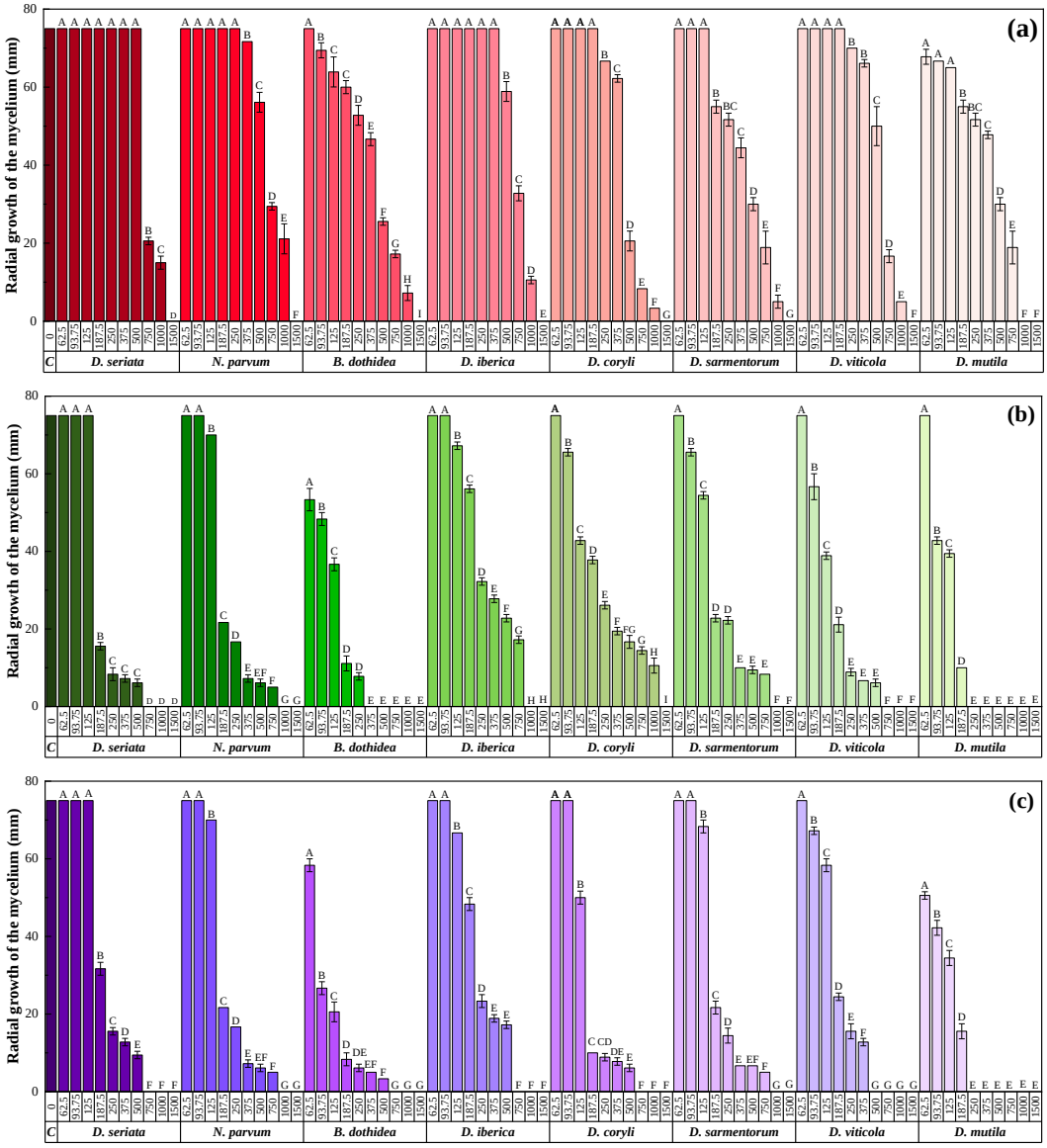

Figure 2. Radial growth of the mycelium for the eight *Botryosphaeriaceae* species under study obtained in in vitro tests conducted in PDA medium with different concentrations (62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000 and 1500 μg·mL^{-1}) of: (**a**) chitosan oligomers (COS); (**b**) COS-*U. dioica* extracts conjugate complex; and (**c**) COS-*E. arvense* extracts conjugate complex. The same letters above concentrations mean that they are not significantly different at $p < 0.05$. Error bars represent standard deviations. 'C' in the leftmost column refers to the control (PDA-only, without any amendment) plates. Only one control plate is plotted for the sake of readability, although there was one control plate per isolate (as shown in the bottom row of Figures S3–S5).

To facilitate comparisons between treatments, the effective in vitro concentrations are summarized in Table 2 (effective concentrations for the *E. arvense* and *U. dioica* extracts alone are not presented, provided that—as mentioned above—full inhibition was not attained even at the highest assayed concentration, so a reliable fitting could not be obtained). In line with the observations made upon comparison of the series of compound dosages (Figure 2), a clear synergistic effect was observed for the two COS-plant extract conjugate complexes in all cases, except for *D. coryli*, in which the EC_{90} for COS alone was lower than that of COS-*E. arvense* formulation. The efficacy for the COS-horsetail and COS-nettle extract treatments were similar in most cases (except against *D. coryli*, in which the performance of COS-nettle extract was noticeably better).

Table 2. EC_{50} and EC_{90} effective concentrations for the different treatments, expressed in $\mu g \cdot mL^{-1}$.

Treatment		*D. seriata*	*N. parvum*	*B. dothidea*	*D. iberica*	*D. coryli*	*D. sarmentorum*	*D. viticola*	*D. mutila*
COS	EC_{50}	744.4	680.2	362.8	706.6	472.2	398.7	554.3	343.7
	EC_{90}	1179.9	1326.6	1191.6	1196.4	972.4	1075.9	1138.7	1196.8
COS-*E. arvense*	EC_{50}	173.9	214.1	109.4	304.1	155.3	198.2	148.2	118.6
	EC_{90}	429.0	637.1	267.1	817.3	999.0	669.0	351.1	208.3
COS-*U. dioica*	EC_{50}	211.5	215.2	72.6	253.0	162.9	203.0	175.3	100.3
	EC_{90}	483.5	650.2	334.4	625.8	411.6	533.0	379.7	227.1

3.3. In Planta Assays

Statistically significant differences were found in terms of the lengths of the vascular necroses between treated and non-treated plants for both fungal pathogens. In addition, visual comparison of the lengths observed after sectioning grapevine plants between treated plants and controls (those plants inoculated only with the pathogens or with the control products, respectively) corroborated statistical results (Figure 3). As regards differences among treatments, in the case of *N. parvum* the performance of the three assayed formulations (and unlike what was observed in in vitro tests) was found to be similar (Table 3), while in the case of *D. seriata* the synergistic behavior observed in the in vitro tests was evidenced (Table 4), with a higher efficacy of the treatments based on conjugate complexes than that of COS alone, which was not significantly different from the control. It was also observed that, in general terms, treatments based on conjugate complexes were slightly more effective when used against *D. seriata*.

Table 3. Kruskal-Wallis test and multiple pairwise comparisons using the Conover-Iman procedure for the lengths of the vascular necroses for *N. parvum*.

Sample	Frequency	Sum of Ranks	Mean of Ranks	Groups		
COS negative control	48	3366.000	70.125	A		
COS-*U. dioica* negative control	48	3458.500	72.052	A		
COS-*E. arvense* negative control	40	3444.500	86.113	A		
COS-*E. arvense*	64	15017.000	234.641		B	
COS-*U. dioica*	72	17119.500	237.771		B	
COS	64	16600.000	259.375		B	
Positive control	64	21194.500	331.164			C

Treatments/controls labelled with the same letters are not significantly different at $p < 0.05$.

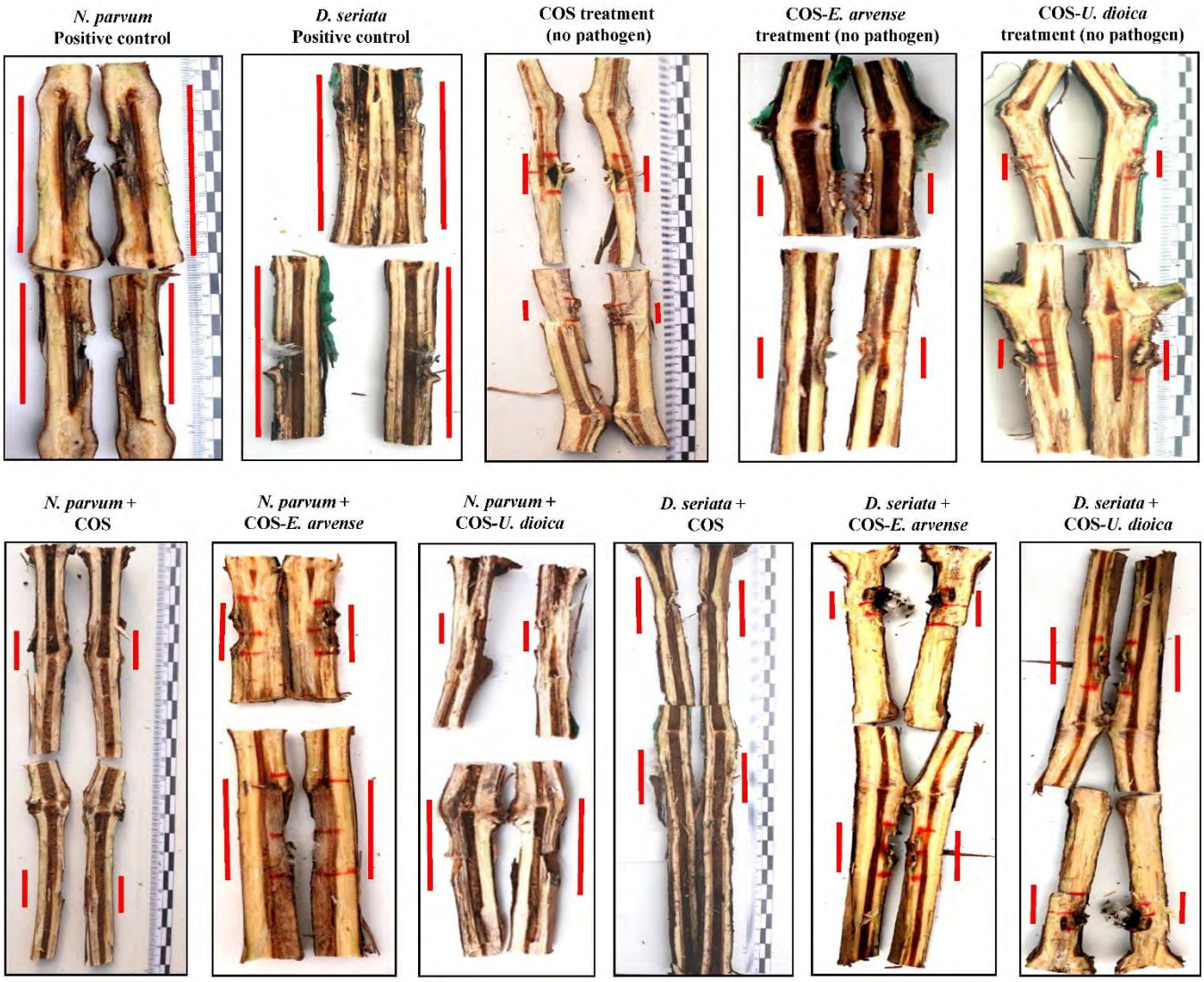

Figure 3. Vascular necroses observed after removal and sectioning of grapevine plants artificially inoculated with both pathogens and control products. Top row, from left to right: vascular necroses produced by *N. parvum*, *D. seriata*, COS treatment, COS-*E. arvense* extract and COS-*U. dioica* extract; Bottom row, from left to right: vascular necroses produced by *N. parvum* + COS, *N. parvum* + COS-*E. arvense* extract, *N. parvum* + COS-*U. dioica* extract, *D. seriata* + COS, *D. seriata* + COS-*E. arvense* extrat and *D. seriata* + COS-*U. dioica* extract, respectively. Red lines delimit the extent of lesions.

Table 4. Kruskal-Wallis test and multiple pairwise comparisons using the Conover-Iman procedure for the lengths of the vascular necroses for *D. seriata*.

Sample	Frequency	Sum of Ranks	Mean of Ranks	Groups		
COS-*U. dioica* negative control	48	4216.500	87.844	A		
COS negative control	48	4255.000	88.646	A		
COS-*E. arvense* negative control	40	4504.500	112.613	A		
COS-*E. arvense*	80	16097.000	201.213		B	
COS-*U. dioica*	80	18098.500	226.231		B	
COS	64	20311.000	317.359			C
Positive control	56	19253.500	343.813			C

Treatments/controls labelled with the same letters are not significantly different at $p < 0.05$.

4. Discussion

4.1. Efficacy Comparisons

Regarding chitosan oligosaccharides-based treatments, Cobos, et al. [25] found a complete inhibition of *D. seriata* and *B. dothidea* at 1000 $\mu g \cdot mL^{-1}$; and EC_{90} values in the 967–1270 $\mu g \cdot mL^{-1}$ range for *N. parvum*, in the 1121–1360 $\mu g \cdot mL^{-1}$ range for *D. seriata* and of 1339 $\mu g \cdot mL^{-1}$ for *B. dothidea* were obtained for the same strains in [30,31] (vs. 1326, 1180, 1192 $\mu g \cdot mL^{-1}$ in this work, respectively). Differences in the inhibitory concentrations may be ascribed to the existence of different isolate-dependent susceptibility profiles or to slight variances in the molecular weight or deacetylation degree of COS, which influence its efficacy.

In connection with *E. arvense* and *U. dioica* extracts, a summary of their effectiveness against various polyphagous phytopathogenic fungi (*Phytophthora infestans, Fusarium* spp., *Aspergillus* spp., *Alternaria* spp., etc.), including grapevine pathogens (*Botrytis cinerea, Plasmopara viticola*) and wood decay fungi (*Phanerochaete chrysosporium, Ceriporiopsis subvermisphora, Gloeophyllum trabeum, Trametes versicolor, Oligoporus placenta, Pleurotus ostreatus* and *Coniophora puteana*) is presented in Table S4. It is worth noting that, although 100% inhibition has been attained against some fungal pathogens by using concentrations of *E. arvense* extract of 3% [40,41] and of *U. dioica* extract of 0.9% [23], such concentrations exceed the limit allowed by the European Union (0.2%). In the studies in which this latter concentration was tested, the inhibition was moderate: for instance, for *E. arvense* extract, La Torre, et al. [28] reported a 32.4% effectiveness against *P. viticola*, and Sen and Yalcin [24] found inhibitions of 25% against *P. chrysosporium, G. trabeum, P. ostreatus* and *C. puteana* for *U. dioica* extracts. These results are comparable to the inhibition found in the present study (<25%).

Concerning COS-based conjugate complexes, EC_{90} values of 507.5, 580.2 and 497.4 $\mu g \cdot mL^{-1}$ were obtained in previous studies for *N. parvum, D. seriata* and *B. dothidea*, respectively, with a COS-ε-polylysine conjugate [31]. When COS-tyrosine conjugate was used instead, EC_{90} values of 1021.4, 672.1 and 707.7 $\mu g \cdot mL^{-1}$, respectively, were reported [30]. For the COS-E. *arvense* extract and COS-*U. dioica* extract conjugates discussed herein, EC_{90} values of 637–650, 429–483 and 267–334 $\mu g \cdot mL^{-1}$ were registered. While the efficacy against *N. parvum* would be slightly lower than that of COS-ε-polylysine conjugate complex, those against *D. seriata* and *B. dothidea* would be higher, with the additional advantage of using legally-accepted basic substances.

As regards a comparison with the EC_{50} values of technical-grade commercial fungicides (Table S5), the values obtained for the conjugate complexes (173.9−211.5, 214.1−215.2, 72.6−109.4 and 100.3−118.6 $\mu g \cdot mL^{-1}$ against *D. seriata, N. parvum, B. dothidea* and *D. mutila*, respectively) would be in the same order of magnitude of the less effective conventional fungicides (e.g., boscalid, metalaxyl or copper oxychloride), but would exhibit a substantially lower effectiveness than fungicides such as flusilazole, tebuconazole, carbendazim or fludioxonil.

With respect to plant bioassays, comparisons of lengths of vascular necroses measured after the application of the different treatments (COS alone and conjugate complexes) showed that, regardless the pathogen considered, the average lengths of necroses were reduced with the different treatments and that these lengths were statistically different from both those produced in control plants inoculated only with the pathogens and from lesions observed when only control products were incorporated to the artificial wounds (Tables 3 and 4). Visual estimations of this protective effect can be also observed in Figure 3: vascular necroses were clearly lower in treated plants (for the three treatments), at both sides of inoculation points, in comparison with positive controls. In this sense, the statistical analyses indicated that the application of control products in the absence of any pathogen produced very low values of vascular discoloration length, which were similar in the three compounds, probably due to a hypersensitivity reaction restricted to the area occupied by the artificially inflicted wounds (Figure 3). In general terms, the median lengths of vascular necrosis obtained after the application of the control products in the case of *N. parvum* were further away from the values recorded in the case of *D. seriata*. Moreover, the treatment of this latter pathogen with COS did not result in a significant

reduction of vascular necrosis, being in the same range as the pathogen control. The control of these two and other *Botryosphaeriaceae* taxa has been extensively studied in vine plants through the use of fungicidal substances, biocontrol agents or natural products. Rusin, et al. [42], in a study on the control of *Lasiodiplodia theobromae* employing a combined set of BCAs, synthetic fungicides and natural products, found different protection levels depending on the product applied, but in the case of plant extracts (garlic and clove), these authors obtained average length values higher than those obtained with the assayed synthetic fungicides and BCAs. Amponsah, et al. [43] evaluated the sensitivity of certain *Botryosphaeriaceae* taxa (*N. luteum*, *N. australe* and *D. mutila*) against several technical-grade commercial fungicides in potted grapevines treated with chemicals, and reported dieback lesion lengths for *N. luteum* noticeably lower than the ones obtained in our study. Other studies on the control of these botryosphaeriaceous fungi dealing with BCAs [44] or conventional fungicides [45] have shown that, with some exceptions, the degree of protection tends to be higher when conventional fungicidal substances are used instead of microbial antagonists.

4.2. Mechanism of Action

Liu, et al. [46] suggested that fatty acids might be applicable to the integrated control of phytopathogens. They tested fatty acids against *Alternaria solani*, *Fusarium oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *lycopersici* and *Colletotrichum lagenarium*, finding that they had an inhibitory effect both on the mycelial growth and spore germination. The extent of inhibition varied depending upon both the type of fatty acids and the fungal species tested. They concluded that saturated fatty acids, i.e., palmitic acid (found in *E. arvense* extract at high concentrations, see above), showed stronger antifungal activity than unsaturated fatty acids. The main molecular mechanism by which fatty acids are thought to act is through their direct insertion into the fungal plasma membrane, resulting in increased fluidity, deregulation of membrane proteins and altered hydrostatic turgor pressure within the cell, leading to cytoplasmic disorder and ultimately to cell death [47]. According to Pohl, et al. [48], palmitic acid should result in an enhanced antifungal efficiency, which has been demonstrated against *Aspergillus niger, A. terreus* and *Emericella nidulans* by Altieri, et al. [49].

With regard to other active substances present in *E. arvense*, dihydroxyacetone (Figure S2) has been reported to exhibit fungicidal activity in medical contexts [50], and 3-deoxy-d-mannoic lactone (present, for instance, in garlic) also has antimicrobial activity [51].

Concerning carbinols, it is recognized that 2-furylcarbinols serve as versatile building blocks in the synthesis of highly oxygenated natural products, via the oxidative conversion of 2-furylcarbinols to pyranones [52]. Pyranone and furanone derivatives, present in both plant extracts discussed herein, have been shown to possess antifungal activity [53].

Imidazoles, such as 4,5-dihydro-2-methylimidazole present in *U. dioica* extract, block ergosterol synthesis, and thereby fungal growth, by binding in the active site of 14a-demethylase enzyme [54]: the key interaction in the active site is the amidine nitrogen atom (N-3), which is believed to bind to the heme iron of the enzyme. This molecular reaction has led to an extensive use of triazoles (conazoles or imidazoles) as systemic fungicides, e.g., triadimefon, triadimenol, difenoconazole, propiconazole, cyproconazole and tebuconazole [55].

In connection with the observed synergistic behavior for the chitosan oligomers-plant extract conjugate complexes, the enhanced efficacy observed for COS-*U. dioica* may be tentatively explained taking into consideration changes in the unsaturated/saturated fatty acids ratio mediated by the imidazoles. On the one hand, it is well-established—on the basis of fatty acid analyses—that plasma membranes of chitosan-sensitive fungi have lower levels of unsaturated fatty acids than chitosan-resistant fungi [56]; and, on the other hand, it has been reported that imidazole antifungal agents at concentrations able to inhibit ergosterol biosynthesis (0.1 μM) decrease the ratio of unsaturated to saturated fatty acids [57]. Hence, the presence of 4,5-dihydro-2-methylimidazole in the conjugate complex would increase the sensitivity of the fungal membrane to COS. This hypothesis would be

supported by a recent study by Lo, et al. [58], who found a synergistic antifungal activity of chitosan with fluconazole against *Candida* spp.

With reference to the synergism observed for COS-E. *arvense*, it may be hypothesized that it would be related to the high content in saturated fatty acids (and particularly palmitic acid) in *E. arvense* extract, which would unbalance the unsaturated/saturated ratio, promoting a higher sensitivity of the fungal membrane to COS. Moreover, palmitic acid would also act as a facilitating factor of the interaction, conferring higher solubility to COS: it has been shown that amphiphilic chitosan derivatives synthesized through grafting of palmitic acid onto chitosan can dissolve in water at concentrations up to 0.35% giving colorless solutions, whereas chitosan is insoluble in water at neutral pH [59].

However, further research is needed to understand the exact mechanism of action and to confirm (or discard) the proposed hypotheses.

4.3. Significance of the Reported Findings, Limitations of the Study and Further Research

The present study deals with the potential of certain phytochemicals or basic substances to control the development of one of the most important fungal group involved in the so-called grapevine trunk diseases, and more specifically one of these mycoses, the so-called "Black Dead Arm" or "Botryosphaeria dieback" [60,61]. At least 20 taxa of this group of ascomycetous fungi have been found to cause wood symptoms in grapevine [61], although some of them have a higher incidence in young grapevine plants coming from nursery [62]. Those include taxa like *N. parvum*, *D. seriata*, *L. theobromae*, *B. dothidea* or *D. mutila*, most of them included in the present study. Some of these species have been associated not only with *Vitis vinifera*, but also with many other plant hosts [63–65], where they can induce cankers, diebacks and fruit rots. Furthermore, one of these taxa studied here, viz. *N. parvum*, is considered nowadays as one of the main mycoses associated with propagation material in Spain, being directly involved in a large percentage of the basal infections observed in grafted plants (from their natural infection in rootstock mother fields [62]) and being ultimately responsible for the failure of young grafted plants, 2–5 years after their plantation. Moreover, this group of *Botryosphaeriaceae* species represents a potential threat to numerous crops in the Mediterranean environment, being linked to woody species that usually share habitat and bioclimatic conditions in large areas of the Mediterranean basin. Taking into consideration that in recent years there has been a drastic reduction in the number of legal active ingredients available for the treatment of these wood pathologies (current legislation recommends the universal adoption of the use of alternative methods and substances for disease control), the testing and evaluation of the protective capacities of certain simple compounds or phytochemicals of natural origin constitute a promising approach for the integrated management of this type of crops.

One clear limiting factor found in the in planta control bioassay deals with the fact that, although the vine plants were artificially infected with two pathogens of economic importance and relevance in the nursery, *N. parvum* and *D. seriata*, these were acquired with a significant baseline level of pre-existing pathologies in the commercial material. Thus, a first phytopathological analysis of some plants that did not sprout in the first days of the trial revealed the presence of previous wood pathologies such as vascular rot present both above the grafting point and in the environment of the root crown, and attributed to species such as *Ilyonectria liriodendri*, *Dactylonectria macrodidyma*, *Rhizoctonia solani* or *N. parvum* itself. Later and at the end of the trial, the processing of the plants that completed the entire bioassay evidenced the presence of these previous pathologies in a large percentage of them. Due to this and related to a second limiting factor in our investigation that had to do with the lack of correlation between foliar symptoms and vascular symptoms, some of the control plants of the trial exhibited symptoms (intra and inter-nerval foliar necrosis or decay of young shoots) not expected based on the absence of pathogenic inoculation. Other aspects susceptible to improvement would be associated with the dispersion medium (calcium alginate) chosen for the in planta assays, that −due to the type of polymerization reaction required for the formulation of the hydrogel beads−

restricts the amount of active ingredient in the bioactive solution that can be incorporated to the matrix to <20%. Furthermore, subsequent experimental designs for testing the germicidal potential of these and other bioactive compounds should include different genotypes for each tested pathogen, to take into account the presumed dose/response variability when establishing sensitivity profiles for each species [66].

Given the speed at which the different fungicidal substances of chemical origin are being banned or withdrawn from their use against this type of wood pathologies, research on the potentialities offered by a wide range of alternative products is increasingly urgent and necessary. All these investigations to be carried out in the coming years must necessarily be based on the discovery, optimization and commercialization of a series of products and formulations based either on the use of substances of natural origin, alone or in combinations of several of them, or on antagonistic microbial agents, all in combination with a less intensive and stressful management of the crop.

5. Conclusions

The antifungal activity of the phytochemicals identified in the extracts of *E. arvense* and *U. dioica*, which may be referred to both their shared constituents (carbinols and other building blocks) and their specific phytochemicals (saturated fatty acids in the case of *E. arvense* and imidazoles in the case of *U. dioica*), was found to be modest in the absence of chitosan oligomers. Nonetheless, for the conjugate complexes of COS with the extracts of the two plants, a clear synergistic behavior was observed, both in vitro—against eight *Botryosphaeriaceae* fungi, with EC_{90} values in the 208–999 μg/mL range—and in vivo—with statistically significant differences in the vascular necroses caused by *N. parvum* and *D. seriata* in artificially inoculated grapevine plants. Such synergism may be ascribed to the contribution of saturated fatty acids to an enhanced sensitivity of the fungal membrane to chitosan, either directly—in the case of *E. arvense* extract—or mediated by imidazoles—in the case of *U. dioica*. Even though larger scale field trials are needed to further confirm the results presented herein, a combined use of these basic substances may be put forward as a promising treatment against GTDs either in organic Viticulture or as a substitute for treatments based on chemical synthesis fungicides in conventional management.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050976/s1, Table S1: Repetitions for each of the plant/treatment combinations in the greenhouse bioassay. Each grafted plant was inoculated at two sites below grafting point; Table S2: GC-MS analysis of *E. arvense* aqueous extract; Table S3. GC-MS analysis of *U. dioica* aqueous extract; Table S4: Examples of application of *E. arvense* and *U. dioica* extracts against phytopathogenic fungi reported in the literature; Table S5: In vitro EC_{50} sensitivity values of some *Botryosphaeriaceae* species to technical-grade fungicides; . FTIR spectra of COS, *E. arvense* and *U. dioica* extract and COS-*E. arvense* and COS-*U. dioica* conjugate complexes; Figure S2. Chemical structures of phytochemicals with potential antifungal activity identified by GC-MS in *E. arvense* and *U. dioica* aqueous extracts; Figure S3. Growth inhibition for the eight *Botryosphaeriaceae* species under study with the chitosan oligomers (COS) treatment; Figure S4. Growth inhibition for the eight *Botryosphaeriaceae* species under study with the COS-*E. arvense* extract conjugate complex treatment; Figure S5. Growth inhibition for the eight *Botryosphaeriaceae* species under study with the COS-*U. dioica* extract conjugate complex treatment.

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ARTÍCULOS SOBRE FUSARIOSIS

Artículo #5 (revista Q1, factor de impacto JCR: 3.893)

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Antifungal Activity against *Fusarium culmorum* of Stevioside, *Silybum marianum* Seed Extracts, and Their Conjugate Complexes

Laura Buzón-Durán; Jesús Martín-Gil; María del Carmen Ramos-Sánchez; Eduardo Pérez-Lebeña; José Luis Marcos-Robles; Ángel Fombellida-Villafruela; Pablo Martín-Ramos

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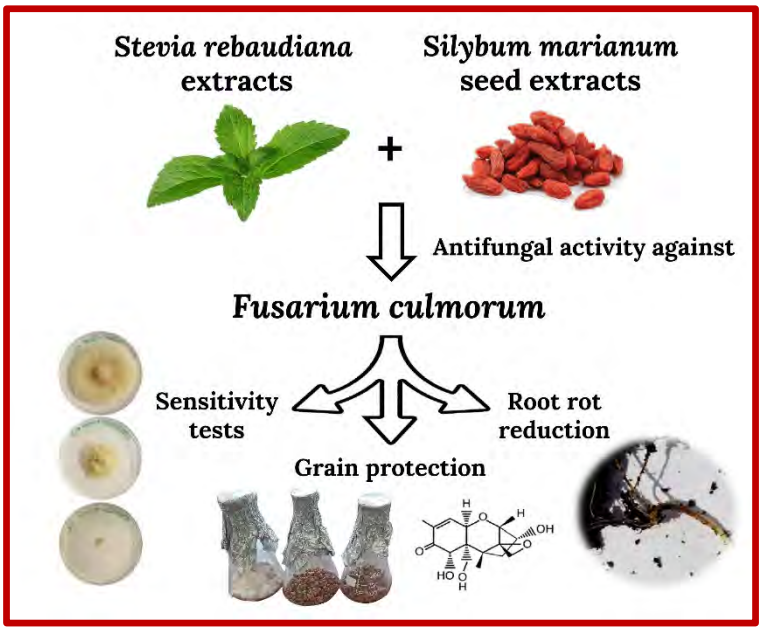

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Antifungal Activity against *Fusarium culmorum* of Stevioside, *Silybum marianum* Seed Extracts, and Their Conjugate Complexes

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Abstract: Fusarium head blight (FHB) is a disease that poses a major challenge in cereal production that has important food and feed safety implications due to trichothecene contamination. In this study, the effect of stevioside—a glycoside found in the leaves of candyleaf (*Stevia rebaudiana* Bertoni)—was evaluated in vitro against *Fusarium culmorum* (W.G. Smith) Sacc., alone and in combination (in a 1:1 molar ratio) with polyphenols obtained from milk thistle seeds (*Silybum marianum* (L.) Gaertn). Different concentrations, ranging from 32 to 512 μg·mL^{-1}, were assayed, finding EC_{50} and EC_{90} inhibitory concentrations of 156 and 221 μg·mL^{-1}, respectively, for the treatment based only on stevioside, and EC_{50} and EC_{90} values of 123 and 160 μg·mL^{-1}, respectively, for the treatment based on the stevioside–polyphenol conjugate complexes. Colony formation inhibition results were consistent, reaching full inhibition at 256 μg·mL^{-1}. Given that synergistic behavior was observed for this latter formulation (SF = 1.43, according to Wadley's method), it was further assessed for grain protection at storage, mostly directed against mycotoxin contamination caused by the aforementioned phytopathogen, confirming that it could inhibit fungal growth and avoid trichothecene contamination. Moreover, seed tests showed that the treatment did not affect the percentage of germination, and it resulted in a lower incidence of root rot caused by the pathogen in Kamut and winter wheat seedlings. Hence, the application of these stevioside–*S. marianum* seed extract conjugate complexes may be put forward as a promising and environmentally friendly treatment for the protection of cereal crops and stored grain against FHB.

Keywords: antifungal; candyleaf; deoxynivalenol; milk thistle; seedling blight; trichothecene

1. Introduction

Pathogenic microorganisms present in the environment are a menace for crops. At a worldwide level, diseases caused by plant pathogenic fungi significantly contribute to overall losses in terms of crop yield. To face this challenge, the use of traditional pesticides entails disadvantages related to handling hazards, cost, residues, and threats to human health and the environment. Consequently, European Directive 2009/128/EC established a framework to achieve their sustainable use, promoting integrated pest management and other alternative approaches such as the use of natural products [1].

Recent regulatory changes (PFC 6 within Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019) have led to a renewed interest in the valorization of the aforementioned natural products as antimicrobial agents, given that new preparations based on natural products are now contemplated as fertilizers (and not as phytosanitary products). In addition to posing a more environmentally friendly alternative to synthetic pesticides, they would also be suitable for application in organic Farming.

A promising source of bioactive products is *Stevia rebaudiana* (Bert.) Bertoni (commonly known as candyleaf), a perennial herbaceous plant of the Asteraceae family. Its leaves contain steviol glycosides (including stevioside, rebaudioside (A to F), steviolbioside, and isosteviol, among which stevioside, rebaudioside A, and rebaudioside C are the major metabolites [2]), polyphenols, chlorophylls, and carotenoids [3]. Such extractives have been suggested to exert beneficial effects on human health [4–6], and several studies have covered their antimicrobial activity [7–10]. Nonetheless, a literature survey revealed that their antifungal/fungistatic activity has been little studied [11–14]. With regard to phytopathogenic fungi, promising results were reported by Shukla et al. [15] and Ramírez et al. [16] against *Fusarium oxysporum*.

Specific and strong inhibitory activities against other *Fusarium* species have also been reported for phenolic and polyphenolic natural compounds [17], but their applicability is limited by their low solubility and bioavailability [18]. Nonetheless, they may be improved through the formation of inclusion compounds or conjugate complexes with, for instance, terpene glycosides [19], as shown by the enhanced in-vitro antifungal activities against *F. culmorum* attained with composites consisting of polyphenol inclusion compounds and silver nanoparticles [20]. The latter soil-borne fungus is one of the most important Fusarium head blight (FHB) causal agents (together with *F. graminearum* Schwabe [21]), and it poses problems in agriculture and the food industry as it not only leads to yield losses of up to 50% but also to trichothecene contamination [22–24].

The aim of the study presented herein is to investigate the antifungal activity of the aforementioned natural bioagents against *F. culmorum*, exploring the presumable synergist effects that could result from the combination of stevioside and milk thistle (*Silybum marianum* (L.) Gaertn) seed extracts, which have been reported to exhibit high total phenolic content [25].

2. Results

2.1. Sensitivity Tests

Two antifungal susceptibility testing experiments were conducted. The antifungal effect was first evaluated by the so-called "poisoned food method", in which the inoculation is done by a mycelial disc (hyphae + spores) that is deposited in the center of the agar plate [26], and then confirmed by determining the inhibition rate percentage of the number of colonies formed, using an inoculum mainly composed of spores [27].

For the first in-vitro experiment, the mycelial growth inhibition obtained for each of the treatments and concentrations after 7 days is shown in Figure 1, and the values across the three replicates and two repeats are summarized in Figure 2.

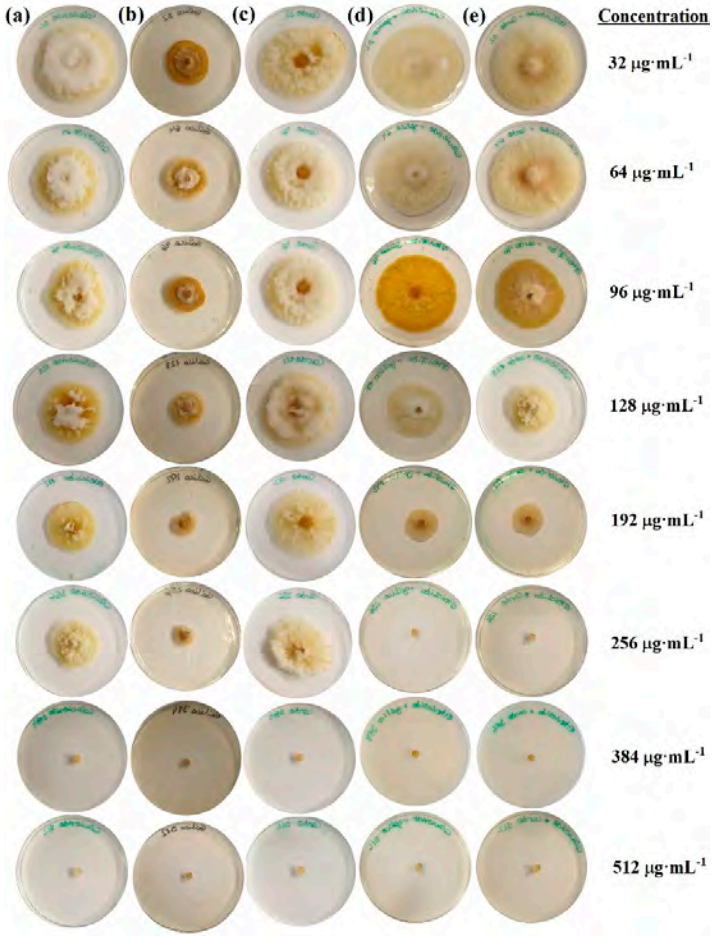

Figure 1. *F. culmorum* radial growth inhibition assays ("poisoned food method") for the treatments based on (**a**) stevioside, (**b**) gallic acid, (**c**) milk thistle seed extracts, (**d**) stevioside–gallic acid, and (**e**) stevioside–milk thistle seed extracts. Only one replicate per treatment and concentration is shown. The control (PDA only) is not shown (radial growth = 60 mm).

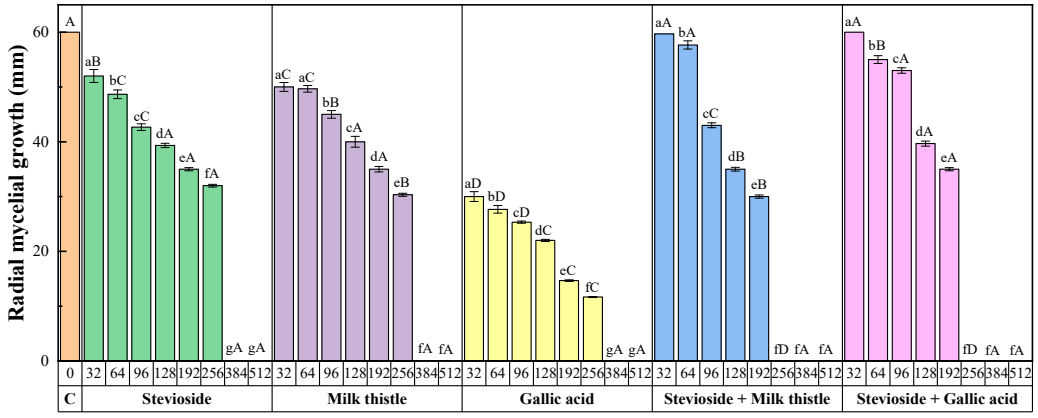

Figure 2. Radial growth values of *F. culmorum* in the presence of stevioside, gallic acid, milk thistle seed extracts, and the stevioside–polyphenol (i.e., stevioside–gallic acid and stevioside–milk thistle seed extract) conjugate complexes at different concentrations (expressed in $\mu g \cdot mL^{-1}$). Concentrations labeled with the same lowercase letters are not significantly different at $p < 0.05$ by Tukey's test. Treatments labeled with the same uppercase letters are not significantly different at $p < 0.05$ at the indicated dose. All values are presented as the average of three replicates and two repeats. Error bars represent the standard deviation.

The increase in the treatment dose from 32 to 512 μg·mL^{-1} resulted in a reduction in the radial growth of the mycelium in all cases, with statistically significant differences amongst the various concentrations (Figure 2). Full inhibition was attained at the two highest doses (384 and 512 μg·mL^{-1}) for all treatments.

Statistically significant differences between treatments were observed at lower concentrations. However, those found at the 256 μg·mL^{-1} dose were particularly interesting: while the treatments based on stevioside–polyphenol conjugate complexes resulted in 100% inhibition, the treatments based on stevioside, milk thistle seeds extracts, and gallic acid led to inhibition percentages of 46.7%, 49.4%, and 80.5%, respectively.

The efficacy of the treatments may also be compared by expressing the sensitivity test results in terms of the concentrations that reduced mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively), summarized in Table 1. The sensitivity of the isolate was very similar for the two treatments based on conjugate complexes, which showed a noticeably better inhibition than the stevioside or the polyphenols-only based treatments. According to the method of Wadley [28] for the quantification of the level of interaction, synergy factors (SFs) of 1.43 and 1.41 (>1.0) were obtained for the stevioside–milk thistle and the stevioside–gallic acid conjugate complexes, respectively, pointing to a synergistic interaction between the fungicides.

Table 1. Effective concentrations that inhibited mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively).

Concentration (μg·mL^{-1})	Stevioside	Milk Thistle	Gallic Acid	Stevioside + Milk Thistle	Stevioside + Gallic Acid
EC_{50}	155.92 ± 26.25	170.08 ± 22.82	150.16 ± 20.13	123.15 ± 14.79	125.33 ± 17.22
EC_{90}	221.01 ± 52.73	237.01 ± 42.74	228.94 ± 42.58	159.70 ± 24.64	160.03 ± 29.39

In the second in-vitro antifungal susceptibility testing experiment, the number of colonies after incubation on the bioactive product-supplemented media was found to be clearly dependent on the bioactive product dose (Figure 3). Full inhibition was reached at 512, 256, and 256 μg·mL^{-1} for the stevioside, stevioside–milk thistle seed extract, and stevioside–gallic acid treatments, although the number of colonies was decreased with all concentrations in comparison with the control. Hence, the results on the inhibition of the number of colonies formed were consistent with the radial growth inhibition tests reported above.

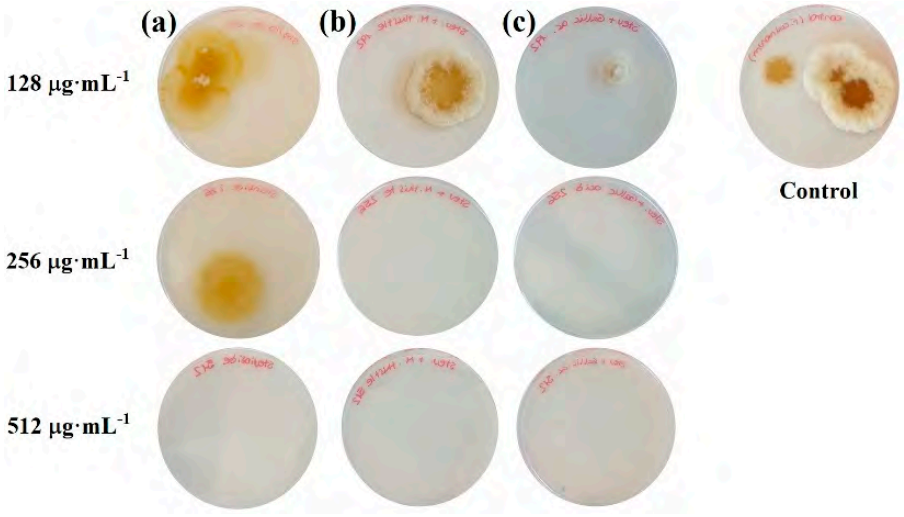

Figure 3. *F. culmorum* colonies formed after 5 days for the (**a**) stevioside, (**b**) stevioside–milk thistle seed extract, and (**c**) stevioside–gallic acid treatments. Only one replicate per treatment and concentration is shown.

2.2. Effect of the Stevioside–*S. marianum* Seed Extract Conjugate Complex on Deoxynivalenol Production

After incubation of the Kamut and winter wheat grain samples inoculated with *F. culmorum* for 28 days, complete inhibition was observed for the grains treated with the stevioside–milk thistle seed extract conjugate complex at a concentration of 512 $\mu g \cdot mL^{-1}$. No deoxynivalenol (DON) was detected (<LOD), while DON contents of 38.5 ± 2.1 and 53.2 ± 0.7 $\mu g \cdot g^{-1}$ were registered for the winter wheat and Kamut positive controls.

2.3. Seedling Tests

No significant differences were observed between the negative control and the noninoculated seeds treated with the conjugate complex (97% and 96% germination rates, respectively, for winter wheat seeds; 98% and 96% germination rates, respectively, for Kamut seeds) in the germination tests (Figure 4). The germination rate for the positive control (inoculated and not treated seeds) was noticeable lower (75% and 72% for winter wheat and Kamut, respectively), but it was clearly improved for the inoculated and treated seeds (92% and 85% for winter wheat and Kamut, respectively).

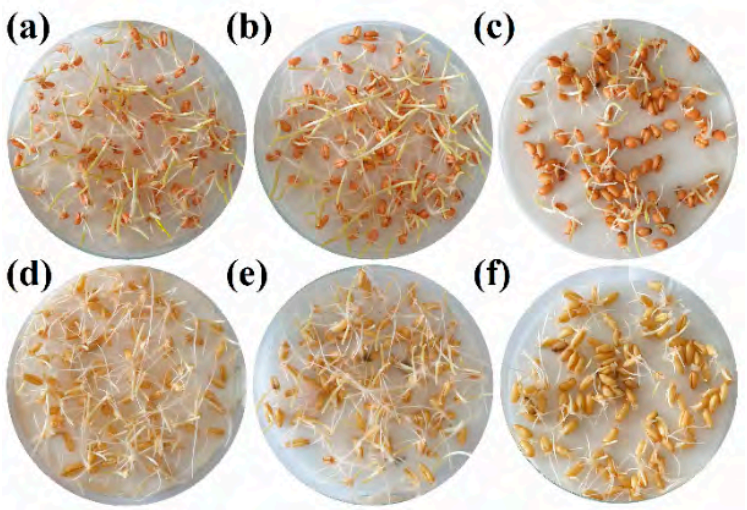

Figure 4. Germination tests for winter wheat seeds—(**a**) negative control; (**b**) treated seeds; (**c**) positive control—and Kamut seeds—(**d**) negative control; (**e**) treated seeds; (**f**) positive control—. Only one replicate is shown.

With regard to root rot symptoms in the seedlings after 2 weeks, no symptoms were observed in the negative control for winter wheat and Kamut. In the positive control, some seedlings showed clear wilting and browning of coleoptiles and roots (Figure 5), particularly for Kamut, and the average disease severity was 10% for winter wheat and 14% for Kamut (with some seedlings reaching a 3 in the 0 to 5 degree of attack scale). In the artificially inoculated seedlings treated with the conjugate complex, the average disease severity was 4% in both cases. In other words, the disease index (DI) was reduced from 50% to 20% for winter wheat and from 35% to 20% for Kamut. Consequently, the control efficacy of the treatment could be deemed as moderate–high (60% and 71.4% for winter wheat and Kamut, respectively).

Regarding the activities of some enzymes used as stress markers, namely, guaiacol peroxidase (POD) and ascorbate peroxidase (APX), or involved in defense-induced metabolic changes, namely, polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL), no statistically significant differences were observed between treated and nontreated artificially inoculated seedlings for winter wheat or Kamut.

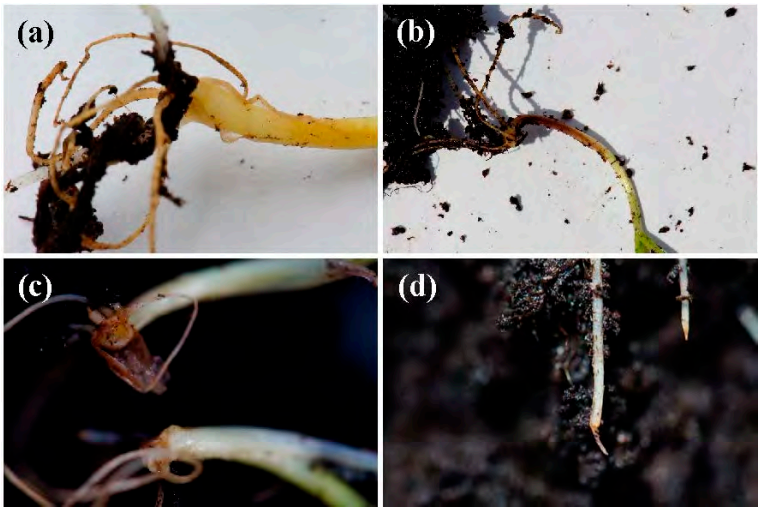

Figure 5. Different root rot symptoms observed in the artificially inoculated seedlings: (**a**) healthy coleoptile and roots; (**b**) necrotic lesions on the coleoptile; (**c**) necrotic lesions on the lower coleoptile, with scarce root development (top) vs. healthy seedling (bottom); (**d**) root apex browning.

3. Discussion

3.1. Efficacy of the Treatments

The stevioside-only treatments showed high inhibitory activity, comparable to that of the polyphenol-only based treatments. This finding supports the antifungal activity of stevia leaves advocated by Shukla et al. [15], which would mainly be ascribed to stevioside and, to a lesser extent, rebaudoside and docosahexaenoic acid [29]. These authors found a MIC of 2 mg·mL^{-1} for *F. oxysporum*, much higher than the one reported herein. Ramírez et al. [16] evaluated the antifungal activity of extracts of different polarity obtained from *S. rebaudiana* leaves against *F. oxysporum* as well, finding that the hexane extract at a concentration of 833 ppm (five times higher than the one reported herein for stevioside) only inhibited mycelial growth by around 50%. The differences may be ascribed to both the sensitivity of the pathogen under study and the use of stevia leaf extract instead of the purified stevioside.

A thorough bibliographical survey yielded only one study (by our group) on the effects of milk thistle seed extracts on *Fusarium* spp. In that study, composites based on stevioside:silymarin inclusion compounds (in a 5:1 molar ratio) combined with chitosan oligomers in hydroalcoholic solution or in ChCl:urea deep eutectic solvent media were assayed. EC_{90} values of 1241 and 830 μg·mL^{-1} (without and with silver nanoparticles, respectively) were obtained in the first dispersion medium, and 394 and 327 μg·mL^{-1} (without and with silver nanoparticles, respectively) in the second dispersion medium [20]. The values reported herein for the stevioside–milk thistle seed extract conjugate complex (1:1 molar ratio) are noticeably better and involve a much simpler preparation procedure.

Concerning the efficacy of gallic acid (taken as a reference in this study) against *Fusarium* spp., Nguyen et al. [30] tested the antifungal activity of gallic acid from *Terminalia nigrovenulosa* Pierre bark against *F. solani*, reporting an 81% rate of disease suppression for a dose of 1000 μg·mL^{-1}. Although, in this case, differences in the efficacy of gallic acid may be ascribed to different *Fusarium* species, conflicting results are observed upon comparison with the work by Pani et al. [17]. In this latter study, the EC_{50} values for the different polyphenols assayed against *F. culmorum* were not reported, but they found that fungal growth was only slightly inhibited when 1.5 mM gallic acid (255 mg·mL^{-1}) was added to the liquid culture, while at 1000× lower concentrations, 80% inhibition was observed in our study. Even if the prediction of the antifungal susceptibility of a single strain is difficult (see [31] and references therein), provided that the *Fusarium* spp. do not have normal minimum inhibitory

concentration (MIC) and minimum effective concentration (MEC) distributions, the difference in intrinsic resistance between the two strains is striking.

It is worth noting that the synergy factors for the stevioside–milk thistle seed extract and the stevioside–gallic acid conjugate complexes were almost identical (1.43 and 1.41, respectively), which suggests that the GAE in milk thistle seed extracts was estimated in an accurate manner and that its poorer solubility problem (as compared to that of gallic acid), which could be responsible for its lower efficacy when used alone, would be solved by conjugation with the glycoside.

Regarding the grain protection trials, it is worth noting that the application of wheat straw vinegar (the main components of which are phenolics and acetic acid), diluted 200-fold, has been reported to significantly decrease wheat *F. graminearum* infection rates and DON content by 66% and 69%, respectively [32]. In a similar fashion, Scaglioni et al. [33] found that microalgal phenolic extracts of *Nannochloropsis* sp. and *Spirulina* sp. were able to control *F. graminearum* development and limit DON contamination (−97% for *Nannochloropsis* and −62% for *Spirulina*). Hence, the performance of the stevioside–milk thistle seed extract conjugate complex was better and comparable to those attained with several essential oils (from oregano, cinnamon, palmarosa, orange, spearmint, verbena, fennel, and rosewood) by Perczak et al. [34].

As regards the seedling tests, the absence of significant differences in the germination rates between the negative control and the noninoculated seeds treated with the conjugate complex suggests that a phytotoxic effect of the formulation could be discarded at the concentration used in this experiment. The disease severity results were consistent with the findings of Wiwart et al. [35], who reported that the response to *F. culmorum* infection was weaker in winter wheat than in *T. polonicum* breeding lines and Kamut. In relation to the absence of statistically significant differences in enzymatic activity, in the case of PAL, it may be readily explained because it would only be evident 2 days after inoculation [36]. In the case of PPO, POD, and APX, our negative results would be consistent with those reported by Orzali et al. [36], who found that the enzymes did not vary significantly among treatments in the case of durum wheat treated with chitosan against *F. graminearum*.

3.2. Mechanism of Action

The antifungal action of the composites may be ascribed to both the phenolic compounds in milk thistle seed extracts and to stevioside. With regard to the former, the inhibitory behavior arises from their ability to disrupt the integrity of the plasma membrane and mitochondrial dysfunction, inducing metabolic stagnation [37]. Gallic acid exhibits both antioxidant as well as pro-oxidant characteristics, which turns it into an efficient apoptosis-inducing agent [38]. In turn, milk thistle seed extracts target the plasma membrane. As explained by Yun et al. [39,40], silymarin increases the permeability of and physically perturbs the plasma membrane, resulting in its malfunction (with depolarization, K^+ leakage, and decrease in membrane fluidity), and induces intracellular reactive oxygen species (contributing to the peroxidation of membrane lipids). Moreover, it is known that phenolic acids and flavonoids bind to adhesins [41], i.e., proteins located on the surface of fungal cells that allow fungi to colonize various substrates and to bind to host tissues.

Concerning the antifungal activity of stevioside (the major constituent of *S. rebaudiana* extract), it is mediated by the isosteviol beta OH derivatives (7β-, 11β-, 12β-, and 17β-hydroxyisosteviols) [42] that result from the stevioside–fungi interaction. In a first step, fungi-mediated hydrolysis of stevioside leads to the aglycon steviol or its rearranged derivative, isosteviol (*ent*-16-ketobeyeran-19-oic acid). Subsequently, fungi metabolize isosteviol into beta OH derivatives through a stereoselective introduction of OH groups at positions C-7, C-11, C-12, and C-17, as well as C-1, C-6, C-15, and further ketonization at the C-1 and C-7 positions [43]. The evidence suggests that the action mechanism of these molecules is related to the uncoupling of mitochondrial oxidative phosphorylation or the permeabilization of the cell membrane as it occurs with 7β-hydroxy-kaurenoic acid (kaurens and beyerans are closely related) [44].

In the case of *F. verticilioides* (the only species of the *Fusarium* genus studied in the literature), the biotransformation of isosteviol leads to *ent*-7β-hydroxy-16-ketobeyeran-19-oic and *ent*-12α-hydroxy-16-ketobeyeran-19-oic as main metabolites (Figure 6) [45,46]. The selective hydroxylation of the ketobeyeran nucleus of these metabolites is similar to the one which has led to the enhanced activity of the metabolites identified by Lin et al. [47], with hydroxylation at the 7β-, 12α-, and 14α-positions and oxidation of the skeleton at the 16-position. There is a presumption that some of these metabolites may be involved in the transformation of isosteviol by *F. culmorum* and that the observed antifungal efficacy may be referred to them.

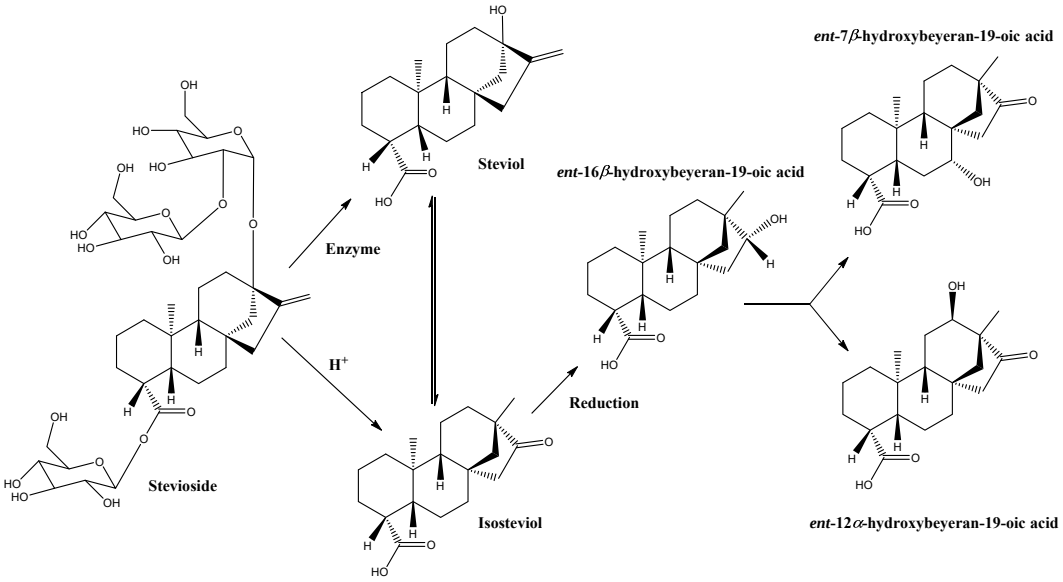

Figure 6. Structures of diterpenoids isolated from microbial transformations of isosteviol. Adapted with permission from Lin et al. [47].

4. Materials and Methods

4.1. Reagents and Fungal Isolate

Stevioside (CAS 57817-89-7, 99%) was purchased from Wako Chemicals GmbH (Neuss, Germany). Powdered milk thistle extract (CAS 22888-70-6; 30% silybin, 98% HPLC) was purchased from KingHerbs Ltd. (Yongzhou, Hunan, China). Gallic acid (CAS 149-91-7; anhydrous, for synthesis), ethanol (CAS 64-17-5; ACS reagent grade), and Tween® 20 (CAS 9005-64-5) were supplied by Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were supplied by Becton, Dickinson & Company (Franklin Lakes, NJ, USA).

Fusarium culmorum strain CECT 20486 was supplied by the Spanish Type Culture Collection (CECT; Valencia, Spain).

4.2. Preparation of the Bioactive Solutions

One treatment based only on stevioside, two treatments based only on polyphenols (either milk thistle extract or pure gallic acid), and their combinations were assayed. It should be clarified that gallic acid was tested as a reference, as materials of plant origin are usually characterized by high variability of phytochemical composition as a result of both genetic variability and environmental variability (influence of weather and soil fertility on the content of active substances).

Ultrasonication-assisted aqueous biphasic system separation was used to prepare the stevioside–polyphenol conjugate complexes in a 1:1 molar ratio. Briefly, 50 mL of an aqueous solution of stevioside (126 mg, MW = 804.87 g·mol^{-1}, 0.156 mM) was mixed with a 50-mL ethanol solution of either gallic acid (26.5 mg, MW = 170.12 g·mol^{-1}, 0.156 mM) or milk thistle seed extract

(250 mg, out of which 75 mg of silybin, MW = 482.44 g·mol^{-1}, 0.156 mM). The polyphenol contents in the initial solutions were 0.26 and 0.29 mg GAE·gDW^{-1}, respectively, as confirmed by Singleton's method, using the Folin–Ciocalteu reagent, in good agreement with Mhamdi et al. [25]. The solutions were sonicated with a probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) for 15 min, keeping the temperature below 60 °C.

4.3. In Vitro Tests of Mycelial Growth Inhibition

The biological activity of the treatments was determined using the agar dilution method, incorporating aliquots of stock solutions into the PDA medium to provide final concentrations of 32, 64, 96, 128, 192, 256, 384, and 512 μg·mL^{-1}. These concentrations, instead of the usual ones defined in CLSI or EUCAST standard antifungal susceptibility testing procedures, were chosen so as to have a true quantitative effect, provided that no growth was observed at concentrations ≥500 μg·mL^{-1}. Mycelial disks of the pathogen (5 mm in diameter) from the edges of a 7-day old culture were transferred to plates filled with these media (three plates per treatment and concentration, with two repeats), using plates containing only the PDA medium as the control.

Radial mycelial growth was determined by the calculation of the average of two perpendicular colony diameters for each replicate. Mycelial growth inhibition for each treatment and concentration after 7 days of incubation, at 25 °C in the dark, was calculated according to the following formula: $((d_c - d_t)/d_c) \times 100$, where d_c is the average diameter of the fungal colony in the control, and d_t is the average diameter of the fungal colony treated with the tested composite.

Results were also expressed as EC_{50} and EC_{90} effective concentrations, estimated by fitting the radial growth inhibition values (%) with a DoseResp function, using an orthogonal distance regression (ODR) algorithm.

4.4. Preparation of Inoculum and Inhibition of Colonies Formation

F. culmorum conidial suspensions were obtained according to the procedure described by Khan et al. [48], with minor modifications. Conidia were harvested from 7-day-old PDB cultures (200 mL cultures, incubated at 25 °C under constant stirring at 140 rpm in a Labolan (Esparza de Galar, Navarra, Spain) orbital stirrer incubator (model ECOLAN 60). The conidial suspension was filtered through two layers of sterile muslin to remove hyphal fragments, and the conidial concentration was first determined using a Weber Scientific International Ltd. (Teddington, Middlesex, UK) Neubauer chamber and then adjusted to a final concentration of 5×10^4 or 1×10^5 conidia·mL^{-1} (depending on the experiment to be conducted afterwards), adding 0.2% Tween® 20.

In vitro tests aimed at determining the inhibition rate percentage of the number of colonies formed were carried out according to the procedure reported by Kheiri et al. [49]. Briefly, 0.5 mL of the conidial suspension (5×10^4 conidia·mL^{-1}) was mixed with different concentrations (128, 256, and 512 μg·mL^{-1}) of the solution of stevioside, stevioside–milk thistle seed extract, or stevioside–gallic acid conjugate complexes to a final volume of 2 mL. The conidial suspension was also prepared with distilled water and 0.5% *v*/*v* acetic acid aqueous solution as the control. The resulting solutions were incubated at 25 °C for 24 h. Aliquots of 50 μL of each dilution were spread on PDA with a Drigalski spatula and incubated at 25 °C, counting the number of colonies formed after 5 days. The test was repeated twice, and each treatment had 3 replicates. The percent inhibition rate was estimated as

$$\% \text{ Inhibition rate} = \frac{\text{number of colonies formed in control plate} - \text{number of colonies formed in treated plates}}{\text{number of colonies formed in control plate}} \times 100 \quad (1)$$

4.5. Effect of Conjugate Complex on DON Production and DON Determination

The effect of the stevioside–milk thistle seed extract conjugate complex on the growth of *Fusarium* fungi on Kamut (*Triticum turgidum* subsp. *turanicum* (Jakubz.) Á.Löve) and winter wheat (*Triticum aestivum* L.; cv. Sofru) grain was investigated using the method described by Perczak et al. [34]. Briefly, 5 mL of the conjugate complex solution, at a concentration of 512 μg·mL^{-1}, was mixed with

25 g of sterile grain in an Erlenmeyer flask. The mixture was vigorously stirred, and three rings (6 mm) of solid culture of *F. culmorum* were then added to each Erlenmeyer flask and further mixed. Solutions of Tween® 20 and deionized water were added to the negative and positive control samples (without the addition of the bioactive compound). Next, the prepared mixtures were stored in the dark at 25 °C for 28 days. After incubation, samples were dried, milled, homogenized, and prepared for chromatographic analysis.

The organic extracts were obtained by soaking of the samples in a mixture of water, methanol, and acetonitrile in a 10:10:30 *v/v* ratio, followed by sonication for 15 min in five 3-min periods. The supernatant solution was filtered with Whatman nº 4 paper and stored at 4 °C until the analytical determinations were carried out. The determination of DON was outsourced to the Laboratorio de Técnicas Instrumentales (University of Valladolid, Valladolid, Spain). The analyses were carried out according to the procedure recommended by Jerome Jeyakumar et al. [50], using a QTOF X500R (Ab Sciex Spain S.L., Madrid, Spain) mass spectrometer coupled to a 2D-UHPLC EXION LC series system and taking a deoxynivalenol solution (CAS 51481-10-8; certified reference material; Sigma-Aldrich, Madrid, Spain) as a reference.

4.6. Seedling Tests

The potential ability of the conjugate complex treatment to induce resistance in seedlings of Kamut and winter wheat against *F. culmorum* was evaluated following the methodology reported by Orzali et al. [36], with minor modifications. Kamut and winter wheat grains were first surface-sterilized for 3 min by immersion in 2% NaOCl and then rinsed with water. The seed treatments (100 seeds per treatment) were performed by immersion in 100 mL of conjugate complex solution (at a concentration of 512 μg·mL^{-1}, with 0.2% Tween® 20) at room temperature, under vigorous stirring for 1 h. Distilled water with 0.2% Tween® 20 was used in the negative and positive controls. The seeds were then air-dried for 30 min, and inoculated by immersion in 100 mL of the 1×10^6 conidia·mL^{-1} suspension, with 0.2% Tween® 20, for 30 min. The seeds were finally air-dried again for 30 min.

The physiological quality of the seeds for each treatment (negative control, positive control, phytotoxicity test, and treatment with the bioactive formulation) was evaluated by germination, as described in the International Rules for Seed Testing. For each treatment, 3 replicates of 100 seeds were placed on glass plates, using the between-paper method, and kept under constant humid conditions. Germination was evaluated after 4 days in such a way that a seed was considered germinated if it produced a well-developed seedling, with three roots and a shoot present.

The efficacy of the antifungal treatment was then assessed by planting the seeds in pots filled with autoclaved peat-based substrate, with a procedure similar to those described by Lozano-Ramírez et al. [51] and Koch et al. [52]. Seedlings were grown in greenhouse conditions at 25 °C, extracted after 2 weeks, and symptoms in the roots and the internode were visually evaluated using a 0 to 5 scale [53]. The protection function was described using the disease index [54] (Equation (2)) and by applying Abbott's formula to determine the efficacy percentage [55] (Equation (3)):

$$\text{Disease index (DI)} = \frac{\sum (\text{disease grade} \times \text{no. of plants in each grade})}{(\text{total no. of plants})\ (\text{highest disease grade})} \times 100 \quad (2)$$

$$\text{Efficacy percentage} = \left(\frac{\%\ \text{infection control} - \%\ \text{treated infection}}{\%\ \text{infection control}}\right) \times 100 \quad (3)$$

The enzymatic activities of some enzymes involved in defense mechanisms (either used as stress markers, i.e., guaiacol peroxidase, ascorbate peroxidase, or involved in phenylpropanoid metabolism, i.e., polyphenol oxidase and phenylalanine ammonia lyase), were extracted from the hypocotyls of the wheat seedlings and analyzed according to the procedures described by Orzali et al. [36].

4.7. Statistical Analyses

Data were subjected to analysis of variance (ANOVA) in IBM SPSS Statistics v.25 software (IBM, Armonk, NY, USA) after checking normality and homoscedasticity assumptions. The Tukey's HSD test at a 0.05 probability level ($p < 0.05$) was used for the post hoc comparison of means.

5. Conclusions

The potential synergistic effect in terms of antifungal activity resulting from the combination of aqueous solutions of stevioside and alcoholic solutions of milk thistle seeds extracts (rich in polyphenols), preconized as the working hypothesis, was evidenced against *F. culmorum* (with a synergy factor of 1.4). Silybin enhanced the antifungal efficacy of stevioside, which, on the other hand, proved to be sufficiently bioactive: EC_{50} and EC_{90} values of 123.2 and 159.7 $\mu g \cdot mL^{-1}$, respectively, were obtained for the stevioside–milk thistle seed extract conjugate complex vs. 155.9 and 221 $\mu g \cdot mL^{-1}$, respectively, for stevioside. The choice of the 1:1 molar ratio noticeably improved the antifungal activity as compared with previously reported inclusion compounds of stevioside and polyphenols in a 5:1 molar ratio. Full inhibition of colony formation was confirmed at 256 $\mu g \cdot mL^{-1}$ for the conjugate complex-based treatment, which was also tested for grain protection at storage, finding that at a concentration of 512 $\mu g \cdot mL^{-1}$, it fully inhibited fungal growth and thus prevented trichothecene production. Assays conducted with Kamut and winter wheat seed tests indicated that the bioactive formulation at 512 $\mu g \cdot mL^{-1}$ reduced seedling root rot (by 71% and 60%, respectively) and did not affect the germination rate. These results suggest that these stevioside–*S. marianum* extract conjugate complexes may hold promise as antifungal agents for FHB treatment.

6. Patents

The work reported in this manuscript is related to Spanish patent P201731489.

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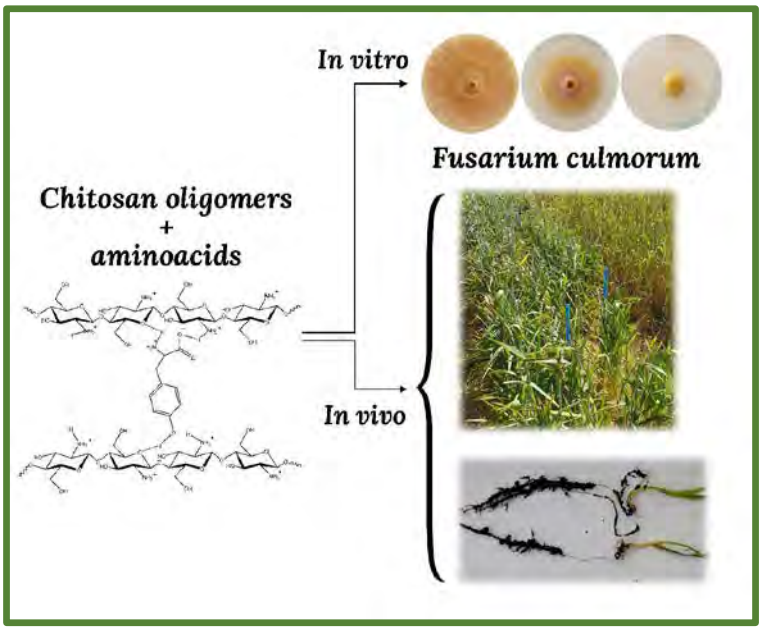

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Abstract: Fusarium head blight (FHB) is a complex disease of cereals caused by *Fusarium* species, which causes severe damages in terms of yield quality and quantity worldwide, and which produces mycotoxin contamination, posing a serious threat to public health. In the study presented herein, the antifungal activity against *Fusarium culmorum* of chitosan oligomers (COS)–amino acid conjugate complexes was investigated both in vitro and in vivo. The amino acids assayed were cysteine, glycine, proline and tyrosine. In vitro tests showed an enhancement of mycelial growth inhibition, with EC_{50} and EC_{90} effective concentration values ranging from 320 to 948 $\mu g \cdot mL^{-1}$ and from 1107 to 1407 $\mu g \cdot mL^{-1}$ respectively, for the conjugate complexes, as a result of the synergistic behavior between COS and the amino acids, tentatively ascribed to enhanced cell membrane damage originating from lipid peroxidation. Tests on colonies showed a maximum percentage reduction in the number of colonies at 1500 $\mu g \cdot mL^{-1}$ concentration, while grain tests were found to inhibit fungal growth, reducing deoxynivalenol content by 89%. The formulation that showed the best performance, i.e., the conjugate complex based on COS and tyrosine, was further investigated in a small-scale field trial with artificially inoculated spelt (*Triticum spelta* L.), and as a seed treatment to inhibit fungal growth in spelt seedlings. The field experiment showed that the chosen formulation induced a decrease in disease severity, with a control efficacy of 83.5%, while the seed tests showed that the treatment did not affect the percentage of germination and resulted in a lower incidence of root rot caused by the pathogen, albeit with a lower control efficacy (50%). Consequently, the reported conjugate complexes hold enough promise for crop protection applications to deserve further examination in larger field trials, with other *Fusarium* spp. pathogens and/or *Triticum* species.

Keywords: amino acids; cereal; chitosan oligomers; FHB; fungicide; synergism; wheat

1. Introduction

Fusarium head blight (FHB) is a devastating fungal disease that affects wheat and other small-grain cereals worldwide, caused by several species belonging to the genus *Fusarium*. Besides causing significant yield losses and reducing grain quality [1], these species are also able to biosynthesize mycotoxins harmful to both humans and animals [2,3]. *F. culmorum* (W. G. Smith) Sacc. and *F. graminearum* Schwabe are generally considered the two most important FHB causal agents worldwide [4], but *F. poae*, *F. asiaticum*, *F. boothii*, *F. vorosii* and *F. cortaderiae* also pose a serious threat [5].

Depending on their chemotype, infection by *F. culmorum* and *F. graminearum* can result in cereal grain contamination with different Type B trichothecenes: nivalenol (NIV) and its acetylated derivatives, deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON), or DON and 15-acetyldeoxynivalenol (15-ADON) [6,7]. Nonetheless, some recently identified *F. graminearum* strains produce Type A trichothecenes (NX-2 and NX-3), similar in structure to DON and 3-ADON, but differing in the presence of a ketone at C_8 [8].

At present, no complete FHB-resistant wheat varieties are commercially available, so, in order to control the disease and limit mycotoxin contamination, the use of less susceptible genotypes in combination with the application of fungicides is used worldwide. However, the extensive use of tebuconazole, metconazole, prothioconazole, prochloraz and other commonly used fungicides of wheat is exerting a selective pressure and is influencing population dynamics of *Fusarium* species [5]. Moreover, the European legal framework (Article 14 in European Directive 2009/128/EC) enforces their use in a sustainable way, but low concentrations of the aforementioned fungicides may cause an incomplete reduction of fungal development [9]. Hence, alternative control strategies have become the subject of intense research, including biocontrol agents [10], the selection of resistant cultivars [11,12], or agronomic practices (e.g., influence of tillage and cover crop [13]).

Another approach to address this challenge involves the replacement of conventional antifungals with novel preparations from natural products. This latter option would be favored by the recent regulatory change at a European level, given that new preparations based on natural products are contemplated in Product Function Category (PFC) 6 within the Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019, which requalifies 'biostimulants' as fertilizers (and not as phytosanitary products). It should also be taken into consideration that, according to Regulation 834/2007 on organic production and labeling, as well as Regulation 889/2008, which establishes provisions for the application of Regulation 834/2007, and its subsequent modifications, products and by-products of plant origin are considered as substances suitable for application in Organic Farming. For example, chitosan, common horsetail (*Equisetum arvense* L.) and common nettle (*Urtica dioica* L.) vegetable extracts, rich in polyphenolic compounds, are widely used as agricultural biostimulant products.

As a response to this regulatory change, investigations on the efficacy against FHB of aromatic carboxylic acids present in barley-root exudates [14], commercial essential oils (garlic, grapefruit, lemon grass, tea tree, thyme, verbena, cajeput and *Litsea cubeba* Pers.) [15], essential oils from lemon-scented gum (*Corymbia citriodora* (Hook.) K.D. Hill and L.A.S. Johnson) [16], essential oils of Moroccan wormseed (*Dysphania ambrosioides* (L.) Mosyakin and Clemants) [17], walnut (*Juglans regia* L.) green husk extracts [18], extracts from buckwheat grain and hulls [19], phenolic-rich bee products (propolis, bread and pollen) [19], or polyphenol inclusion compounds and conjugate complexes [20,21], among others, have been recently reported.

In the work presented herein, novel formulations based on chitosan (which has been previously assayed against *Fusarium* spp. [22–25], and which has been put forward as a sustainable alternative in crop protection [26]) in combination with amino acids have been tested against *F. culmorum*, both in vitro and in vivo, with a view to assessing if an enhanced behavior resulting from synergies between these natural products can be attained.

2. Materials and Methods

2.1. Reagents and Fungal Isolates

High molecular weight chitosan (CAS No. 9012-76-4; 310,000–375,000 Da) was purchased from Hangzhou Simit Chemical Technology Co., Ltd. (Hangzhou, China). Citric acid (CAS 77-92-9; 99.5%) and Tween® 20 (CAS 9005-64-5) were supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Neutrase® 0.8 L enzyme was supplied by Novozymes (Bagsvaerd, Denmark). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Becton, Dickinson and Company

(Franklin Lakes, NJ, USA). Cysteine (Cys, CAS No. 52-90-4), glycine (Gly, CAS No. 56-40-6), proline (Pro, CAS No. 147-75-3) and tyrosine (Tyr, CAS No. 60-8-4), all with 99% purity, were purchased from Panreac S.L.U (Barcelona, Spain).

The *Fusarium culmorum* strain used in the present study (CECT 20493) was obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain). The chemotype was 3-ADON [7].

2.2. Preparation of Chitosan Oligomers and Bioactive Solutions

Chitosan oligomers (COS) were prepared following the procedure described by Buzón-Durán et al. [27]. The amino acid-only bioactive solutions were prepared by dissolving the amino acids in distilled water, without further purification, at an initial concentration of 3000 $\mu g \cdot mL^{-1}$. The COS–amino acid conjugate complexes (viz., COS–cysteine, COS–glycine, COS–proline and COS–tyrosine) were prepared by mixing in a 1:1 (v/v) ratio of the respective solutions (COS solution at 1500 $\mu g \cdot mL^{-1}$ + amino acid solution at 1500 $\mu g \cdot mL^{-1}$), followed by sonication with a probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) for 15 min in five 3-min periods, controlling the temperature so as to keep it below 60 °C.

2.3. In Vitro Tests of Mycelial Growth Inhibition

The biological activity of the treatments was determined using the agar dilution method (or "poisoned food method") [28], incorporating aliquots of stock solutions into the PDA medium to provide final concentrations of 62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000 and 1500 $\mu g \cdot mL^{-1}$, i.e., the usual ones defined in the Clinical & Laboratory Standards Institute (CLSI) or in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard antifungal susceptibility testing procedures. Mycelial disks of *F. culmorum* (5 mm in diameter) from the edges of a 7-day-old culture were transferred to plates filled with these media (in triplicate), using plates containing only the PDA medium as the control.

Radial mycelial growth was determined by calculation of the average of two perpendicular colony diameters for each replicate. Mycelial growth inhibition for each treatment and concentration after 7 days of incubation, at 25 °C in the dark, was calculated according to the formula:

$$((d_c - d_t)/d_c) \times 100 \quad (1)$$

where d_c is the average diameter of fungal colony in the control and d_t is the average diameter of the fungal colony treated with the tested composite.

Results were also expressed as half maximal and 90% maximal effective concentrations (EC_{50} and EC_{90}, respectively), estimated by regressing the radial growth inhibition values (%) against the $\log_{10}$ values of the treatment concentrations.

2.4. Preparation of Inoculum

Conidial suspensions were obtained following the procedure described by Khan et al. [29], with minor modifications. *F. culmorum* conidia were harvested from 7-day-old PDB cultures (200 mL cultures incubated in the dark at 25 °C, 140 rpm in an ECOLAN 60 (Labolan; Esparza de Galar, Navarra, Spain) orbital stirrer incubator). The suspension obtained was then filtered through two layers of sterile muslin to remove hyphal fragments. Spore concentration was determined using a hemocytometer (Weber Scientific International Ltd., Teddington, Middlesex, UK), and was adjusted to a final concentration of 5×10^4, 1×10^5 or 1×10^6 spores (conidia) mL^{-1}, with 0.2% Tween® 20, depending on the experiment to be conducted afterwards.

2.5. Inhibition of Colonies Formation

In vitro tests aimed at determining the inhibition rate percentage of the number of colonies formed were carried out according to the procedure reported by Kheiri et al. [30]. 0.5 mL of the

conidial suspension (5×10^4 conidia·mL^{-1}) was mixed with different concentrations (500, 1000 and 1500 μg·mL^{-1}) of the solution of COS–tyrosine conjugate complex (for which the lowest EC_{90} value was obtained, as discussed below) to a final volume of 2 mL. Conidial suspension was also prepared with distilled water and 0.5% v/v acetic acid aqueous solution as the control. The resulting solutions were incubated in the dark at 25 °C for 24 h. Aliquots of 50 μL of each dilution were spread on PDA and incubated at 25 °C, counting the number of colonies formed after 5 days. Each treatment was replicated 3 times. The percent inhibition rate was estimated as: % Inhibition rate = (number of colonies formed in control plate − number of colonies formed in treated plates)/number of colonies formed in control plate × 100.

2.6. Effect of Conjugate Complexes on Mycotoxin Production and Mycotoxin Chemical Analysis

The effect of the conjugate complex with the best performance on the growth of *Fusarium* fungi on wheat grain was investigated using the method described by Perczak et al. [31]. 5 mL of the COS–tyrosine solution at a concentration of 1500 μg·mL^{-1} was mixed with 25 g of sterile spelt grain in an Erlenmeyer flask. The mixture was vigorously stirred, and three rings (6 mm) of solid culture of *F. culmorum* were then added to each Erlenmeyer flask and mixed. Solutions of Tween® 20 and deionized water were added to the control sample, without the addition of bioactive compound. Next, the prepared mixtures were stored in the dark at 25 °C for 28 days. After incubation, samples were dried, milled, homogenized and prepared for chromatographic analysis.

The organic extracts were obtained by soaking of the samples in a mixture of water, methanol and acetonitrile in a 10:10:30 v/v ratio, followed by sonication for 15 min in five 3-min periods. The supernatant solution was filtered with Whatman nº 4 paper and stored at 4 °C until the analytical determinations were carried out. The determination of mycotoxins was carried out according to the procedure recommended by Jeyakumar et al. [32], using a X500R (Ab Sciex Spain S.L., Madrid, Spain) quadrupole time-of-flight mass spectrometer (QTOF-MS) coupled to an ExionLC series two-dimensional ultra-high performance liquid chromatography (2D-UHPLC) system, at the Laboratorio de Técnicas Instrumentales facilities (Universidad de Valladolid, Valladolid, Spain).

2.7. In Vitro Seedling Tests

The potential ability of the conjugate complex treatment to induce resistance in seedlings of spelt against *F. culmorum* was evaluated following the methodology reported by Orzali et al. [33], with minor modifications. Spelt grains were first surface-sterilized for 3 min by immersion in 2% NaOCl and then rinsed with water three times. The seed treatments (100 seeds per treatment) were performed by immersion in 100 mL of conjugate complex solution (at a concentration of 1500 μg·mL^{-1}, with 0.2% Tween® 20) at room temperature, under stirring for 1 h. Distilled water with 0.2% Tween® 20 was used in the positive and negative controls. The seeds were then air-dried for 30 min, and inoculated by immersion in 100 mL of the 1×10^6 conidia·mL^{-1} suspension, with 0.2% Tween® 20, for 30 min. The seeds were finally air-dried again for 30 min.

The physiological quality of the seeds for each treatment (negative control, positive control, phytotoxicity test and treatment with the bioactive formulation) was evaluated by germination, as described in the International Rules for Seed Testing [34]. For each treatment, 3 replicates of 100 seeds were placed in glass plates, using the between paper method, and kept under constant humid conditions. Germination was evaluated after 4 days, in such a way that a seed was considered germinated if it produced a well-developed seedling with three roots and a shoot present.

The efficacy of the antifungal treatment was then assessed by planting the seeds in pots filled with autoclaved peat-based substrate, with a procedure similar to those described by Lozano-Ramírez et al. [35] and Koch et al. [36]. Seedlings were grown in greenhouse conditions at 25 °C. After 2 weeks, they were removed from the substrate and, based on their appearance, classified as healthy or diseased. Symptoms in the roots and the internode were visually evaluated, using a 0 to 5 scale [37].

2.8. Field Trials

Small-scale field trials with spelt plants were carried out in micro-plots (8 × 1 m) located in the ETSIIAA facilities (University of Valladolid, Palencia, Spain). The soil is classified as Eutric Fluvisol, commonly known as 'meadow' soil, easy to till, with good permeability, with a balanced and uniform texture along the profile, good depth and pH above 7. Sowing was conducted in mid-November 2019 with a trial sowing machine, at a dose of 250 kg/ha. The rest of the agricultural practices were the usual ones in the area. At the beginning of spring, an herbicide treatment was applied, with a mixture of Bromoxinil, Typhensulfuron and Diflufenican. A NPK(S) 20:10:10(7.5) fertilizer with 3,4-dimethylpyrazole phosphate (DMPP) nitrification inhibitor was used for fertilization in a single application, at a rate of 350 kg/ha, in mid-February 2020.

Climatologically, the agricultural year was marked by frequent rainfall in the first half of spring. Until then, the rainfall was that of the average year, with normal nascence. Since April 2020, the rainfall was higher than in the average year, with mild temperatures and almost no frost, resulting in good ear formation and—in absence of artificial inoculation—good grain filling.

Artificial inoculation tests were conducted in agreement with the procedure described by Brennan et al. [38], with minor modifications. At growth stage (GS) 65 (mid-anthesis; 21 May), 100 heads (1 head per plant) were sprayed to run-off (ca. 4 mL/ear)—using a hand-held atomizer—with the COS–tyrosine solution (at a 1500 $\mu g \cdot mL^{-1}$ concentration) containing 0.2% Tween® 20. After 24 h, the same 100 heads were artificially inoculated with *F. culmorum* by spraying the prepared conidial suspension (1×10^5 conidia$\cdot mL^{-1}$) onto ears at a rate of ca. 4 mL of spore suspension per ear. 100 heads were sprayed with distilled water containing 0.2% Tween® 20 (negative control), 100 heads with the conidial suspension and 0.2% Tween® 20 (positive control) and another 100 heads with the COS–tyrosine solution (phytotoxicity test). Heads subjected to treatments were secondary heads. All plants were enclosed for 24 h using clear polythene bags to increase humidity and promote disease development.

The FHB disease symptoms (% infected spikelets per head) were visually assessed at GS73, 77, 83 and 87 (soft dough). Severity was assessed using a scale similar to that of Parry et al. [39], and by applying the following formula: number of heads in each class × each evaluation class/total number of heads. Efficacy percentage was determined by applying Abbott's formula: (% infection control − % treated infection)/(% infection control) × 100.

2.9. Statistical Analyses

Data were subjected to analysis of variance (ANOVA), followed by post hoc comparison of means through Tukey's HSD (honest significant difference) test at $p < 0.05$. SPSS Statistics v.25 software (IBM; Armonk, NY, USA) was used.

3. Results

3.1. In Vitro Tests of Mycelial Growth Inhibition

The results of the sensitivity tests are summarized in Figure 1. The treatments based solely on amino acids showed a lower performance than the treatments including COS, either alone or in combination with the amino acids (Figure 2). Upon increase of the concentration, higher inhibition was attained for all treatments, except for the glycine only treatment, in which fungal inhibition remained below 30%.

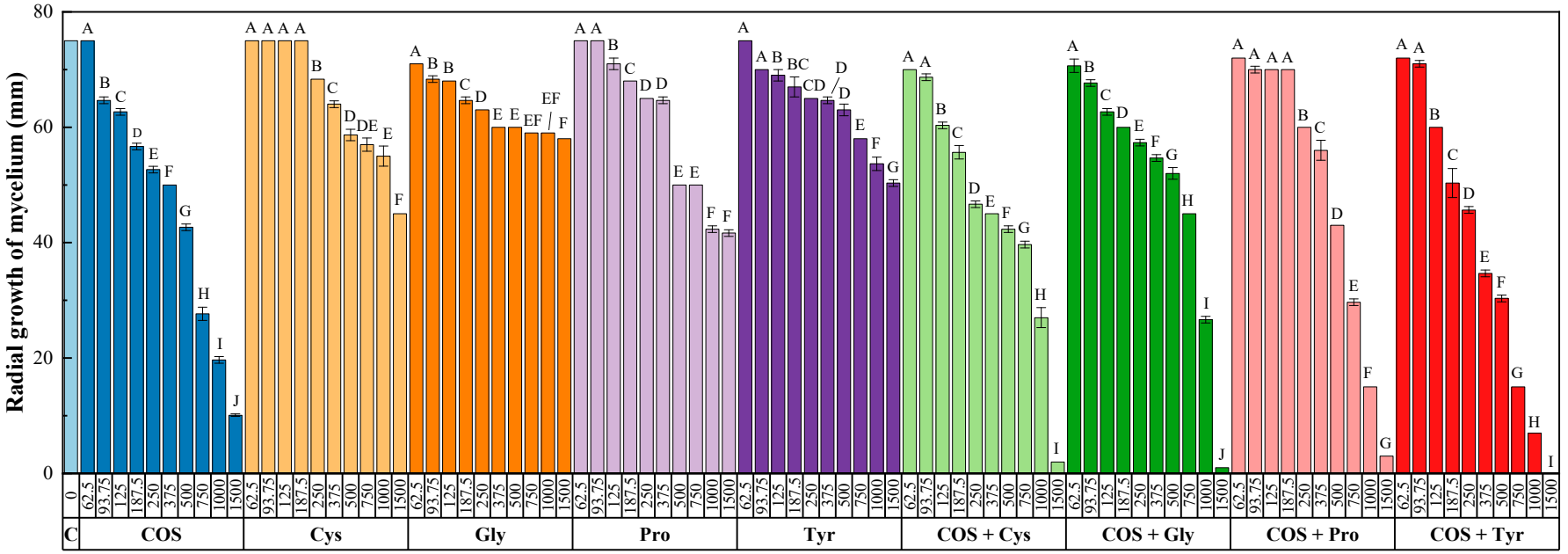

Figure 1. Radial growth values of *F. culmorum* in the presence of the different treatments, which consisted of chitosan oligomers (COS), amino acids (cysteine, Cys; glycine, Gly; proline, Pro; tyrosine, Tyr), and the conjugate complexes consisting of COS–amino acids (1:1 v/v). A 75 mm radial growth was obtained for the potato dextrose agar (PDA) control (C). Concentrations labelled with the same letters are not significantly different at $p < 0.05$ by Tukey's test. All values are presented as the average of three repetitions. Error bars represent the standard deviation across three replicates.

Figure 2. Sensitivity test. Radial growth of mycelium for: (**a**) control (PDA), (**b**) COS only, (**c**) COS–Cys, (**d**) COS–Gly, (**e**) COS–Pro, (**f**) COS–Tyr. From top to bottom: 62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000 and 1500 $\mu g \cdot mL^{-1}$. Only one replicate is shown.

The effective concentrations are presented in Table 1 for comparison purposes. In view of the EC_{90} values, a synergistic behavior was observed for all the conjugate complexes, which was particularly evident for tyrosine. In fact, the lowest EC_{50} and EC_{90} values were obtained for this amino acid, which was then used in the rest of the experiments.

Table 1. Effective concentrations that inhibited mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively).

Concentration ($\mu g \cdot mL^{-1}$)	COS	Cys	Gly	Pro	Tyr	COS–Cys	COS–Gly	COS–Pro	COS–Tyr
EC_{50}	680.27	1516.7	-	726.7	3524.8	820.45	948.39	675.69	320.46
EC_{90}	2230.26	8150.2	-	3460.3	79,197.5	1406.87	1359.08	1372.58	1106.87

COS = chitosan oligomers; cysteine = Cys; glycine = Gly; proline = Pro; tyrosine = Tyr.

3.2. Inhibition of Colonies Formation

The number of colonies were counted five days after incubation on the COS–tyrosine supplemented medium, which showed various levels of inhibition of the number of colonies as a function of the bioactive product dose (Figure 3). The number of colonies was decreased with all concentrations in comparison with the control, reaching full inhibition at a concentration of 1500 μg·mL^{-1}. The percentage of reduction of the number of colonies for 500 and 1000 μg·mL^{-1} concentrations was 55.6% and 66.7%, respectively.

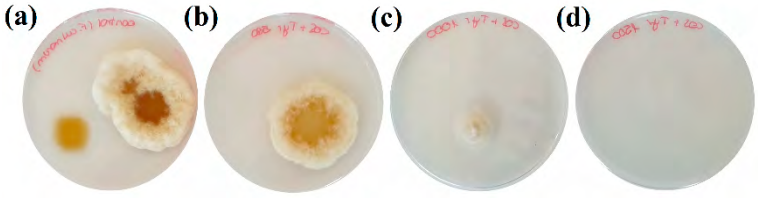

Figure 3. Effect of the COS–tyrosine conjugate complex treatment on the number of colonies formation of *F. culmorum* after 5 days. Suspension of fungal spores soaked for 24 h at different concentration of conjugate complex, and 50 μl of treated spores were spread on PDA medium. (**a**) control, (**b**) 500 μg·mL^{-1}, (**c**) 1000 μg·mL^{-1} and (**d**) 1500 μg·mL^{-1}. Only one replicate is shown.

3.3. Effect on Mycotoxin Production

After incubation of the grain samples inoculated with *F. culmorum* for 28 days, differences in the growth of the mycelium were observed for the grains treated with COS–Tyr, as compared to control trials (Figure 4). The treatment, at a concentration of 1500 μg·mL^{-1}, clearly had an inhibitory effect. From the analyses, DON content was reduced by ca. 91% vs. The control sample (in which DON content reached 8.12 ± 1.53 μg·mL^{-1}).

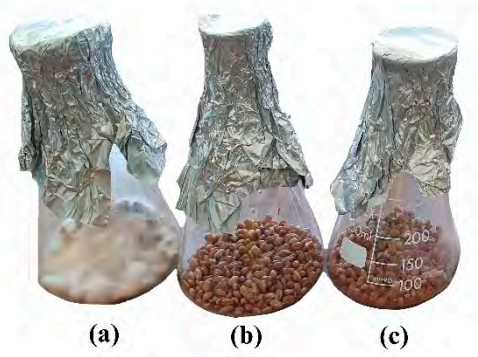

Figure 4. Effect of the application of COS–tyrosine conjugate complex on the growth of *F. culmorum* on spelt grain: (**a**) positive control, (**b**) negative control, (**c**) treated grain at a dose of 1500 μg·mL^{-1}.

3.4. Seedling Tests

Concerning the germination tests (Figure 5), no significant differences were observed between the negative control and the seeds treated with the conjugate complex and not inoculated with *F. culmorum* (100% and 99% germination percentages, respectively), thus suggesting that the bioactive product based on chitosan would not be phytotoxic. The germination rate for the positive control (inoculated and not treated seeds) was noticeably lower (48%), but it was clearly improved for the inoculated and treated seeds (89.5%).

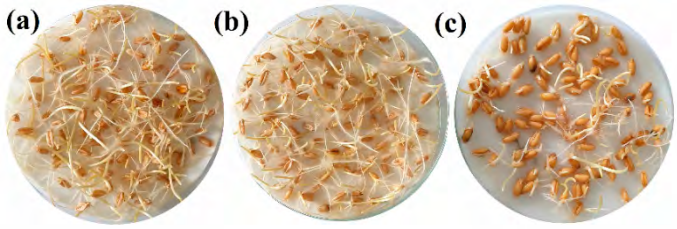

Figure 5. Germination tests: (**a**) negative control, (**b**) treated seeds, (**c**) positive control. Only one replicate is shown.

With regard to seedling symptoms after two weeks, in the negative control, no root rot symptoms were observed; in the positive control, the average disease severity was 28% (with some seedlings reaching a 3 in the 0 to 5 scale, showing clear wilting and browning of coleoptiles and roots, Figure 6), and in the artificially inoculated seedlings treated with the COS–Tyr conjugate complex, the average disease severity was close to 14%. Hence, the control efficacy of the treatment was moderate (50%).

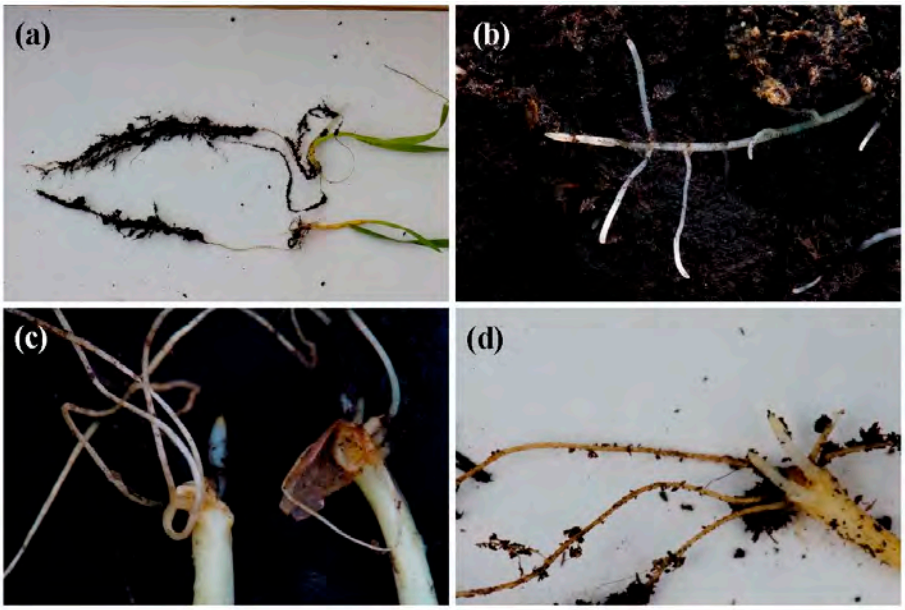

Figure 6. (**a**) Healthy (top) vs. infected (bottom) seedlings, (**b**) healthy seedling roots, (**c**) root rot symptoms, (**d**) root shortening.

3.5. Field Trials

No disease symptoms were observed in the negative control heads, and no signs of phytotoxicity were observed in the heads that had been treated with the COS–Tyr solution. In GS 87, the average disease severity reached 45.2% for the positive control plants (Figure 7), while in the treated plants, it was noticeably lower (only 7.45%), thus indicating a good control efficacy (83.5%) of the chosen treatment.

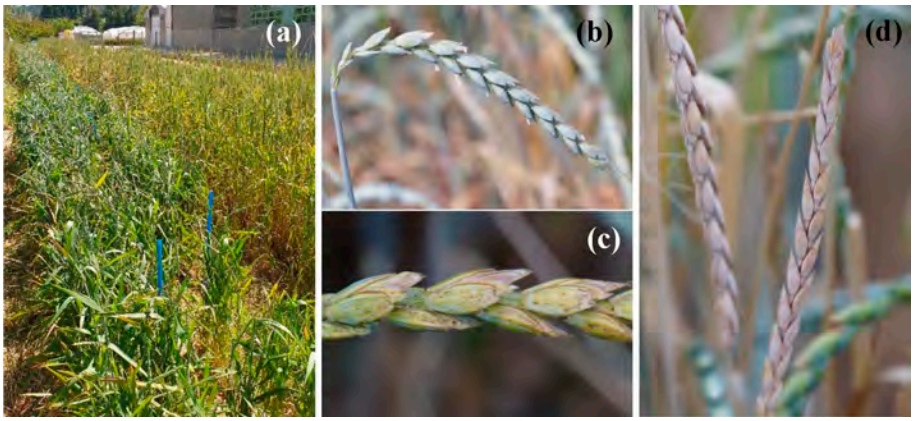

Figure 7. (**a**) Microplots used in the field trials, with spelt plants in growth stage (GS) 65, (**b**) healthy treated ear, (**c**) ear with attack of intermediate severity, (**d**) positive control, entirely affected.

4. Discussion

4.1. Comparison of Treatment Efficacy

Fusarium spp. do not have a normal minimum inhibitory concentration (MIC) and minimum effective concentration (MEC) distribution, so prediction of the antifungal susceptibility of a single strain is difficult ([40] and references therein). Moreover, as the susceptibility profile is isolate-dependent, comparisons of the effective concentrations below should be taken with caution.

A thorough bibliographical survey yielded no studies on the effects of amino acids on *Fusarium* spp., but the results presented herein may be compared with those of other treatments based on chitosan. In relation to the in vitro mycelial growth inhibition tests, contrasting results have been reported: while Xing et al. [25] found no mycelial growth inhibition against *F. culmorum* at concentrations of up to 2 mg·mL^{-1}, Al-Hetar et al. [24] reported an EC_{50} of 1.4 mg·mL^{-1} and a maximum inhibition of 76.36% at 8 mg·mL^{-1} for *F. oxysporum* f. sp. *cubense*. Park et al. [23] obtained EC_{50} values in the 1.5–4.0 and 1.8–3.2 mg·mL^{-1} range for *F. graminearum* and *F. oxysporum* respectively, and Kheiri et al. [30] reported that ≥85% inhibition of *F. graminearum* on PDA was attained with 5 mg·mL^{-1} of chitosan nanoparticles. The EC_{50} value reported herein for COS (0.68 mg·mL^{-1}) was approximately half of those reported by Al-Hetar et al. and Park et al., and the EC_{90} value was half of that reported by Kheiri et al., but it should be stressed that the pathogen was different.

Concerning the inhibition of colonies of *F. graminearum*, Kheiri et al. [30] reported 43.95% and 72.8% inhibition of colonies treated with 5 mg·mL^{-1} of chitosan nanoparticles and chitosan, respectively. In our study, for *F. culmorum*, full inhibition was reached at a lower dose (1.5 mg·mL^{-1}).

Regarding in vivo tests, Khan et al. [41] evaluated the ability of chitosan to inhibit *Fusarium* seedling blight disease of wheat (cv. GK-Othalom) caused by *F. culmorum* (strain FCF 200). Regarding its effect on the germination of wheat seedlings, they found that wheat seeds inoculated with *F. culmorum* failed to germinate, and that treatment with chitosan did not ameliorate the effect of *F. culmorum* inoculation on wheat seedling germination (in contrast with our results, in which almost half of the positive control spelt seeds germinated and the treatment had a marked positive effect on the germination rate). Nonetheless, it is worth noting that the authors claimed that, when used as a stem base treatment, chitosan (at 1 mg·mL^{-1}) was among the most effective treatments to reduce *Fusarium* seedling blight disease symptoms (by 89% vs. The positive control).

Orzali et al. [33] evaluated the effect of chitosan seed treatment as an elicitor of resistance to *F. graminearum* in wheat (cv. Simeto and cv. Creso) in greenhouse. They found that wheat seed treatments with different concentrations of chitosan led to no significant changes in germination and vigor index values compared with the untreated control, confirming that chitosan is not phytotoxic for the seeds (in good agreement with the results obtained in this study). The treatment with chitosan at

a concentration of 5 mg·mL^{-1} resulted in a disease severity in the seedling roots of 21% and 22.3% vs. 38.4% and 39.5% for the positive controls (for cv. Simeto and cv. Creso, respectively). The disease severity observed in our study for spelt (28% and 14% for the positive control and the treated seeds) was lower, but differences may be ascribed to differences in the pathogen and in the sensitivity of the cultivar (*T. spelta* has been reported to have a weaker response to *F. culmorum* infections than other *Triticum* spp. [42]). If efficacies are compared, those attained by Orzali et al. were 45.3% and 43.5%, lower than the one obtained herein (50%) at a significantly lower bioactive product dose (1.5 vs. 5 mg·mL^{-1}).

With reference to the field trials, Khan et al. [29] also studied the efficacy of chitosan for the control of FHB and associated mycotoxin contamination of grain. Spraying of winter wheat (cv. GK-Othalom) heads with chitosan (at 1 mg·mL^{-1}) resulted in an 81% and 76% reduction in FHB disease symptom development in greenhouse trials and in small-scale field trials respectively, slightly lower than the reduction reported herein (83.5%). Chitosan also significantly reduced the concentration of DON under both glasshouse and field conditions (≥74% reduction). The reduction in our in vitro experiments was higher (89%), albeit lower than that reported by Perczak et al. [31], who obtained trichothecenes concentration reductions in the 94.51–100% range by using essential oils.

In greenhouse experiments, Kheiri et al. [30] reported that spraying with chitosan nanoparticles and chitosan (at 1 mg·mL^{-1}) resulted in disease severity percentages of 26.87% and 28.74% respectively, three weeks after fungus inoculation. These results apparently are much higher than those reported herein (only 7.45%), but the severity in the positive controls was also higher (94.25% vs. 45.2%). Upon comparison of efficacies calculated with Abbott's formula (71.5% and 69.5% for chitosan nanoparticles and chitosan in the study by Kheiri et al. [30] respectively, and 83.5% in this study), the difference is reduced, but the complex conjugate still seems to have a better performance than the chitosan-only treatment.

4.2. Mechanism of Action

The inhibition mode of chitosan is based on three main mechanisms, according to Ing et al. [43]: (i) the interaction of its positive charge with the negatively charged phospholipid components of the fungal membrane, which results in an increase in its permeability and in the leakage of cellular contents, (ii) its behavior as a chelating agent, given that its binding to trace elements causes the unavailability of the essential nutrients needed for normal growth of fungi and (iii) its ability to penetrate the fungal cell wall and to bind to its DNA, which inhibits mRNA synthesis and affects essential proteins and enzymes production.

The existence of reactive groups in COS has been explored for the conjugation with other small chemical groups that primarily act by modulating the physicochemical properties of the molecule. Conjugation with amino acids entails the formation of hydrogen bonds (Figure 8), already observed in the interaction of chitosan and chitosan oligomers with the hydrophobic amino acid DL-Tyrosine [44].

The conjugation of COS and Tyr (mainly, L-Tyr) reduces the crystallinity of COS and (in a spontaneous and endothermic process) generates a rearrangement of their chains, a decrease of the intercatenary spacing and, mainly, an increase of their cationic surface charge. We believe that the occurrence of this latter feature enhances the linkage to the negatively charged site-specific binding receptors on the fungal membrane through electrostatic interactions.

Whereas chitosan-resistant fungi such as *Pochonia chlamydosporia* or *Beauveria bassiana* have low-fluidity membranes (enriched on saturated free fatty acids, FFA), the membranes of chitosan-sensitive fungi such as *F. oxysporum* or *Neurospora crassa* are highly fluid (rich in polyunsaturated FFA, such as linolenic acid) [45] and more susceptible to peroxidation. The lipid peroxidation in the fungal membrane is enhanced with the concomitant presence of amino acids, which have been shown to possess potential pro-oxidant capacity in linoleic acid. This pro-oxidative activity could be attributed to the presence of the α-amino group in the form H3-N-R, and the difference in this activity would mainly be due to the functional groups attached to β-carbon in the amino acid molecules.

In fact, a linear relation between concentration of hydroperoxides and time during the early stages of oxidation has been reported for cysteine [46], and a similar behavior is expected for tyrosine. Thus, the enhancement of mycelial growth inhibition of *F. culmorum* observed, which evidences a synergistic behavior between COS and amino acids, can be referred to as enhanced damage (permeabilization) of the fungal membrane via lipid peroxidation induction. Future studies should investigate the generation of oxylipins, metabolites derived from lipid peroxidation [47].

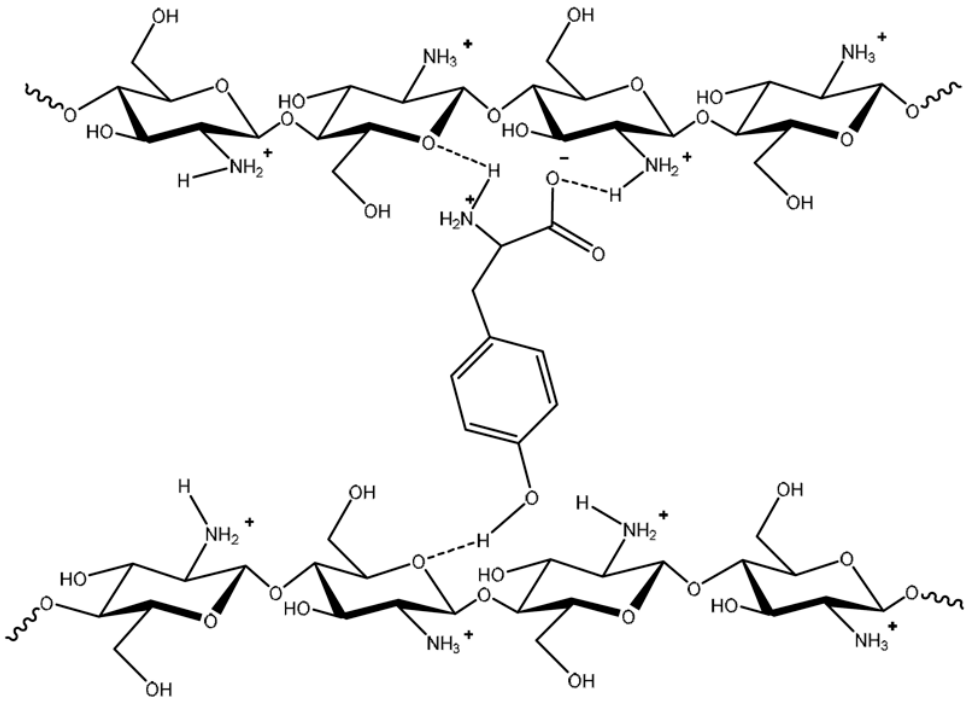

Figure 8. Hydrogen bonding in the COS–tyrosine conjugate complex.

4.3. Significance of the Reported Findings

Although follow-up studies will be necessary to draw firm conclusions on the effectiveness of the application of the proposed treatments (see Section 4.4 below), the fact that the conjugate complexes reached higher mycelial growth inhibition than other chitosan-based treatments makes them promising candidates for the effective control of FHB.

It is also worth noting that *F. culmorum* is not only a pathogen of wheat, but also of other commercially important cereal crops, such as barley, corn, sorghum, oats or rye. In addition, it has been isolated from sugar beet, flax, carnation, bean, pea, asparagus, red clover, hop, leeks, Norway spruce, strawberry and potato tuber [48]. Consequently, the results of this study may also find application in other pathosystems, resulting in a higher ecological and economic impact.

4.4. Limitations of the Study and Further Research

Although the preliminary results may be suggestive of a noticeable antifungal activity of the proposed conjugate complexes against *F. culmorum*, further research is needed before a conclusion can be made on their real applicability for wheat crop protection applications. Apart from the caveats noted above about the differences in resistance among different *Fusarium* species and strains, field tests on other *Triticum* spp. genotypes with different ploidy levels would also need to be carried out, provided that their responses to FHB infection have been reported to vary in a significant manner [42]. Moreover, the impact of the treatments on other wheat diseases (caused by, for instance, *Puccinia* spp., *Tilletia* spp., etc. [49]) should also be taken into consideration if traditional fungicides are to be replaced with

this natural product-based alternative, although it is worth noting that in our field tests, the chosen formulation appeared to control *Alternaria* spp. infection too.

In addition, a detailed assessment the effect of timing of fungicide application and dose rate on the development of FHB and the accumulation of DON (similar to that reported in References [50,51]) would be required before other key practical aspects (e.g., cost, degradation tolerance and efficacy of protection over time, etc.) can be factored in.

5. Conclusions

From in vitro mycelial growth inhibition tests, conjugate complexes of chitosan oligomers and amino acids were found to feature an enhanced antifungal behavior, tentatively ascribed to enhanced damage of the fungal membrane via lipid peroxidation (due to the pro-oxidative activity of the amino acids). The best treatment, based on the COS–tyrosine, with EC_{50} and EC_{90} values of 320 and 1107 $\mu g \cdot mL^{-1}$, was further tested for the inhibition of colonies formation and to avoid mycotoxin production in grain, with positive results: full inhibition of colonies was attained at a concentration of 1500 $\mu g \cdot mL^{-1}$, and the same dose reduced DON content by 89%. In planta assays, conducted both in greenhouse trials with *T. spelta* seedlings and in small-scale field experiments, showed that the chosen formulation had no phytotoxic effects and significantly reduced the severity of FHB symptom development (by 50% in seedling roots after two weeks, and by 83.5% in artificially inoculated heads after three weeks). These promising results call for further studies with other fungal pathogens and *Triticum* species to elucidate the potential of these conjugate complexes as an alternative to conventional fungicides.

6. Patents

The work reported in this manuscript is related to the following Spanish patents: Universidad de Valladolid. Solución acuosa que comprende un complejo de inclusión, método de obtención y su uso para aplicación en cultivos y la mejora de su rendimiento [Aqueous solution comprising an inclusion complex, its method of obtention and its use for crop application and yield improvement]. Application number P201931118, 17 December 2019; Universidad de Valladolid. Complejo conjugado para el tratamiento de agentes patógenos presentes en la agricultura y la naturaleza [Conjugate complex for the treatment of pathogens present in agriculture and nature]. Application number P201831106, 15 November 2018.

Author Contributions: Conceptualization, J.M.-G. and E.P.-L.; Formal analysis, J.M.-G. and P.M.-R.; Investigation, L.B.-D. and J.M.-G.; Methodology, L.B.-D., J.M.-G., J.L.M.-R. and Á.F.-V.; Project administration, J.M.-G.; Resources, J.M.-G., J.L.M.-R., Á.F.-V. and P.M.-R.; Supervision, J.M.-G. and P.M.-R.; Validation, J.L.M.-R., Á.F.-V. and E.P.-L.; Visualization, L.B.-D. and P.M.-R.; Writing—original draft, L.B.-D., J.M.-G. and P.M.-R.; Writing—review and editing, L.B.-D., J.M.-G. and P.M.-R. All authors have read and agreed to the published version of the manuscript.

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Artículo #7 (revista indexada en Emerging Sources Citation Index)

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In Vitro Antifungal Activity of Chitosan-Polyphenol Conjugates against *Phytophthora cinnamomi*

Petruta Mihaela Matei; Laura Buzón-Durán; Eduardo Pérez-Lebeña; Jesús Martín-Gil; Beatrice Michaela Iacomi; M. Carmen Ramos-Sánchez; Pablo Martín-Ramos

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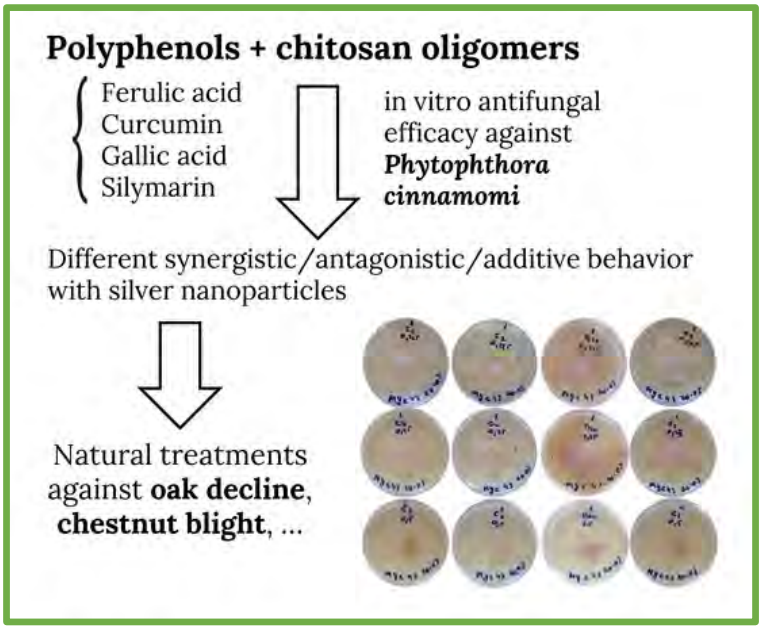

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In Vitro Antifungal Activity of Chitosan-Polyphenol Conjugates against *Phytophthora cinnamomi*

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Abstract: *Phytophthora cinnamomi* is responsible for radical rot in a wide range of hosts, resulting in large economic and ecological losses worldwide. In Spain, it is responsible for diseases such as the oak decline or the chestnut blight. In this study, different polyphenol-stevioside inclusion compounds dispersed in a hydroalcoholic solution of chitosan oligomers have been investigated, with a view to their application as natural bioactive complexes to replace conventional systemic fungicides against this fungus. The polyphenols tested in vitro were curcumin, ferulic acid, gallic acid and silymarin. Three concentrations (125, 250 and 500 $\mu g \cdot mL^{-1}$) were assayed, with and without silver nanoparticles (AgNPs), and notable differences were found in the inhibition of mycelium growth, with EC_{50} and EC_{90} values ranging from 171 to 373.6 $\mu g \cdot mL^{-1}$, and from 446.2 to 963.7 $\mu g \cdot mL^{-1}$, respectively. The results obtained showed that the addition of AgNPs, despite their antimicrobial activity, did not always lead to synergies. In the case of *P. cinnamomi*, an unexpected antagonistic behavior was found for two of the polyphenols (curcumin and silymarin), while an additive behavior for ferulic acid and a synergistic behavior for gallic acid were attained. In view of their inhibitory power, the preparations based on ferulic acid with AgNPs and on silymarin without AgNPs are proposed for applications in crop and forests protection against *P. cinnamomi*.

Keywords: chitosan oligomers; fungicide; phenolic compounds; silver nanoparticles; synergism

1. Introduction

Phytophthora cinnamomi is an oomycete that lives on the ground nourishing itself thanks to decomposing matter. This pathogen, responsible for "root rot" or "regressive death", is one of the most invasive species worldwide. It infects close to 5000 species of plants [1], affecting a variety of plant families: conifers, grasses, ferns, ornamental plants and food crops such as pineapple or avocado [2]. Its expansion has been attributed to the phenomenon of climate change, given that the potential disease range is influenced by winter temperature (disease development is strongly hampered by cold winters) and other climatic variables, such as summer temperatures and hydrologic variables. For instance, in Mediterranean forests, the initiation of the disease at the root level occurs during temporal waterlogging, caused by the higher frequency of extreme rain events [3]. Symptoms of *P.*

cinnamomi infection include: wilting, reduced fruit size, death of young shoots, chlorosis of leaves and stem cankers [4].

In the Iberian Peninsula, *P. cinnamomi* is responsible for diseases such as the oak decline, with an enormous impact on the *dehesa* or *montado* ecosystem (a "man-made" ecosystem characterized by a savannah-like physiognomy that occupies extensive areas in Southern Spain and Portugal), or of chestnut blight [5].

At present there is no treatment able to eradicate regressive death by *P. cinnamomi*, although the stem injection of phosphites (which are elicitors that indirectly behave as fungicides) [6] and foliar spraying with metalaxyl + mancozeb (fungicides with contact and systemic activity) have been tested.

As an alternative to these conventional treatment agents, it is possible to use natural bioactive complexes extracted from plants, such as phenolic acids, flavonoids, tannins or lignans. In particular, polyphenols have been shown to feature a remarkable antimicrobial activity [7–10]. However, their applicability is limited by their low solubility and bioavailability [11], which may be improved through the formation of inclusion compounds with, for instance, terpene glycosides [12]. In this study, polyphenol-stevioside inclusion compounds dispersed in a hydroalcoholic solution of chitosan oligomers have been assayed. The polyphenols selected for the in vitro tests were curcumin, ferulic acid, gallic acid and silymarin, at different concentrations, with and without silver nanoparticles (AgNPs). The aim of the research has been to investigate the activity of the different chitosan-polyphenol conjugates against *P. cinnamomi* and to study synergistic effects upon addition of AgNPs to the conjugates.

2. Materials and Methods

2.1. Reagents and Preparation of the Bioactive Conjugates

Curcumin (CAS No. 458-37-7), ferulic acid (CAS No. 1135-24-6), gallic acid (CAS No. 149-91-7) and silymarin (MDL number MFCD01776359) were supplied by Sigma-Aldrich (Merck, Darmstadt, Germany); stevioside (CAS No. 57817-89-7) was supplied by Wako (Osaka, Japan); and medium molecular weight chitosan (MMWC) was purchased from Hangzhou Simit Chemical Technology Co. Ltd. (Hangzhou, China).

The preparation of chitosan oligomers from MMWC was carried out according to the methodology reported by Sun et al. [13]. Microwave-assisted aqueous biphasic system separation was used to prepare the polyphenol inclusion compounds. The conjugates with chitosan oligomers in hydroalcoholic solution medium were prepared according to the procedure previously reported in Matei et al. [14], and were characterized by infrared spectroscopy (FTIR), SEM and TEM microscopy techniques to ensure the reproducibility of the results presented in patent P201731489 [15].

It should be clarified that the antifungal efficacy assays reported herein and those reported in Matei et al. [14] (focused on the comparison between two dispersion media and on differences between polyphenols, but with AgNPs in all cases) were run in parallel. Since they were conducted at the same time and in the same conditions, the impact of physiological changes of the fungus or differences in abiotic conditions may be excluded.

2.2. Fungal Isolate, Growth Conditions and In Vitro Tests of Mycelial Growth Inhibition

The fungal isolate of *Phytophthora cinnamomi* used for the in vitro sensitivity assays (MYC43) was provided by ICMC-IPROCOR (CICYTEX, Mérida, Spain) and was maintained in potato-dextrose-agar (PDA) culture medium at 4 °C. Fresh subcultures to obtain the inoculum for the tests were obtained by transferring hyphae plugs to Petri dishes containing PDA medium, which were incubated at 25 °C in the dark for 7 days.

To test the antifungal activity of the conjugates, the agar dilution method was used. Final concentrations of 125, 250 and 500 $\mu g \cdot mL^{-1}$ were obtained by adding aliquots of the stock solutions discussed in previous subsection to the PDA medium. Eight-mm mycelial disks from the

margins of these fresh subcultures were then placed in these PDA plates. Pure PDA culture medium was used as the control. Three experiments, with three replicates per experiment, were carried out.

After 7 days of incubation at 25 °C in the dark, mycelial growth inhibition for each treatment and concentration was determined according to the formula: $((d_c - d_t)/d_c) \times 100$, where d_c and d_t are the average diameters of the control and treated fungal colonies, respectively [16].

The concentrations that reduced mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively) were determined by regressing the values of radial growth inhibition (%) against the $\log_{10}$ values of the concentration of the conjugates.

To assess the joint action of the bioactive products in mixtures, Wadley's method was used. In this method, if the synergy factor (SF) is 1, the hypothesis of similar joint action (additivity) can be accepted; if SF > 1, there is synergistic action; and if SF < 1 there is antagonistic action between the bioactive products [17].

2.3. Statistical Analyses

Data from the results obtained herein and from those reported in a previous study with AgNPs [14] were subjected to analysis of variance (ANOVA), followed by post hoc comparison of means through Tukey's HSD (honest significant difference) test at $p < 0.05$. SPSS Statistics v.25 software (IBM; Armonk, NY, USA) was used.

3. Results and Discussion

The radial growth of the mycelium was monitored to study the in vitro activity of the different treatments. The results of the sensitivity assays are shown Figure 1 and Figure S1, in which one may observe that a reduction in the radial growth of the mycelium was attained in all cases when the concentration of the conjugates was increased from 125 to 500 $\mu g \cdot mL^{-1}$.

It is worth noting that the addition of AgNPs only resulted in a noticeable enhancement of activity for the conjugates based on gallic acid. For the conjugates based on ferulic acid the improvement was statistically significant only at the lowest dose; and for the conjugates based on curcumin or silymarin, the addition of AgNPs led to a lower performance (particularly evident for the conjugate with silymarin at 500 $\mu g \cdot mL^{-1}$).

The results from the factorial ANOVA (Table S1) indicated statistically significant one-way, two-way and three-way interaction effects. A classification of the treatments, both with and without AgNPs, according to Tukey's HSD test is shown in Table S2.

The effective concentrations EC_{50} and EC_{90} for each treatment are summarized in Table 1. The presence/absence of AgNPs in the conjugates and the phenolic compound had a noticeable influence on the sensitivity of the isolate. While the presence of AgNPs barely modified the EC_{90} values for curcumin and ferulic acid, it significantly improved those of gallic acid conjugates (by 34% and 112% for EC_{50} and EC_{90}, respectively).

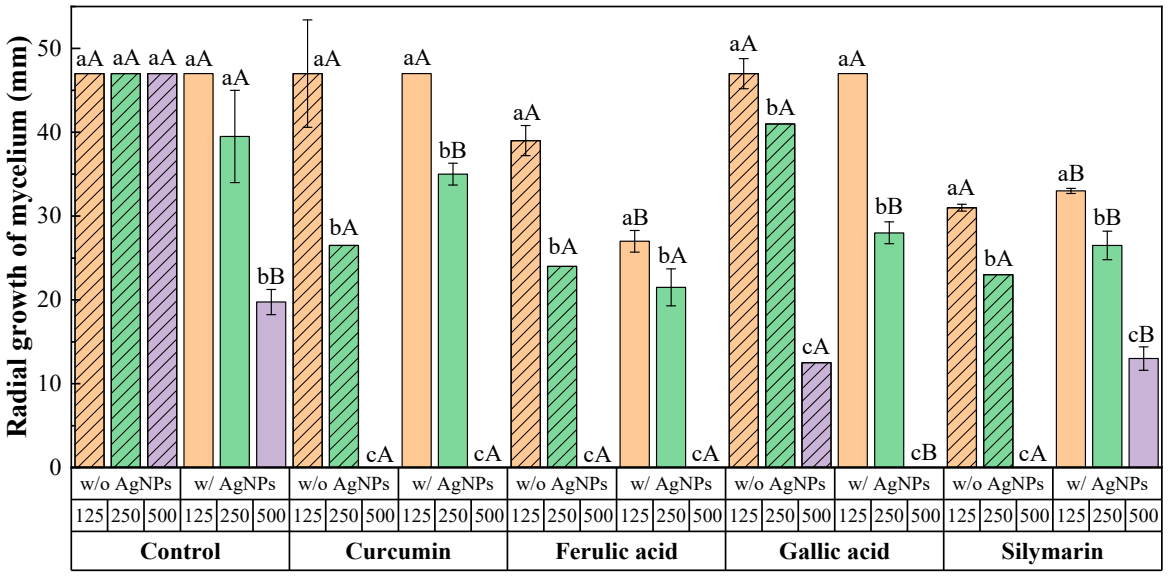

Figure 1. Radial growth values of *P. cinnamomi* mycelium in the presence of the conjugates, composed of various polyphenol inclusion compounds with (w/) or without (w/o) silver nanoparticles (AgNPs) [14] at different concentrations (in $\mu g \cdot mL^{-1}$). For each treatment, concentrations labelled with the different lowercase letters are significantly different at $p < 0.05$ by Tukey's test. For treatments with the same polyphenol and at the same dose, different uppercase letters indicate that the absence/presence of AgNPs resulted in significant differences at $p < 0.05$ by Tukey's test. Mean values of three experiments with three replicates are presented, with error bars representing the standard deviation.

Table 1. Effective concentrations of the conjugates that inhibited mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively).

Treatment	EC_{50} ($\mu g \cdot mL^{-1}$)		EC_{90} ($\mu g \cdot mL^{-1}$)	
	w/o AgNPs	w/AgNPs	w/o AgNPs	w/AgNPs
Control	–	458.4	–	1192.8
Curcumin	257.5	279.9	448.3	487.4
Ferulic acid	228.7	171.6	446.2	450.4
Gallic acid	373.6	261.3	795.3	455.6
Silymarin	195.5	261.8	453.1	963.7

In relation to the influence of the polyphenol, for the treatments without AgNPs, both of on the basis of the least square means and the EC_{50} values, the efficacy would follow the sequence: silymarin > ferulic acid > curcumin > gallic acid. On the basis of the EC_{90} values, the sensitivities of *P. cinnamomi* to curcumin, ferulic acid and silymarin would be similar, and gallic acid would be the least preferred choice.

According to Wadley's method [17] for quantification of the level of interaction, a synergy factor FS = 1.7 was obtained for gallic acid, indicative of a synergistic interaction with AgNPs; for ferulic acid, FS = 1.0 was found, indicative of an additive behavior between the two antifungal products; and for silymarin and curcumin, FS values of 0.5 and 0.9 were calculated, respectively, indicative of an antagonistic behavior with the AgNPs.

Although rare in the literature, it should be clarified that, for example, cases of antagonistic behavior of AgNPs with amoxicillin versus methicillin-resistant *Staphylococcus aureus* (MRSA) have been reported [18]. These authors, in trials with 7 organisms and 19 antibiotics, completed 96 tests, finding 5 combinations with synergistic behavior, 89 with additive behavior and 2 with antagonistic

behavior. This work would provide evidence of the existence of analogous complex interactions with AgNPs in the case of phenolic complexes.

4. Conclusions

The in vitro tests led to notable differences in the inhibition of mycelium growth of *P. cinnamomi*, observing a superior performance of the preparations based on ferulic acid with AgNPs and silymarin without AgNPs (thus evidencing that the addition of AgNPs, in spite of their antimicrobial activity, does not always result in synergies). In fact, an unexpected antagonistic behavior was detected for two of the polyphenols (curcumin and silymarin), an additive behavior was observed for ferulic acid and a synergistic behavior was found for gallic acid. In any case, in view of the EC_{50} and EC_{90} concentrations, these preparations can be promising bioactive products for protection applications of crops and forests against *P. cinnamomi*.

Supplementary Materials: The following are available online at http://www.mdpi.com/2624-7402/2/1/5/s1, Figure S1: Example of sensitivity test. Radial growth of mycelium for the control and treatments with curcumin, ferulic acid, gallic acid and silymarin; Table S1: Test of between-subjects effects; Table S2: Categories as a function of radial growth values for each polyphenol*dose*AgNPs combination, with a confidence interval of 95%, by Tukey's HSD test.

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ARTÍCULOS PENDIENTES DE PUBLICACIÓN

Artículo #8 (enviado a *Phytopathologia Mediterranea*, Q1; factor de impacto JCR: 2.037)

Rutin–stevioside conjugates for the control of grapevine trunk diseases

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Rutin–stevioside conjugates for the control of grapevine trunk diseases

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Summary. Flavonoids and phenolic acids play a major role in grapevine defence against grapevine trunk disease (GTD) agents. In this work, rutin –one of the main flavonoids present in the vegetative organs of the grapevine plant, which features advantages in terms of non-toxicity and non-oxidizability over other flavonoids– was assayed against two Botryosphaeriaceae taxa, both *in vitro* and *in vivo*. Its limited bioavailability problem was circumvented by conjugation with stevioside, a glycoside obtained from *Stevia rebaudiana*. A clear synergistic effect was observed for the stevioside-rutin adduct, resulting in EC_{50} and EC_{90} values of 275.03/236.75 and 845.83/716.44 $\mu g \cdot mL^{-1}$ against *Neofusicoccum parvum*/*Dothiorella viticola*, respectively. In greenhouse experiments, moderate and complete inhibition of *N. parvum* and *D. viticola* fungal growth, respectively, was found. These results were better than those attained for ferulic acid, chosen as a reference in the experiments given that it is considered the most effective phenolic acid against GTDs. Conjugation with stevioside thus provides a key for rutin solubility enhancement and paves the way to the design of glycodrugs based on rutin-rich plant extracts as promising antifungals against GTDs.

Keywords. Antifungal; Botryosphaeria dieback; candyleaf; GTDs; rutoside.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) cultivars are greatly affected by a large number of pathogens (fungi, bacteria, oomycetes or viruses). The so-called grapevine trunk diseases (GTDs) have been responsible for a long time of significant economic losses worldwide, and some of them are well known since at least one century. The interest of the viticulture sector in this group of mycoses has been growing in the last three decades as a result of two fundamental facts. On the one hand, in the last 10-20 years a noticeable increase in the mortality of young vine plants from the nursery has been detected, especially in the first 1-3 years after their planting in the field. On the other hand, the progressive suspension of the use of fungicides of chemical origin has resulted in a progressive increase in the incidence and losses due to these diseases. Current agricultural policies are promoting eco-friendly crop management, a scenario that has provoked intense interest in developing alternative, natural-derived anti-fungal products for the sustainable management of grapevine diseases. Along with these facts, it is

currently accepted that there are other factors directly or indirectly involved in the spread of these pathologies, both in young individuals and in adults. Some of these are related with a series of changes in cultural practices, such as less protection in wounds generated by pruning work or the reduction of sanitary control measures in certified propagation material (Graniti *et al.*, 2000). In addition, it is commonly accepted that a correlation exists between the increase in the incidence of decay of young vine plants and the increase in the demand of new plantings or replacements in the different world producing areas. This vision has led to advocate a model in which infected material from producer nurseries is currently considered as the primary source of inoculum in the dynamics of the disease. Thus, there are numerous studies (Surico, 2001; Fourie and Halleen, 2004) that have correlated the presence of certain fungal species causing decay in the propagation material in nurseries with the dead of grapevine plants in the very first years after planting.

Natural compounds tested up to date against the three main grapevine trunk diseases (Botryosphaeria dieback, Esca complex, Eutypa dieback) include chitosan, garlic extract, tea tree (*Melaleuca alternifolia* (Maiden & Betche) Cheel) oil, green coffee extract, lemon peel extract, honey, propolis, seaweed extract and saponins, according to the comprehensive review paper by Mondello *et al.* (2018). Nonetheless, few studies have focused on the specific phytochemicals behind the bioactivity of these natural compounds, which may result in a more efficient approach if one takes into consideration that materials of plant origin are usually characterized by a high variability of phytochemical composition, resulting from both genetic variability and environmental variability (due to the influence of weather and soil fertility on the content of active substances).

Phytoalexins, which in vines are phenolic compounds that include tannins, phenolic acids, flavonoids and stilbenes, are involved in grapevine defence mechanisms, increasing plant resistance (Del Río *et al.*, 2004): for instance, progression of fungal pathogens along the wood is inhibited by the formation of polyphenol-rich reaction zones (Fontaine *et al.*, 2016). The possible role of phenolics in grapevine defence against GTD casual agents was studied by Lambert *et al.* (2012a), who analysed the *in vitro* effect of 24 grapevine phenolic compounds (8 phenolic acids, 3 flavan-3-ols, 2 flavonols, and 11 stilbenoids) on 6 taxa belonging to the *Botryosphaeriaceae* family. They reported that these pathogens exhibited a common susceptibility to phenolics that was found to be more or less significant, and concluded that ferulic acid (i.e., *trans*-4-hydroxy-3-methoxycinnamic acid) was one of the most active molecules, with an inhibition effectiveness comparable to that of stilbenoids such as *ε*-viniferin, vitisin A and B, or *trans*-pterostilbene. However, in that study, no inhibitory activity on any wood disease fungi was found for flavonols (kaempferol and quercitin) and flavan-3-ols [(+)-catechin, (−)-epicatechin, epicatechin-3-*O*-gallate] and, in some cases, they even enhanced the growth of some of the assayed fungi. This is a surprising result, given that other authors have reported that, for instance, catechin inhibited fungi involved in Petri disease and in other trunk grapevine diseases (e.g., *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams, *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai , *Eutypa lata* (Pers.) Tul & C. Tul and *Stereum hirsutum* (Willd.) Pers.) (Del Río *et al.*, 2004). Furthermore, the antifungal efficacy of flavonoids is well-established, as discussed in the recent review papers by Al Aboody and Mickymaray (2020) and Jin (2019).

To gain further insight into these conflicting results, in this work we have compared the effectiveness of a flavonoid-3-*O*-glycoside, viz. rutin (also known as rutoside,

phytomelin or quercetin 3-*O*-rutinoside), with that of aforementioned successful ferulic acid. Rutin is one of the most abundant polyphenols (excluding stilbenes) in the vegetative organs of grapevine plants, found at mean concentrations of 257 $mg \cdot kg^{-1}$ (Goufo *et al.*, 2020), and is known to possess a significant antimicrobial activity (Ganeshpurkar and Saluja, 2017). Its use is advantageous over other flavonoids as it is consider as a non-toxic and non-oxidizable molecule (Sharma *et al.*, 2013).

However, the low water-solubility of phenolic acids (e.g., ferulic acid (Shakeel *et al.*, 2017)) and flavonoids (Chebil *et al.*, 2007) can limit their bioavailabity and applicability (Hussain *et al.*, 2017). This obstacle may be circumvented through the formation of inclusion compounds or conjugate complexes with terpene glycosides (Nguyen *et al.*, 2017). For instance, stevioside [the major constituent of *Stevia rebaudiana* (Bertoni) Bertoni extract] may be a suitable option to form those conjugate complexes, resulting in an enhanced antifungal activity; in this sense, a clear synergistic effect has been previously reported upon conjugation of phenolic acids with stevioside against *Fusarium culmorum* (Wm.G.Sm.) Sacc. (Buzón-Durán *et al.*, 2020) and *Phytophthora cinnamomi* Rands (Matei *et al.*, 2018b).

The goal of the study presented herein has been to assess the antifungal activity –both *in vitro* and *in vivo*– of rutin against two taxa of the *Botryosphaeriaceae* family that affect with highest incidence grapevine worldwide, specially young plants in the first years after planting, alone and in a conjugate complex with stevioside, taking ferulic acid as a reference substance for comparison purposes. This information should be useful with a view to the selection of promising plants for the valorisation of their extracts as natural antifungal products in organic or integrative Viticulture.

MATERIALS AND METHODS

Reagents

Rutin hydrate (CAS 207671-50-9, ≥94%), ferulic acid (CAS 537-98-4, European Pharmacopoeia reference standard), sodium alginate (CAS 9005-38-3) and calcium carbonate (CAS 471-34-1, ≥99.0%) were supplied by Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). Stevioside (CAS 57817-89-7, 99%) was purchased from Wako Chemicals GmbH (Neuss, Germany). Potato dextrose agar (PDA) was supplied by Becton, Dickinson & Company (Franklin Lakes, NJ, USA).

Fungal isolates

Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & A.J.L.Phillips (code ITACYL_F111; isolate Y-091-03-01c; isolated from 'Verdejo' cultivar grapevines in a nursery in Navarra, Spain, in 2006) and *Dothiorella viticola* A.J.L.Phillips & J.Luque (code ITACYL_F118; isolate Y-103-08-01; isolated from grapevines in Extremadura, Spain, in 2004) were supplied as lyophilized vials (later reconstituted and refreshed as PDA subcultures) by the Agricultural Technological Institute of Castilla and Leon (ITACYL, Valladolid, Spain) (Martin and Cobos, 2007).

Preparation of the bioactive formulations

Treatments based on pure stevioside, rutin and ferulic acid were prepared by dissolving the respective reagents in Milli-Q water as supplied, without further purification.

Ultrasonication-assisted aqueous biphasic system separation was used to prepare the stevioside–polyphenol conjugate complexes in a 1:1 molar ratio. 50 mL of an aqueous solution of stevioside (126 mg, MW=804.87 g·mol^{-1}, 0.156 mM) were mixed with a 50 mL ethanol solution of either ferulic acid (95.2 mg, MW=610.517 g·mol^{-1}, 0.156 mM) or rutin (75.3 mg, MW=482.44 g·mol^{-1}, 0.156 mM). The solutions were sonicated with a probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) for 15 min, keeping the temperature below 60 °C.

For the *in vivo* experiments, the conjugate complexes were dispersed in a calcium alginate matrix, in the form of hydrogel beads. The beads were prepared as follows: each bioactive product was firstly added to a 3% sodium alginate solution in a 2:8 ratio (20 mL bioactive product:80 mL sodium alginate), and this solution was then dispensed drop by drop onto a 3% calcium carbonate solution to spherify (polymerize) it. Beads of ⌀ = 0.4-0.6 cm containing the different treatments were obtained.

In vitro tests of mycelial growth inhibition

The biological activity of the treatments was determined using the agar dilution method, incorporating aliquots of stock solutions into a PDA medium to provide final concentrations of 62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000 and 1500 µg·mL^{-1}. Mycelial plugs of each pathogen (⌀ = 5 mm) from the edges of a 7-day old cultures were transferred to plates filled with these media (3 plates per treatment and concentration, with 2 repeats). Plates containing only the PDA medium without any amendment were used as controls.

Radial mycelial growth was determined by calculating the average of two perpendicular colony diameters for each replicate. Mycelial growth inhibition for each treatment and concentration after 7 days of incubation, at 25 °C in the dark, was calculated according to the formula: $((d_c - d_t)/d_c) \times 100$, where d_c and d_t are the average diameter of fungal colony in the control and of the fungal colony treated with the tested composite, respectively.

Fitting the radial growth inhibition values (%) with a DoseResp function, using an orthogonal distance regression (ODR) algorithm, allowed to express the results in terms of the 50% and 90% effective concentrations (EC_{50} and EC_{90}, respectively).

As regards the level of interaction, synergy factors (S.F.) were estimated according to Wadley's method (Levy *et al.*, 1986).

Greenhouse bioassays on grafted plants

The protective capabilities of the most promising formulations and dosages according to the *in vitro* mycelial growth inhibition experiments were further assayed in grafted plants against the two selected *Botryosphaeriaceae* species. Plant material consisted of 68 plants, half of which were 2-year old "Tempranillo" cultivar vines (CL. 32 clone) grafted on 775P rootstock, and the rest 1-year old "Garnacha" cultivar (VCR3 clone) vines grafted on 110R rootstock. Plants were grown on 3.5 L plastic pots containing a moss peat–sterilized natural soil (75:25) mixed substrate, to which a slow release fertilizer was incorporated when needed. Plants were kept in a greenhouse with drip irrigation and anti-weed ground cover for 6 months (from June to December 2020).

One week after placing them in the greenhouse, grapevine plants were artificially inoculated with the two *Botryosphaeriaceae* pathogens (*N. parvum* and *D. viticola*) and

simultaneously treated with either the stevioside−rutin or the stevioside−ferulic acid treatment. Inoculations of both pathogens and bioactive products were carried out directly on the trunk of the living plants at two sites per plant individual –at least 5 cm apart from each other– below the grafting point (and not reaching the root crown). For the pathogens, agar plugs coming from 5-days-old fresh PDA cultures of each species were used as the fungal inoculum. In the mentioned two inoculation points of each grapevine plant, slits (⌀ ≈ 15 mm, 5 mm deep) were done with a scalpel. Agar plugs (⌀ nes ADDIN EN.CITE ADDIN EN.CITE.DATA (Agrelli et al., 2009; Amalfitano et al., 2011) ,while and ; then, the beads containing the bioactive product were placed at both sides of the agar plug, and both the agar plugs and the beads were covered with cotton soaked in sterile bi-distilled water and sealed with Parafilm™ tape.

Five repetitions were arranged for each pathogen/bioactive product and plant (cultivar/rootstock) combination; in addition to 4 positive controls/(pathogen*cultivar) and 3 negative controls (incorporating only the bioactive product) for each treatment were disposed (Table 1).

Table 1. Repetitions for each of the plant/treatment combinations arranged in the greenhouse bioassay. Each grafted plant was inoculated at two sites below grafting point.

Plant	Treatment	Pathogen	Number of replicates
'Tempranillo' (CL. 32 clone) on 775P rootstock	Stevioside-ferulic acid	*N. parvum*	5
		D. viticola	5
		None (negative control)	3
	Stevioside-rutin	*N. parvum*	5
		D. viticola	5
		None (negative control)	3
	None (positive control)	*N. parvum*	4
		D. viticola	4
'Garnacha' (VCR3 clone) on 110R rootstock	Stevioside-ferulic acid	*N. parvum*	5
		D. viticola	5
		None (negative control)	3
	Stevioside-rutin	*N. parvum*	5
		D. viticola	5
		None (negative control)	3
	None (positive control)	*N. parvum*	4
		D. viticola	4

During the assay period, cuprous oxide 75% was applied in mid-July to control downy mildew outbreaks, together with a first sprouting (followed by periodic sprouting). Grapevine plants were visually examined during the whole assay period for the presence of foliar symptoms (including both internerval and nerval necroses) on a weekly basis.

At the end of the experiment, plants were removed and two transversal sections of the inoculated stems between the grafting point and the root crown were prepared and sectioned longitudinally. The effect caused by the fungal pathogens inoculated was evaluated by measuring the length of the vascular necroses longitudinally on upper and lower directions from the inoculation point for both halves of the longitudinal cut.

Samples from the assayed grapevine plants were further processed to re-isolate the different pathogenic taxa previously inoculated. Thus, in order to fulfil Koch's postulates, 5 mm-long wood chips exhibiting vascular necroses (1–2 cm around the wounds) were washed, surface sterilized, placed in PDA plates amended with streptomycin sulphate (to avoid bacterial contamination) and incubated at 26 °C in the

dark for 2–3 days in a culture chamber. Emerging colonies were identified on the basis of their morphological characters.

Statistical analyses

Given that the homogeneity and homoscedasticity requirements were satisfied, according to Shapiro–Wilk and Levene tests, the results of the *in vitro* inhibition of mycelial growth were statistically analyzed using one-way analysis of variance (ANOVA), followed by *post hoc* comparison of means through Tukey's test at $p < 0.05$. In the case of greenhouse assay results, since the normality and homoscedasticity requirements were not met, Kruskal-Wallis non-parametric test was used instead, with Conover-Iman test for *post hoc* multiple pairwise comparisons. R statistical software was used for all the statistical analyses (R Core Team, 2020).

RESULTS

In vitro tests of mycelial growth inhibition

From the results of the *in vitro* tests (Figure 1 and Figure S1), a higher antifungal efficacy could be observed for stevioside and rutin alone than for ferulic acid alone against both *Botryosphaeriaceae* taxa, especially in the case of *N. parvum*. Upon application of the stevioside–rutin and stevioside–ferulic acid conjugate complexes, a significant improvement was observed in both cases. For the former adduct, full (or almost full) inhibition was attained at 1000 and 750 μg·mL^{-1} against *N. parvum* and *D. viticola*, respectively, while a dose of 1500 μg·mL^{-1} was needed when stevioside or rutin were separately tested. In the case of the ferulic acid adduct, its efficacy again was slightly lower than that of its rutin counterpart: concentrations of 1500 and 1000 μg·mL^{-1} were required to attain full inhibition against *N. parvum* and *D. viticola*, respectively (while inhibition percentages of 63.6 and 73.8%, respectively, were registered at 1500 μg·mL^{-1} for the ferulic acid-only treatment).

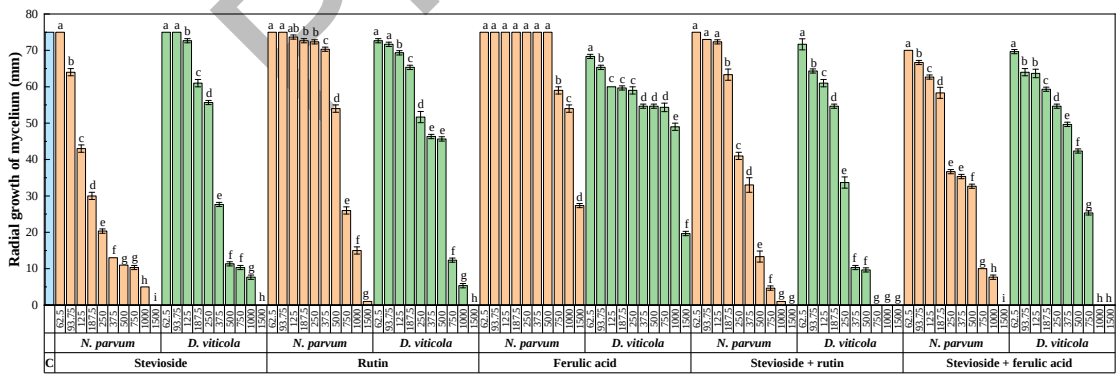

Figure 1. Colony radial growth values of *N. parvum* and *D. viticola* strains when cultured in PDA plates containing the various control products (viz. stevioside, rutin, ferulic acid, stevioside–rutin and stevioside–ferulic acid conjugate complexes) at concentrations in the 62.5–1500 μg·mL^{-1} range. The same letters above concentrations indicate that they are not significantly different at $p < 0.05$. Error bars represent standard deviations.

A comparison of the 50% and 90% maximal effective concentrations (Table 2) corroborated the comments made above, with lower EC values for rutin than for ferulic

acid, especially against *D. viticola*. As regards the presence of a synergistic behaviour, differences were found between the two fungi: in the case of *N. parvum*, synergy factors for ferulic acid were close to one, suggesting that the hypothesis of similar joint action (or additivity) could be accepted; while for rutin a SF of 1.23 was obtained for the EC_{90} value, pointing to synergistic action. On the other hand, SFs >1 were obtained in all cases against *D. viticola*, with values close to 1.5 and 1.3 for the rutin and ferulic acid adducts, respectively, evidencing clear synergism and differences in the response between the two fungi.

Table 2. EC_{50} and EC_{90} effective concentrations, expressed in $\mu g \cdot mL^{-1}$.

Pathogen	Effective Concentration	Stevioside	Rutin	Ferulic acid	Stevioside–rutin	S.F.	Stevioside–ferulic acid	S.F.
N. parvum	EC_{50}	194.86	529.19	1339.41	275.03	1.04	335.26	1.01
	EC_{90}	723.82	1834.36	3230.15	845.83	1.23	1132.15	1.04
D. viticola	EC_{50}	306.91	395.52	1387.22	236.75	1.46	385.39	1.32
	EC_{90}	917.08	1281.12	3921.26	716.44	1.49	1421.75	1.24

S.F. = synergy factor

Greenhouse bioassays on grafted plants

Statistically significant differences were found in terms of both the lengths of the vascular necroses observed between treated and non-treated plants for both fungal pathogens and visual estimations. In the case of *N. parvum* (Figure 2), the performance of the two assayed formulations was found to be similar (Table 3). On the other hand, in the case of *D. viticola*, a higher efficacy of the stevioside-rutin conjugate complex than that of the stevioside-ferulic acid conjugate complex –previously observed in the *in vitro* tests– was evidenced (

Table **4**). Moreover, the lengths of the necroses for the treated plants were not significantly different from those of the negative controls (i.e., those recorded in plants treated with conjugate complexes without pathogens), pointing to a high inhibition of this pathogen; furthermore, the lesions observed for the two negative controls were similar to each other.

An unexpected result was that the mean of ranks value for the stevioside–rutin treated plants was even lower than that of its negative control, i.e., the length of the necrosis in wounds artificially inoculated with *D. viticola* and treated with the rutin conjugate complexes was shorter than the length of the callus tissue developed on the wound exposed only to the bioactive product. This may be tentatively explained by slight differences in the length of the inflicted wounds, but suggests that an almost complete inhibition of the pathogen must have been attained so that necrosis length values were comparable to the hypersensitivity reaction caused by exposure of the plant tissues to this treatment (which was small).

Table 3. Kruskal-Wallis test and multiple pairwise comparisons using the Conover-Iman procedure for the lengths of the vascular necroses for *N. parvum*. Treatments/controls labelled with the same letters are not significantly different at $p < 0.05$.

Sample	Frequency	Sum of ranks	Mean of ranks	Groups		
Stevioside-rutin negative control	48	2043.500	42.573	A		
Stevioside-ferulic acid negative control	40	1927.500	48.188	A		
Stevioside-rutin	48	6991.500	145.656		B	
Stevioside-ferulic acid	40	6225.500	155.638		B	
Positive control	64	11732.000	183.313			C

Table 4. Kruskal-Wallis test and multiple pairwise comparisons using the Conover-Iman procedure for the lengths of the vascular necroses for *D viticola*. Treatments/controls labelled with the same letters are not significantly different at $p < 0.05$.

Sample	Frequency	Sum of ranks	Mean of ranks	Groups			
Stevioside-rutin	72	6514.000	90.472	A			
Stevioside-rutin negative control	48	5319.000	110.813	A	B		
Stevioside-ferulic acid negative control	40	4948.500	123.713		B	C	
Stevioside-ferulic acid	72	10485.500	145.632			C	
Positive control	64	16689.000	260.766				D

Figure 2. Foliar symptoms and vascular necroses observed in grapevine plants artificially inoculated with *N. parvum* and treated with the two conjugate complexes of natural products. Top row, from left to right; general aspect of plants treated with *N. parvum* (positive control), stevioside–ferulic acid, stevioside–rutin, *N. parvum* + stevioside–ferulic acid and *N. parvum* + stevioside–rutin respectively; Bottom row; vascular lesions observed after sectioning of those grapevine plants.

DISCUSSION

Comparison with antifungal efficacies reported in the literature for these bioactive substances

When comparing results referring to the sensitivity of a certain fungal pathogen to the exposure against certain fungicidal compound, it should be taken into account that the susceptibility profile in these microorganisms is usually species- and even isolate-dependent, so comparisons of the effective concentrations discussed below should be taken with caution.

Previous works have advocated ferulic acid as the phenolic acid with the strongest anti-fungal activity (Lambert *et al.*, 2012a; Sabel *et al.*, 2017; Zabka and Pavela, 2013). Concerning studies of its efficacy against GTDs, Lambert *et al.* (2012b), assaying concentration of 500 μM (97 $\mu g \cdot mL^{-1}$), found *in vitro* inhibition growth percentages of 23% and 35% for ferulic acid against *N. parvum* PER20 and *N. parvum* Bp0014 strains, respectively, but no MIC or EC values were reported. In addition, EC_{50} values of 3530 and 4740 $\mu g \cdot mL^{-1}$ were reported for ferulic acid against *Botryosphaeriaceae* sp. and *Phaeoacremonium minimum* by Gómez *et al.* (2016). In the study by Dekker *et al.* (2002), 62% inhibition was reported for ferulic acid against *Botryosphaeria* sp. at a concentration of 25 mM (4855 $\mu g \cdot mL^{-1}$), with an EC_{50} value of 15 mM (2913 $\mu g \cdot mL^{-1}$). In general terms, the EC_{50} values in aforementioned studies would be 2-3 times higher than the ones reported herein (1340 and 1454 $\mu g \cdot mL^{-1}$). Srivastava *et al.* (2013) tested ten naturally occurring phenolic compounds from plants against various isolates of *Botryosphaeria* genus (viz. *B. rhodina* (syn. *Lasiodiplodia theobromae*), *B. obtusa*, and *B. ribis* (syn. *Neofusicoccum ribis*)), and found that ferulic acid at 25 mM (4855 $\mu g \cdot mL^{-1}$) resulted in ca. 80% and ca. 70% mycelium growth inhibition of *B. rhodina* and *B. ribis*, respectively; and, in the case of *B. obtusa*, 100% inhibition was attained at 20 mM (3885 $\mu g \cdot mL^{-1}$). These concentrations are in the same order of magnitude as the EC_{90} values obtained here against *N. parvum* and *D. viticola* (3230 and 3921 $\mu g \cdot mL^{-1}$, respectively).

In relation to its activity against other fungi, in a comprehensive study of the efficacy of 21 phenolic components of essential oils and plant substances against several toxicogenic filamentous fungi, Zabka and Pavela (2013) reported MIC values >1000 $\mu g \cdot mL^{-1}$ for ferulic acid against *Fusarium oxysporum*, *F. verticillioides*, *Penicillium brevicompactum*, *P. expansum*, *Aspergillus flavus* and *A. fumigatus*. EC_{50} values ranged from 411 (*P. expansum*) to 895 $\mu g \cdot mL^{-1}$ (*A. flavus*). Ferulic acid was also found to inhibit conidial germination of a watermelon soil-borne pathogen, *F. oxysporum* f. sp. *niveum* at concentrations of 800 $\mu g \cdot mL^{-1}$ (Wu *et al.*, 2010). Other researchers observed that ferulic acid at 5000 $\mu g \cdot mL^{-1}$ severely repressed the growth of the lignocellulolytic fungi *Trichoderma harzianum*, *Chaetomium cellulolyticum*, *Phanerochaete chrysosporium*, *Trametes versicolor* and *Pleurotus sajor-caju* (Asiegbu *et al.*, 1996). Ferulic acid or ferulic acid-rich extracts have also been proposed as a natural alternative to reduce post-harvest losses by Hernández *et al.* (2021); these authors reported almost 100% inhibition of *Monilinia fructicola* at a dose of 2 mM (390 $\mu g \cdot mL^{-1}$), and 90% inhibition of *Alternaria alternata* and *Botrytis cinerea* at a concentration of 3 and 7.5 mM (583 and 1457 $\mu g \cdot mL^{-1}$, respectively).

With regard to the antifungal activity of rutin against GTDs, to the best of the authors' knowledge, no data is available. Nonetheless, to date, it is reported that more than 70 plant species contain rutin (Gullón *et al.*, 2017), and different plant extracts with

high contents of rutin have shown inhibitory effects on the growth of other fungal species. For instance, Devi *et al.* (2007) found significant growth inhibition against *F. oxysporum, Curvularia lunata* and *Trichoderma viride* when treated with *Eupatorium birmanicum* DC [*Eupatorium cannabinum* subsp. *cannabinum*] extracts (at 1000, 500 and 100 ppm extract concentration, respectively, depending on the fungal species). Likewise, *Alternanthera maritima* (Mart.) St. Hil ethanolic extracts were assayed against *Candida albicans, C. tropicalis, C. glabrata, C. parapsilosis, Trichophyton mentagrophyte* and *T. rubrum*, finding moderate inhibition (Salvador *et al.*, 2004). Upon testing of pure rutin against the same fungi, MIC values of 500 $\mu g \cdot mL^{-1}$ were reported.

Parvu *et al.* (2015) found rutin contents of 130 and 170 $\mu g \cdot mL^{-1}$ in the flower and fruit extracts, respectively, of *Hedera helix* L. (ivy), and assayed them against *A. niger, B. cinerea, F. oxysporum* f. sp. *tulipae, Penicillium gladioli* and *Sclerotinia sclerotiorum.* Full mycelial growth inhibition was attained at 12, 8, 10, 10 and 8% concentration, respectively, for the flower extracts; and at 14, 19, 12, 12 and 10% concentration, respectively, for the fruit extracts.

Elansary *et al.* (2020a) assayed the stem extracts of six *Ferocactus* species (*F. gracilis, F. pottsii, F. herrerae, F. horridus, F. glaucescens,* and *F. emoryi*), with rutin contents of up to 108 mg/100 g DW against several bacteria and fungi. They found strong antifungal effects against *A. flavus, A. ochraceus, A. niger, C. albicans, Penicillium funiculosum* and *P. ochrochloron* (with MICs in the 100-730 $\mu g \cdot mL^{-1}$ range). Slightly higher rutin concentrations (139 mg/100 g DW) were found by the same group in *Ocimum basilicum* L. (basil), resulting in MIC values over the 290-560 $\mu g \cdot mL^{-1}$ interval against the same fungal pathogens (Elansary *et al.*, 2020d). Much higher rutin concentrations (1533 and 1010 mg/100 g DW) were found in *Acacia saligna* L. and *Ruta graveolens* L. leaves (Elansary *et al.*, 2020b; Elansary *et al.*, 2020c). In the associated *in vitro* assays, conducted for both the leaf methanolic extracts and several pure bioactive compounds detected by high-performance liquid chromatography–diode array detection (HPLC-DAD), the authors found MIC values against the mentioned fungal taxa referred above in the 180-300 $\mu g \cdot mL^{-1}$ range for pure rutoside (comparable to those of Streptomycin), ranging from 300 to 580 $\mu g \cdot mL^{-1}$ for *A. saligna* extract, and over the 330-780 $\mu g \cdot mL^{-1}$ interval for *R. graveolens* extract.

Regarding the antifungal activity of stevioside (*Stevia rebaudiana* extracts), Ghosh *et al.* (2008) and Abou-Arab and Abu-Salem (2010) reported a growth inhibition effect against *A. solani, Helminthosporium solani, A. niger, A. ochraceus, A. parasiticus, A. flavus* and *Penicillium chrysogenum,* but MIC values were not determined. Its antifungal activity (at a concentration of 50000 $\mu g \cdot mL^{-1}$) was also demonstrated against *A. flavus, A. fumigatus, A. niger,* and *Fusarium oxysporum* by Arya *et al.* (2012). MIC values in the 250–300 $\mu g \cdot mL^{-1}$ range were found against *A. flavus, A. ochraceus, A. niger* and *Fusarium moniliforme* by Abdel-Fatt *et al.* (2018). Shukla *et al.* (2013) reported MIC values of 3 and 2 $mg \cdot mL^{-1}$ against *B. cinerea* and *F. oxysporum*, respectively, and Guerra Ramírez *et al.* (2020) found that the hexane extract at a concentration of 833 ppm inhibited mycelial growth of *F. oxysporum* up to 50%.

Concerning the antifungal activity of stevioside-polyphenol conjugate complexes, no data is available –to the best of the authors' knowledge– against fungi associated with GTDs. As regards other phytopathogenic fungi, EC_{50} and EC_{90} values of 123 and 160 $\mu g \cdot mL^{-1}$, respectively, were reported against *F. culmorum* for conjugate complexes based on stevioside and polyphenols present in milk thistle seeds (*Silybum marianum*

(L.) Gaertn) in a 1:1 ratio (Buzón-Durán *et al.*, 2020). Composites based on stevioside:ferulic acid inclusion compounds (albeit in a 5:1 molar ratio), combined with chitosan oligomers in hydroalcoholic solution or in choline chloride:urea deep eutectic solvent media, were assayed against *F. culmorum* (Matei *et al.*, 2018a), obtaining EC_{50} and EC_{90} values in the 175-292 and 377-713 $\mu g \cdot mL^{-1}$ range, respectively, depending on the dispersion medium. Inclusion compounds from stevioside and ferulic acid in 6:1 ratio, dispersed in a hydroalcoholic solution of chitosan oligomers, were also assayed against *Phytophthora cinnamomi*, with EC_{50} and EC_{90} values of 171-229 and 446-450 $\mu g \cdot mL^{-1}$, respectively (depending on the presence/absence of silver nanoparticles) (Matei *et al.*, 2018b; Matei *et al.*, 2020).

Mechanism of action

Antimicrobial activity of ferulic acid can involve various modes of action, mainly related to the destabilization and permeabilization of the cytoplasmatic membrane and to enzyme inhibition by the oxidized products (Borges *et al.*, 2013). Regarding the effect of phenolic acids on physicochemical surface properties of the cells, it has been verified that these compounds are electrophilic and change hydrophobicity. Due to their partially lipophilic character, it is assumed that they cross the cell membrane by passive diffusion in their undissociated form, disturbing the cell membrane structure by localized hyperacidification, and possibly acidifying the cytoplasm and causing protein denaturation. The alteration of cell membrane potential makes it more permeable and results in the leakage of cell constituents, including proteins and nucleic acids. Additionally, ferulic acid (like p-coumaric acid and caffeic acid) affects the cell membrane structure by rigidity and alteration of the dynamics of phospholipid chains (Ota *et al.*, 2011). Other authors have noted that it also leads to significant changes in intracellular ATP concentrations (Shi *et al.*, 2016).

The antifungal mechanism of action of flavonoids has been recently covered in a comprehensive review paper by Al Aboody and Mickymaray (2020). Flavonoids inhibit fungal growth via various underlying mechanisms, including plasma membrane disruption, mitochondrial dysfunction induction, and inhibition of the efflux mediated pumping system, cell division, cell wall formation, and protein and RNA synthesis. In the particular case of rutin, the mechanism of its pharmacological action have been summarized by Koval'skii *et al.* (2014), who also noted that it can interact with various structures at the molecular level (free radicals, protein systems, enzymes, etc.).

In addition to aforementioned direct effect, which results in a reduction of fungal growth by altering hyphal morphology, it has been reported that vine phenolic compounds also exert their action against fungi involved in GTDs through an indirect effect, via the inhibition of the extracellular fungal manganese peroxidase (MnP) enzyme involved in lignin degradation (Gómez *et al.*, 2016). A decrease of laccase production and pectinase activity of *Botryosphaeria* isolates resulting from phenolic compounds was also reported by Srivastava *et al.* (2013).

In connection with the mechanism of action of stevioside, a recent review by Khan *et al.* (2017) on phytoglycosides suggests that their antimycotic activity is mediated through different and multiple targets that are not fully understood, but there appears to be general consensus regarding that the main underlying mechanism is related to their ability to complex with sterols of fungal membranes, which produces spore-like structures that cause pore formations in membrane, loss in membrane integrity and even the rupture of the membrane, leading to fungal cell death.

Solubility and synergistic behaviour

As noted above, the major disadvantage associated with rutin is its poor bioavailability, mainly caused by its low aqueous solubility and poor stability. According to Gullón *et al.* (2017), this is a determinant factor that hinders the *in vivo* biological effects of rutin, in spite of the fact that it may show detectable bioactivity in different *in vitro* systems. Common approaches used to enhance its bioavailability are particle diminution to the submicron range and complexation with cyclodextrins and various metals. Also, various carrier systems for its delivery, namely micro- and nano-emulsions, nanocrystals and nanosuspensions have been proposed (Sharma *et al.*, 2013).

An alternative method is to use steviol glycosides as a natural solubilizer, an approach that has been successfully tested for several natural phenols, such as curcumin (a diarylheptanoid) (Zhang *et al.*, 2011; Nguyen *et al.*, 2017), liquiritin (the 4'-*O*-glucoside of the flavanone liquiritigenin) (Nguyen *et al.*, 2014), and betulinic acid (a pentacyclic triterpenoid) (Zhang *et al.*, 2016). In the particular case of rutin, Ko *et al.* (2016) optimized its solubility by the Box-Behnken design with the aid of microwave-treatment (instead of ultrasonic treatment, as in the study presented herein). Other authors, such as Nguyen *et al.* (2015), have attained similar results for other flavonoids (quercetin), by complexation with rubusoside and rebaudioside, finding that as the glycoside concentration increases, the solubility of quercetin in water increased, without reducing its biological functions (in fact, they found a synergistic behavior in terms of inhibition activity against the $3CL^{pro}$ of SARS). Hence, it may be hypothesized that this solubility optimization would be responsible for the synergistic effect observed when testing conjugates of the two glycodrugs against fungal pathogens involved on the grapevine trunk diseases. The same rationale may be applied to tentatively explain the synergy observed for ferulic acid (albeit weaker than that attained for rutin).

Opportunities for future GTDs treatments

Levels of phenolic compounds have been reported to increase in the discoloured wood of Esca disease-affected grapevines (Agrelli *et al.*, 2009; Amalfitano *et al.*, 2011), while it has been also shown that phenolic compounds play a key role in limiting fungal development in grapevine vascular tissues (Lambert *et al.*, 2012a; Lima *et al.*, 2011). For instance, Spagnolo *et al.* (2014) found the highest levels of total phenolics in the brown striped wood of three cultivars infected with *N. parvum* and *Diplodia seriata*; and stilbene polyphenols such as resveratrol and ε-viniferin increased in wood of vines artificially inoculated with *Phaeomoniella. chlamydospora* (Martin *et al.*, 2009). Quercetin-3-*O*-glucoside and *trans*-caffeoyltartaric acid (analogous to the rutin and ferulic acid pair studied herein) were found to be associated to *Plasmopara viticola* resistance in grapevine leaves (Ali *et al.*, 2012), and an increase of quercetin-3-*O*-galactoside and kaempferol-3-*O*-glucoside has been found in the asymptomatic leaves of plants infected with Bois noir phytoplasma (Rusjan *et al.*, 2012).

Hence, the approach presented herein, based on mimicking grapevine's response via their own phenolic compounds existing inside their tissues (albeit with solubility and bioavailability enhancements), can be regarded as a "natural" and effective way to control the development of certain GTDs pathogens. In spite of the fact that pure reagents were assayed in this study, the promising results attained may serve as guidance for the selection of natural antifungal sources. In the case of rutin, the extracts from *Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli, *Sambucus nigra* L., *Drimys*

winteri J.R.Forst. or *Taraxacum officinale* Weber ex Wiggins (Meinhart *et al.*, 2020) may deserve further attention for larger-scale field experiments. If plants rich in both rutin and ferulic would be preferred, the activity of the extracts from sea buckthorn (*Hippophae Rhamnoides* L.) (Criste *et al.*, 2020), *Rhinacanthus nasutus* (L.) Kurz (Huang *et al.*, 2015), *Artemisia absinthium* L., *Achillea millefolium* L., *Sambucus nigra* L. or *Salvia officinalis* L. (Bljajić *et al.*, 2021) may be explored instead.

CONCLUSIONS

In an attempt to mimic grapevine's defence response against GTDs, rutin efficacy as an antifungal agent was assayed against two *Botryosphaeriaceae* taxa, alone and forming a conjugate complex with stevioside. While the *in vitro* performance of the pure flavonoid-3-*O*-glycoside was moderate, with EC_{90} values of 1834 and 1281 $\mu g \cdot mL^{-1}$ against *N. parvum* and *D. viticola*, respectively, a remarkable improvement was attained for stevioside–rutin, with EC_{90} values of 846 and 716 $\mu g \cdot mL^{-1}$, respectively. The observed synergistic behaviour (with SFs of 1.23 and 1.49) can be tentatively ascribed to solubility and bioavailability optimization. Upon testing of the formulations in greenhouse *in vivo* conditions, moderate inhibition of *N. parvum* and full inhibition against *D. viticola* were attained for the stevioside-rutin treatment. These EC_{90} and *in vivo* results were consistently better than those found for ferulic acid and stevioside–ferulic acid, taken as a reference. The promising results attained in the present approach may serve as guidance for the selection of new plant extracts that can be valorised as antifungals in organic Viticulture.

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Rutin–stevioside conjugates for the control of grapevine trunk diseases

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SUPPORTING INFORMATION

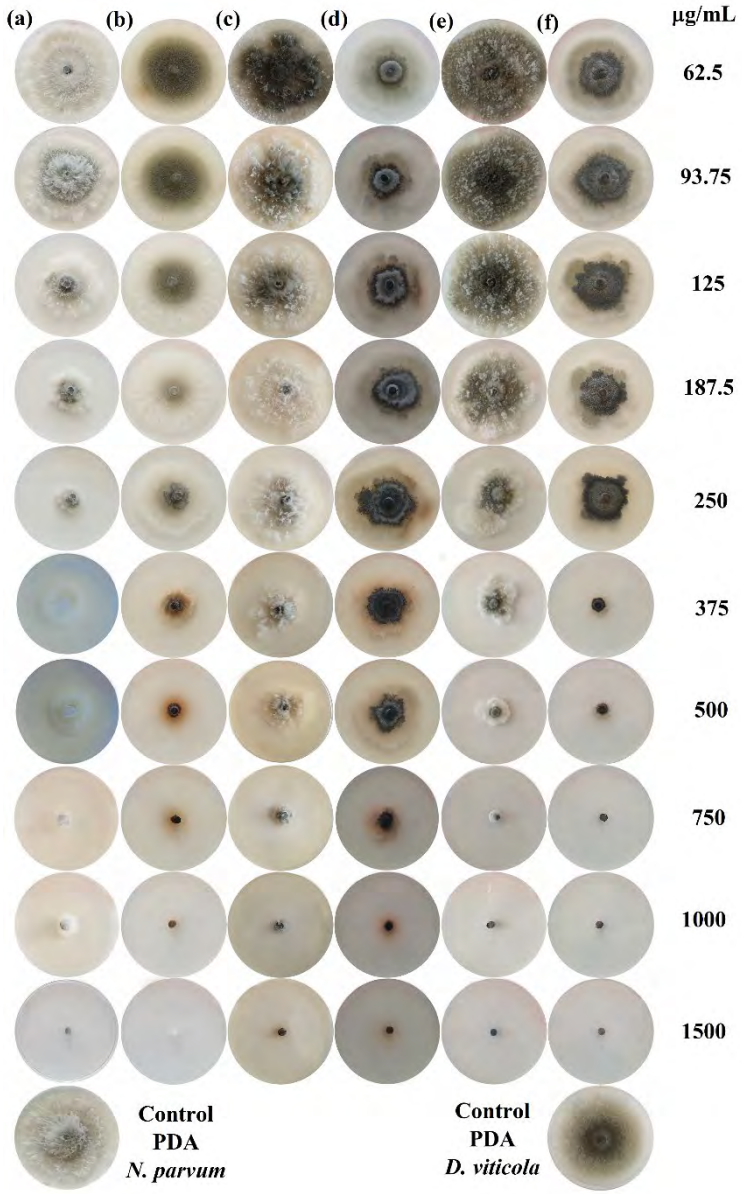

Figure S1. Mycelial growth inhibition of the two phytopathogenic fungi upon treatment with: **(a,b)** stevioside; **(c,d)** stevioside-ferulic acid conjugate complex; **(e,f)** stevioside-rutin conjugate complex. **(a, c and e)** correspond to *N. parvum*, and **(b, d, f)** to *D. viticola*. Only one replicate is shown.

Artículo #9 (enviado a *Plants*, Q1; factor de impacto JCR: 2.762)

Coniferyl alcohol and ferulic acid esters in the hydro-methanolic extract of *Silybum marianum* capitula: activity against Botryosphaeriaceae fungi

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Coniferyl alcohol and ferulic acid esters in the hydro-methanolic extract of *Silybum marianum* capitula: activity against Botryosphaeriaceae fungi

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Abstract
Silybum marianum (L.) Gaertn, viz. milk thistle, has been the focus of research efforts in the past few years, albeit almost exclusively restricted to the medicinal properties of its fruits (achenes). Given that other milk thistle plant organs have been scarcely investigated for bioactive compounds, in this study we present a phytochemical analysis of the extracts of *S. marianum* capitula during the flowering phenological stage (stage 67). Gas chromatography-mass spectroscopy results evidenced the presence of high contents of coniferyl alcohol (45.6%), and secondarily of ferulic acid ester, opening a new valorization strategy of this plant based on the former high-added value component. Moreover, the application the hydro-methanolic extracts as an antifungal agent has been explored. In particular, their activity against three fungi responsible for grapevine wood diseases (*Neofusicoccum parvum*, *Dothiorella viticola* and *Diplodia seriata*) has been assayed both *in vitro* and *in vivo*. From the mycelial growth inhibition assays, the best results (EC_{90} values of 303, 366 and 355 $\mu g \cdot mL^{-1}$ for *N. parvum*, *D. viticola* and *D. seriata*, respectively) were not obtained for the hydroalcoholic extracts, but after their conjugation with stevioside, which resulted in a strong synergistic behavior. Greenhouse experiments confirmed the efficacy of the conjugated complexes, pointing to the potential of milk thistle extracts as a promising plant protection product in organic Viticulture.

Keywords: *Silybum marianum*; coniferyl alcohol; ferulic acid; silymarin; stevioside

1. Introduction

Silybum marianum (L.) Gaertn (syn. *Carduus marianus* L.), commonly known as milk thistle, St. Mary's Thistle or wild artichoke, is an herbaceous plant of the *Asteraceae* family. Native to the Mediterranean area, it is nowadays grown in many countries as a medicinal plant, due to the plurality of biological activities –mostly linked to the hepatoprotective properties and anti-carcinogenic capacity– associated with the main pharmacological active ingredient extracted from its achenes (fruits): silymarin [1,2].

The standardized extract obtained from the dried fruits of *S. marianum* contains 70-80% of silymarin and 20-30% of polymeric and oxidized polyphenolic compounds [3]. Silymarin is a flavonolignan complex of polyphenolic molecules, which includes diastereoisomers silybin A and silybin B (whose mixture in a 1:1 ratio is named silibinin), silydianin, silychristin, isosilychristin, isosilybin A and isosilybin B, and the taxifolin flavanonol [4]. Biosynthesis of silybins from taxifolin and coniferyl alcohol is schematized in Figure S1 [5].

Most research has studied silymarin, or its major compound silybin, instead of the plant as a whole. The concentration of silymarin is organ dependent, and it is only localized in the outer portion of the fruit, which includes all the cell layers from the pericarp epidermis to the albumen,

and embryos [6], accounting for 1.5–4.3% of the fruit weight [7]. Silymarin is not present in flowers, stems or leaves, and is not found in steps before the development of fruit [8,9], which explains while other milk thistle plant organs have been scarcely investigated for bioactive compounds: total polyphenol and flavonoid contents in leaves extracts were studied by Saidi, *et al.* [10]; a phytochemical screening and GC–MS analysis of bioactive compounds present in ethanolic leaves extract was conducted by Mani, *et al.* [11]; and Sulas, *et al.* [12] studied the concentrations of crude protein, fat, total phenolics and total flavonoids in leaves, heads and stems.

To the best of the author's knowledge, an analysis of the phytochemicals present in the capitula in the flowering stage, prior to seed maturation, has not been reported to date. Nonetheless, the existence of some precursors proposed in the bibliography, such as coniferyl alcohol or ferulic acid, may be expected (Figure S2) [5]. Coniferyl alcohol is one of the main monolignols of angiosperm dicots [13], and is distributed throughout the milk thistle plant [8]. It is associated with the defense mechanisms of trees and is known to have inhibitory activity against the growth of fungi [14,15]. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) and its precursors, *p*-coumaric acid, and caffeic acid, are metabolites in the biosynthesis of lignins. These compounds are intermediates in the biosynthesis of some important natural products very often found in plants, such as *p*-coumaryl alcohol, curcumin, chlorogenic acid, diferulic acids, sinapic acid, synapyl alcohol, coniferyl alcohol, vanillin, etc. [16].

As regards the antifungal activity of above cited compounds, the literature indicates that silymarin is effective against *Candida* spp. (*C. albicans, C. krusei* and *C. glabrata*) [17,18], and that coniferyl derivatives are effective against *Cladosporium cucumerinum* and *C. albicans* [19]. Ferulic acid has been reported as an inhibitor of the fungal growth of, for instance, *Pythium* spp. [20], *Fusarium* spp. [21,22], and *Aspergillus* spp. [23,24]. Esters of ferulic acid were found to be more potent antimicrobial agents than amides and anilides, according to Khatkar, *et al.* [25], and their high antimicrobial activity was evidenced by the results of Mahiwal, *et al.* [26].

Concerning the control of Botryosphaeriaceae fungi –which are recognized as aggressive plant pathogens on different types of hosts, from agricultural crops to ornamental and forest hosts– [27], ferulic acid has been assayed against *Diplodia seriata, Neofusicoccum parvum, Eutypa lata, Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* [28-30], but the activity of *S. marianum* extracts or coniferyl alcohol has not been assayed to date, in spite of the importance of these phytopathogens in, for instance, Viticulture [31].

In this study, a phytochemical analysis of the extracts of *S. marianum* capitula during the flowering phenological stage (stage 67) is presented, with the aim of exploring the presence of high-added value components and the potential application the hydro-methanolic extracts as antifungal agents against three Botryosphaeriaceae that play a major role in grapevine trunk diseases (GTDs). Taking into consideration that the solubility of ferulic acid in water is very low Shakeel, *et al.* [32] and that coniferyl alcohol is insoluble in water, which can limit their bioavailability and applicability, conjugate complexes with stevioside will also be assayed.

2. Material and methods

2.1. *Plant Material, Reagents and Fungal Isolates*

The specimens of *S. marianum* under study were collected in the banks of Carrión river as it passes through the town of Palencia (Spain) during stage 67 (or 6N7) according to the extended BBCH scale [33], when the head disk was covered by open florets (i.e., during the flowering stage and before the development of fruit). The capitula of *S. marianum* were first shade-dried, then oven dried (at 105 °C to constant mass) and pulverized to fine powder in a mechanical grinder. Different specimens (n=25) were thoroughly mixed to obtain a composite sample.

Chitosan (CAS 9012-76-4; high MW: 310,000–375,000 Da) was supplied by Hangzhou Simit Chem. & Tech. Co. (Hangzhou, China). Neutrase^{TM} 0.8 L enzyme was supplied by Novozymes A/S (Bagsværd, Denmark). Stevioside (CAS 57817-89-7, 99%) was purchased from Wako

Chemicals GmbH (Neuss, Germany). Coniferyl alcohol (CAS 458-35-5, 98%), ferulic acid (CAS 537-98-4, European Pharmacopoeia Reference Standard), sodium alginate (CAS 9005-38-3), calcium carbonate (CAS 471-34-1, ≥99.0%), and methanol (CAS 67-56-1, UHPLC, suitable for mass spectrometry) were acquired from Sigma-Aldrich Química (Madrid, Spain). PDA (potato dextrose agar) was supplied by Becton Dickinson (Bergen County, NJ, USA).

The three fungal isolates under study (Table 1) were supplied as lyophilized vials (later reconstituted and refreshed as PDA subcultures) by the Agricultural Technological Institute of Castilla and Leon (ITACYL, Valladolid, Spain) [34].

Table 1. Fungal isolates used in the study.

Code	Isolate	Binomial nomenclature	Geographical origin	Host / date
ITACYL_F111	Y-091-03-01c	*Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L.Phillips	Spain (Navarra, nursery)	Grapevine (Verdejo) 2006
ITACYL_F118	Y-103-08-01	*Dothiorella viticola* A.J.L.Phillips & J.Luque	Spain (Extremadura)	Grapevine 2004
ITACYL_F098	Y-084-01-01a	*Diplodia seriata* De Not.	Spain (DO Toro)	Grapevine (Tempranillo) 2004

2.2. *Preparation of Plant Extracts*

Silybum marianum capitula samples were mixed (1:20, w/v) with a methanol/water solution (1:1 v/v) and heated in a water bath at 50 °C for 30 min, followed by sonication for 5 min in pulse mode with a 1 min stop for each 2.5 min, using a probe-type ultrasonicator model UIP1000hdT (Hielscher Ultrasonics, Teltow, Germany; 1000 W, 20 kHz). The solution was then centrifuged at 9000 rpm for 15 min and the supernatant was filtered through Whatman No. 1 paper. Aliquots were lyophilized for infrared spectroscopy analyses.

2.3. *Physicochemical Characterization of S. marianum Extracts*

The infrared vibrational spectra were registered using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 Fourier-transform infrared spectrometer, equipped with an in-built diamond attenuated total reflection (ATR) system. The spectra were collected with a 1 cm^{-1} spectral resolution over the 400–4000 cm^{-1} range, taking the interferograms that resulted from co-adding 64 scans. The spectra were then corrected using the advanced ATR correction algorithm [35] available in OMNIC™ software suite.

The hydroalcoholic plant extracts were studied by gas chromatography-mass spectrometry (GC-MS) at the Research Support Services (STI) at Universidad de Alicante (Alicante, Spain), using a gas chromatograph model 7890A coupled to a quadrupole mass spectrometer model 5975C (both from Agilent Technologies). The chromatographic conditions were: injection volume = 1 µL; injector temperature = 280 °C, in splitless mode; initial oven temperature = 60 °C, 2 min, followed by ramp of 10 °C/min up to a final temperature of 300 °C, 15 min. The chromatographic column used for the separation of the compounds was an Agilent Technologies HP-5MS UI of 30 m length, 0.250 mm diameter and 0.25 µm film. The mass spectrometer conditions were: temperature of the electron impact source of the mass spectrometer = 230 °C and of the quadrupole = 150 °C; ionization energy = 70 eV. NIST11 library and Adams [36] were used for compound identification.

2.4. *Preparation of Chitosan Oligomers and Bioactive Formulations*

Chitosan oligomers (COS) were prepared according to the procedure previously reported in [37].

The stevioside–*S. marianum*, stevioside-coniferyl alcohol and stevioside-ferulic acid conjugate complexes were obtained by mixing of the respective solutions in a 1:1 (v/v) ratio. The mixture was then sonicated for 15 minutes in five 3-minute periods (so that the temperature did not exceed 60 °C) using a probe-type ultrasonicator.

2.5. *Antifungal Activity Assessment*

2.5.1. *In Vitro Tests of Mycelial Growth Inhibition*

The antifungal activity of the different treatments was determined using the agar dilution method according to EUCAST standard antifungal susceptibility testing procedures [38], by incorporating aliquots of stock solutions onto the PDA medium to obtain concentrations in the 62.5–1500 $\mu g \cdot mL^{-1}$ range. Mycelial plugs ($\varnothing = 5$ mm), from the margin of 1-week-old PDA cultures of *D. seriata*, were transferred to plates incorporating the above-mentioned concentrations for each treatment (3 plates per treatment/concentration, with 2 replicates). Plates were incubated at 25 °C in the dark for a week. PDA medium without any amendment was used as the control. Mycelial growth inhibition was estimated according to the formula: $((d_c - d_t) / d_c) \times 100$, where d_c and d_t represent the average diameters of the fungal colony of the control and of the treated fungal colony, respectively. Effective concentrations (EC_{50} and EC_{90}) were estimated using PROBIT analysis in IBM SPSS Statistics v.25 (IBM; Armonk, NY, USA) software. The level of interaction, i.e. synergy factors, were determined according to Wadley's method [39].

2.5.2. *Greenhouse Bioassays on Grafted Plants*

Together with the experiments of mycelial growth inhibition *in vitro*, bioassays with stevioside–*S. marianum* conjugate complexes were performed in living plants in order to scale the protective capabilities of these compounds against two selected *Botryosphaeriaceae* species responsible for grapevine trunk diseases (GTDs) on young grapevine plants. *Neofusicoccum parvum*, *D. viticola* and *D. seriata* were then selected on the basis of their prevalence/frequency of isolation in Spain and adjacent areas [40-42], especially in young grapevine plants coming from nurseries [43]. Plant material consisted of 30 plants each of cultivars "Tempranillo" (CL. 32 clone) (2-years old) and "Garnacha" (VCR3 clone) (one year old) grafted on 775P and 110R rootstocks, respectively. Plants were grown on 3.5 L plastic pots containing a mixed substrate of moss peat and sterilized natural soil (75:25), incorporating slow release fertilizer when needed. Plants were maintained in the greenhouse with drip irrigation and anti-weed ground cover for six months (June-December 2020). One week after placing them in the greenhouse, grapevine plants were inoculated with the mentioned three *Botryosphaeriaceae* taxa together with the stevioside–*S. marianum* treatment. Five repetitions were arranged for each pathogen/control product and plant combination (cultivar/rootstock), together with 4 positive controls per pathogen and cultivar plus 3 negative controls (incorporating only the bioactive product) for each treatment (Table S1). Inoculations of both pathogens and the control product were carried out directly on the trunk of the living plants at two sites per plant stand (separated a minimum of 5 cm among them) below the grafting point and not reaching the root crown. For the pathogens, agar plugs coming from 5-days-old fresh PDA cultures of each species were used as fungal inoculum. In the mentioned two inoculation points of each grapevine plant, slits (made up with a scalpel) of approx. 15 mm in diameter and 5 mm deep were done. After this, 5 mm diameter agar plugs were inoculated and placed in such a way that the mycelium was in contact with vascular tissue in the stem. Calcium alginate beads served as dispersal matrix, including the different control products and conjugates assayed, and beads were placed at both sides of the agar plug. For this, beads were prepared as follows; the control product was added to a 3% sodium alginate solution in a 2:8 ratio (20 mL compound/80 mL sodium alginate). Then, this solution was dispensed drop by drop onto a 3% calcium carbonate solution to spherify (polymerize) in beads of 4-6 mm diameter containing the different control treatments. Finally, both agar plugs and beads were covered with cotton soaked in sterile bi-distilled water and sealed with Parafilm™ tape. During the assay period, application of copper to control powdery mildew outbreaks was performed in mid-July, together with a first sprouting (followed by periodic sprouting). Grapevine plants were visually examined weekly during the whole assay period for the presence foliar symptoms including both internerval and nerval necroses. After six months in the greenhouse, plants were removed and two sections of the inoculated stems between the grafting point and the root crown were prepared, sectioned longitudinally and the length of the vascular necroses (tracheomycosis) caused by the different pathogens evaluated. Thus, the length of the vascular necroses was measured longitudinally on upper and lower directions from the inoculation point for both halves of the longitudinal cut, and the average measures of these statistically analysed and compared depending on the type of

pathogen and product formulation employed. All the data were compared with controls. Finally, grapevine plants removed and measured at the end of the assay were also processed to re-isolate the different pathogenic taxa previously inoculated. Thus, in order to fulfil Koch's postulates, 0.5 cm long wood chips exhibiting vascular necroses (1–2 cm around the wounds) were washed, surface sterilized, placed in PDA plates amended with streptomycin sulphate (to prevent bacterial contamination) and incubated at 26 °C in the dark in a culture chamber for 2–3 days.

2.6. Statistical Analyses

The results of the *in vitro* inhibition of mycelial growth of the three phytopathogens as affected by the different concentrations of the treatments were statistically analyzed using one-way analysis of variance (ANOVA), followed by *post hoc* comparison of means through Tukey's test at $p < 0.05$ (provided that the homogeneity and homoscedasticity requirements were satisfied, according to the Shapiro–Wilk and Levene tests). In the case of the greenhouse assay results, since the normality and homoscedasticity requirements were not met, Kruskal-Wallis non-parametric test was used instead, with Conover-Iman test for *post hoc* multiple pairwise comparisons. R statistical software was used [44].

3. Results

3.1. *Vibrational Characterization*

The assignments of the major absorption IR bands in *S. marianum* extract spectrum (Figure S3) are presented in Table 2. The most prominent bands occurred at 3335, 1651-1602, 1457, 1313, 1242, and 1029 cm^{-1}. The band at 3335 cm^{-1} is attributed to phenolic (OH) vibrations; the multi-peak band at 1651 cm^{-1} to mixed (C=O) amide and (C=C) vibrations; the peak at 1515 cm^{-1} (typical of ferulic acid and vanillin) to >C=C< aromatic; the peak at 1457 cm^{-1} to symmetric aromatic ring stretching vibration (C=C ring); and the peaks at 1030 cm^{-1} and 779 cm^{-1} to C-O stretching and C=C, respectively (both vanillin-related peaks).

Table 2. FTIR spectra of vegetal components of *S. marianum*.

Silybum marianum	**Sylimarin**	**Ferulic acid**	**Assignments**
3335		3331	OH group in phenolic compounds
	3279		
3069			
2918	2932	2926	O-H stretching
1651			skeletal vibration due to aromatic C=C ring
1634		1649	stretch and C=O stretching
1602		1605	aromatic C=C stretching
1558			>C=C< aromatic
1515		1510	symmetric aromatic ring stretching vibration
1457	1458		(C=C ring)
1429	1434		olefinic C-H
1313		1329	C-H vibration of the methyl group
		1275	Carboxylic acid C=O stretching
1242	1257		
	1126		in plane =C-H bending / C=C stretching
1030	1076		C–O stretching / O-H out plane bending
	941		
779			C=C on the aromatic ring
	721	693	methylene rocking vibration

3.2. *Gas Chromatography–Mass Spectrometry (GC-MS)*

GC-MS analyses of the hydro-methanolic extract of *S. marianum* (Figure S4) allowed to identify 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (also named coniferyl alcohol or γ-hydroxyisoeugenol); its analogue *trans*-isoeugenol; 2-propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester (known as ferulic acid methyl ester); 2-methoxyphenol; and 4-hydroxy-3-methylacetophenone as the main components (Table 3, Figure S5).

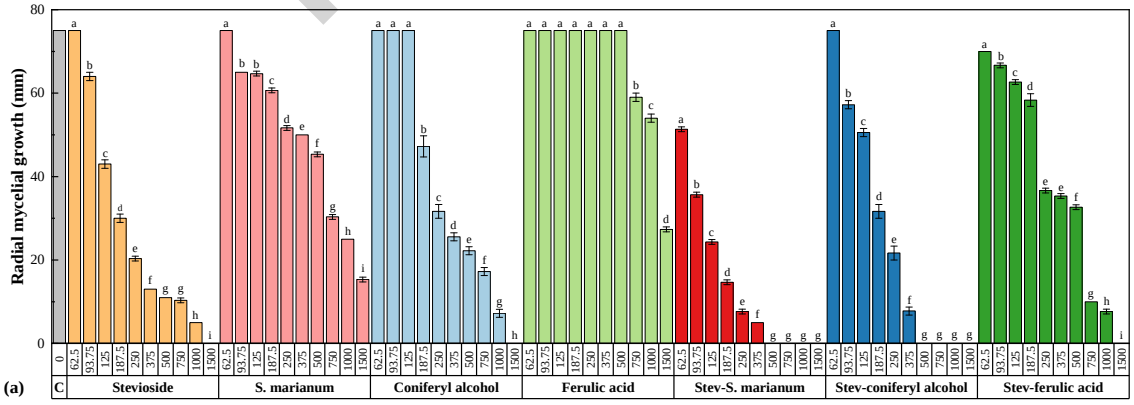
Table 3. Phytochemical compounds identified in the hydromethanolic extract of *Silybum marianum* by GC-MS.

Peak	Rt (min)	Area (%)	Tentative assignments
1	4.8755	2.67	methoxy-phenyl-oxime
2	6.0099	3.50	glycerin
3	7.3293	2.62	hexamethyl-cyclotrisiloxane; tris(tert-butyldimethylsilyloxy)arsane
4	7.6360	7.31	2-methoxy-phenol
5	9.4764	2.48	2,3-dihydro-benzofurane
6	9.6516	1.95	methenamine
7	10.8737	3.92	4-hydroxy-3-methylacetophenone
8	12.0275	1.64	vanillin
9	12.6653	1.51	*trans*-isoeugenol
10	13.0548	1.36	6-methoxy-3-methylbenzofuran
11	15.3139	1.69	4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (also named coniferol or γ-hydroxyisoeugenol)
12	15.5865	0.82	2-hydroxy-4-isopropyl-7-methoxytropone
13	15.9370	1.68	4-hydroxy-3-methoxybenzeneacetic acid, -, methyl ester
14	16.0636	45.64	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol
15	17.1153	14.99	2-propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester (also named ferulic acid methyl ester)
16	17.9234	0.49	ethyl (2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate
17	19.5447	0.67	9,15-octadecadienoic acid, methyl ester, (Z,Z)-
18	21.1027	2.93	2-(1,4,4-trimethyl-cyclohex-2-enyl)-ethanol
19	24.4377	2.13	9,12-octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester

3.3. *Antifungal Activity*

3.3.1. *In vitro Growth Inhibition Tests*

The results of the mycelial growth inhibition tests are summarized in Figure 1. When tested alone, a higher efficacy of coniferyl alcohol as compared to ferulic acid could be observed: full inhibition was only reached for the former in the case of *N. parvum* and *D. viticola*. In the case of *D. seriata*, for which both treatments resulted in full inhibition, it was attained at a lower dose for coniferyl alcohol (1000 *vs.* 1500 μg·mL^{-1}). Hence, the antifungal efficacy found for the extracts should be mostly ascribed to its main constituent. On the other hand, upon conjugation with stevioside, a clear enhancement in terms of efficacy was found in all cases, which was particularly evident in the case of the extracts, for which even higher inhibition than that of the coniferyl alcohol conjugates was attained at almost all concentrations against the three pathogens.



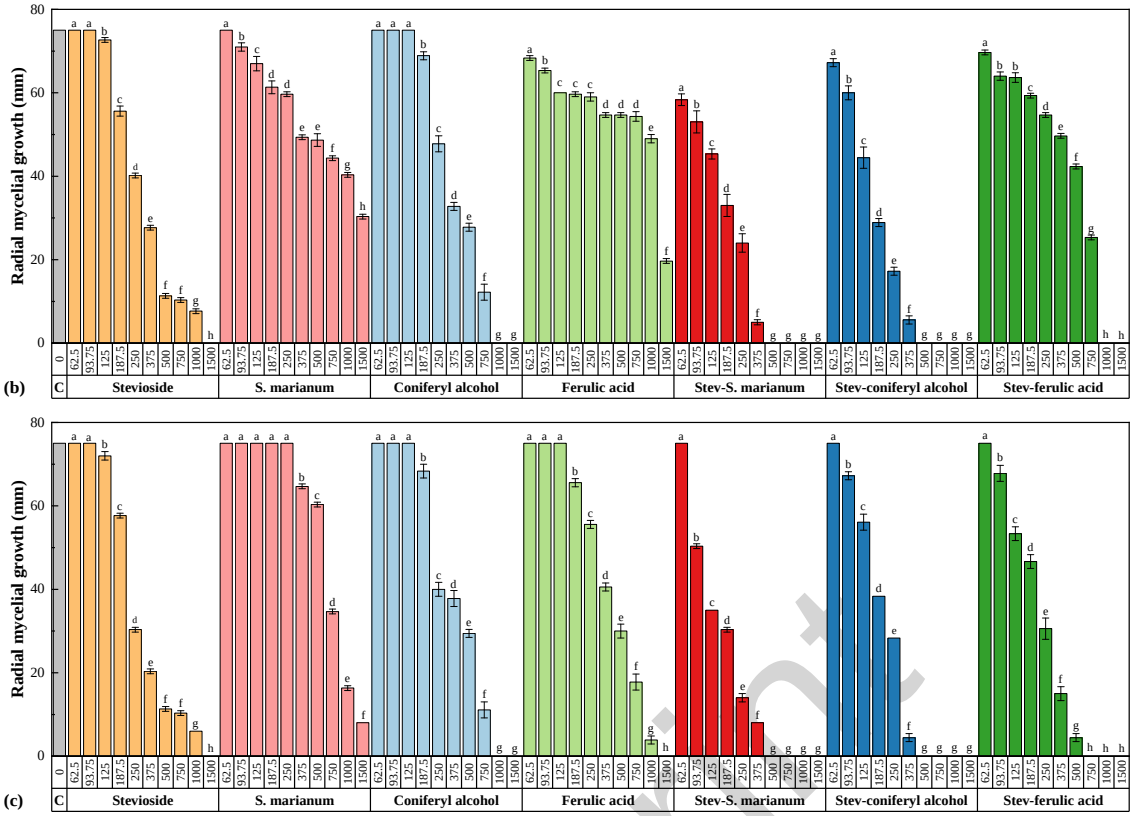


Figure 1. Colony growth values of (**a**) *N. parvum*, (**b**) *D. viticola* and (**c**) *D. seriata* strains when cultured in PDA plates containing the various control products (viz. stevioside, *S. marianum* hydromethanolic extract, coniferyl alcohol, ferulic acid, stevioside–*S. marianum*, stevioside-coniferyl alcohol and stevioside–ferulic acid conjugate complexes) at concentrations in the 62.5–1500 μg·mL^{-1} range. The same letters above concentrations indicate that they are not significantly different at $p < 0.05$. Error bars represent standard deviations.

If effective concentrations are compared (Table 4), differences in the efficacy of the treatments as a function of the pathogen could be observed for some of the treatments in a more clear manner: for instance, a slightly higher efficacy of stevioside and stevioside-*S. marianum* conjugate complex was found against *N. parvum*, and *D. seriata* seemed to be particularly sensitive to ferulic acid and its conjugate. On the other hand, the response of the three fungi to the coniferyl alcohol-based treatments was very similar.

Table 4. EC_{50} and EC_{90} effective concentrations, expressed in μg·mL^{-1}.

		Stevioside	*S. marianum*	Stevioside–*S. marianum*	Coniferyl alcohol	Stevioside–coniferyl alcohol	Ferulic acid	Stevioside–ferulic acid
N. parvum	EC_{50}	195	557	87	325	169	1339	335
	EC_{90}	724	2938	303	1005	384	3230	1132
D. viticola	EC_{50}	307	1088	148	351	154	1387	385
	EC_{90}	917	9942	596	1000	366	3921	1422
D. seriata	EC_{50}	288	703	147	341	186	414	226
	EC_{90}	840	1461	355	1010	401	1041	685

In line with the comments made above, the calculation of synergy factors (Table 5) indicated a strong synergistic behavior for the stevioside-*S. marianum* conjugate, with SF values in the 2.78-3.83 range, higher than those attained for coniferyl alcohol (in the 1.45-2.62 range), and much higher than those found for ferulic acid (with SF values of up to 1.50 in the case of *D. seriata*, but with values close to 1 in the case of *N. parvum*, pointing to a mere additive behavior).

Table 5. Synergy factors for each of the conjugate complexes against the three Botryosphaeriaceae taxa.

Pathogen	EC	Synergy factor		
		Stevioside–*S. marianum*	Stevioside–coniferyl alcohol	Stevioside–ferulic acid
N. parvum	EC_{50}	3.34	1.45	1.01
	EC_{90}	3.83	2.20	1.04
D. viticola	EC_{50}	3.23	2.12	1.32
	EC_{90}	2.82	2.62	1.24
D. seriata	EC_{50}	2.78	1.68	1.50
	EC_{90}	3.01	2.29	1.36

3.3.2. *Greenhouse Bioassays*

The tests conducted in grafted plants with the stevioside-milk thistle treatment confirmed its efficacy in more realistic conditions (i.e., closer to field ones): the application of the conjugate complex resulted in statistically significant differences as compared to the positive controls in all cases (Table 6). It is worth noting that the median lengths of the vascular necroses were higher in the case of *N. parvum* than for the other two fungi, which may be regarded as an unexpected result, provided that the associated EC_{90} value was the lowest in the *in vitro* tests.

Table 6. Kruskal-Wallis test and multiple pairwise comparisons using the Conover-Iman procedure for the lengths of the vascular necroses for the three phytopathogen in greenhouse *in vivo* assays.

Pathogen	Sample	Frequency	Sum of ranks	Mean of ranks	Groups
N. parvum	Stevioside-*S. marianum* negative control	48	1275.500	26.573	A
	Stevioside-*S. marianum*	64	5911.000	92.359	B
	Positive control	64	8389.500	131.086	C
D. viticola	Stevioside-*S. marianum* negative control	48	2174.000	45.292	A
	Stevioside-*S. marianum*	64	4272.000	66.750	B
	Positive control	64	9130.000	142.656	C
D. seriata	Stevioside-*S. marianum* negative control	48	2062.500	42.969	A
	Stevioside-*S. marianum*	72	5641.500	78.354	B
	Positive control	56	7872.000	140.571	C

4. Discussion

4.1. *Valorization of Coniferyl Alcohol and Ferulic Acid*

As expected from the phenological stage in which the plants were collected (flowering, before fruit ripening) and taking into consideration that the entire capitula were used for the extraction (not only the fruits), the panel of extracted components was different from those present in the commercially available milk thistle seed extract: instead of silybin (A and B) and isosilybin (A and B), coniferyl alcohol and other eugenol analogues were identified; and instead of vanillin, the quantitative presence of its precursor (ferulic acid methyl ester) was evidenced.

Coniferyl alcohol is a valuable chemical, which reaches 350 $USD \cdot g^{-1}$ when bought from commercial suppliers such as Sigma-Aldrich. Current approaches to obtain coniferyl alcohol are either inefficient, harmful (*Penicillium simplicissimum* vanillyl alcohol oxidase (PsVAO) can be used to produce it, but it intrinsically produces harmful byproduct H_2O_2), or expensive (its synthesis involves expensive substrates and catalyst and harsh reaction conditions) [45,46]. Other is the case for the results obtained by the ultrasonic-assisted hydro-methanolic extraction of the capitula, reported in this paper, which may allow to obtain the phenylpropanoid coniferyl alcohol with a yield of 50-80%. Alternative extractive approaches, such as the use of ionic liquid analogs (deep eutectic solvents) as extractive solvents [47], microwave-assisted extraction, dynamic maceration process [48], negative pressure cavitation-assisted extraction with macroporous resin enrichment [49], etc. should nonetheless be explored in order to optimize the yield.

In case that the production of silymarin-based drugs is desired, the biotransformation of eugenol and coniferyl alcohol to silybin and isosilybin can be efficiently attained by the oxidation of the precursors by milk thistle ascorbate peroxidase (APX1), as shown in Figure 2*a*.

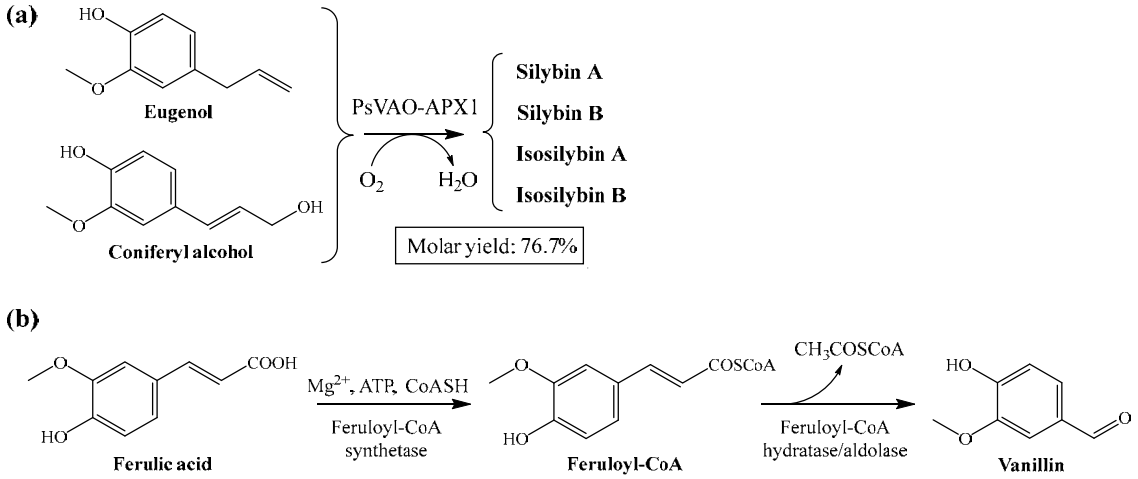


Figure 2. (a) Highly efficient enzymatic cascade engineered for biotransformation of eugenol to silybin and isosilybin. Adapted from [46]. **(b)** Schematic representation of the non-β-oxidative pathway for conversion of ferulic acid into vanillin, according to [50].

In the same way, the finding of a 10:1 ratio for the ferulic acid-vanillin pair confirms that, for *S. marianum* capitula during the flowering phenological stage in a hydro-methanolic medium, the presence of the ferulic acid precursor is enhanced. Should vanillin be the desired chemical to obtain, the quantitative conversion of ferulic acid into vanillic acid could be feasible in presence of *Pseudomonas* spp. [51] (Figure 2*b*). *Pycnoporus cinnabarinus* basidiomycete has also been proposed for the production of vanillin from ferulic acid [52], although the vanillin produced is either rapidly converted to other products or utilized by the microorganism as a source of carbon and energy. Genetic engineering has been applied to produce vanillin from ferulic acid using metabolically engineered *Escherichia coli* [50,53]. Another alternative would be the use of packed bed-stirred fermenters using *Bacillus subtilis* [54].

It should be noted that the extraction of coniferyl alcohol and ferulic acid would not preclude the valorization of the rest of the biomass as a feedstock for bioenergy production [55-57].

4.2. *Efficacy of the Treatments*

Stevioside, a terpene glycoside obtained from *Stevia rebaudiana* (Bertoni) Bertoni showed a high inhibitory activity, comparable to that of coniferyl alcohol. Since −to the best of the authors' knowledge− this is the first time that this compound is assayed against Botryosphaeriaceae fungi, no comparisons with similar taxa in terms of MIC values are available. However, the detected antifungal activity would be in good agreement with the results presented by other authors, who reported an inhibitory effect against other fungi (*Alternaria solani, Helminthosporium solani, Aspergillus* spp., *Fusarium* spp., *Penicillium chrysogenum,* or *Botrytis cinerea,* among others) [58-62], with MIC values varying over a wide range (from 250 to 3000 $\mu g \cdot mL^{-1}$).

As regards the antifungal action of coniferyl alcohol, no data against GTD-related fungi is available in the literature, but −according to Kuc [63]− it has strong antifungal properties. For instance, coniferyl alcohol and its derivatives have been shown to be effective against *Colletotrichum lagenarium, Cladosporium cucumerinum, Melampsora lini* and *C. albicans* [19,64,65].

In relation to ferulic acid, it has been assayed against GTDs, and, according to Lambert, *et al.* [66], it is the phenolic acid with the strongest activity against *D. seriata, N. parvum, E. lata* and *P. chlamydospora*. The same group, in a different study, found inhibition percentages of 23% and 35% for ferulic acid at a concentration of 500 μM (97 $\mu g \cdot mL^{-1}$) against *N. parvum* PER20 and *N. parvum* Bp0014, respectively [67]. EC_{50} values of 3530 and 4740 $\mu g \cdot mL^{-1}$ were reported against *Botryosphaeriaceae* sp. and *Phaeoacremonium minimum* by Gómez, *et al.* [29]. An EC_{50} value of 15 mM (2913 $\mu g \cdot mL^{-1}$) was reported against *Botryosphaeria* sp. by Dekker, *et al.* [30].

Srivastava, *et al.* [68] found that ferulic acid at 25 mM (4855 $\mu g \cdot mL^{-1}$) resulted in ca. 80% and ca. 70% mycelium growth inhibition of *B. rhodina* and *B. ribis*, respectively; and 100% inhibition was attained at 20 mM (3885 $\mu g \cdot mL^{-1}$) in the case of *B. obtusa*. These concentrations are in the same order of magnitude as the EC_{90} values obtained against *N. parvum* and *D. viticola* (3230 and 3921 $\mu g \cdot mL^{-1}$, respectively).

Concerning the conjugate complexes with stevioside, no data is available against GTDs. The most similar assayed product would be the stevioside:silymarin conjugate complexes (in a 1:1 molar ratio) tested against *Fusarium culmorum*, for which an EC_{90} value of 160 $\mu g \cdot mL^{-1}$ and a synergy factor of 1.43 were reported [69]. No antifungal efficacy data is available for stevioside-coniferyl alcohol conjugate complexes, but stevioside-ferulic acid inclusion compounds (with different molar ratios to the one assayed herein, and involving a more complex preparation procedure) have been tested against *F. culmorum* and *Phytophthora cinnamomic*. In the former case, composites based on stevioside:ferulic acid inclusion compounds (in a 5:1 molar ratio), combined with chitosan oligomers in hydroalcoholic solution or in choline chloride:urea deep eutectic solvent media, led to EC_{90} values in the 377-713 $\mu g \cdot mL^{-1}$ range against *F. culmorum* [70], depending on the dispersion medium. In the case of *Phytophthora cinnamomic*, inclusion compounds from stevioside and ferulic acid in 6:1 ratio, dispersed in a hydroalcoholic solution of chitosan oligomers, resulted in EC_{90} values of 446-450 $\mu g \cdot mL^{-1}$ (depending on the presence/absence of silver nanoparticles) [71,72].

5. Conclusion

In the hydromethanolic extract of *Silybum marianum* capitula, during the flowering stage, high contents of coniferyl alcohol derivatives and ferulic acid esters were found, instead of other species such as the sylimarin complex or vanillin. Given the high price of coniferyl alcohol, this may pose an alternative valorization strategy for this weed, compatible with a subsequent valorization for bioenergy purposes. As regards the antifungal activity of the hydroalcoholic extract, the EC_{50} and EC_{90} values obtained against the three studied Botryospheriaceous phytopathogens (*N. parvum, D. viticola* and *D. seriata*) were in the 557–1088 and 1461–9942 $\mu g \cdot mL^{-1}$ range, respectively. However, a significant efficacy enhancement (with EC_{50} and EC_{90} values in the 87–148 and 303–596 $\mu g \cdot mL^{-1}$, respectively) was obtained by formation of conjugate complexes of the hydrometanolic extract of *S. marianum* with stevioside, evidencing a clear synergistic behavior (with synergy factor values of up to 2.62) as a result of from the solubility and bioavailabity improvement. The efficacy of the stevioside–milk thistle conjugate complexes was further assessed in artificially inoculated grafted plants, obtaining significant differences in the vascular necroses lengths *vs*. the positive controls in all cases. The presented results support the possibility of extending the applications of milk thistle to Agriculture as an antifungal agent, in particular for the protection of grapevines against fungal trunk diseases.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1. Repetitions for each of the plant/treatment/pathogen combinations in the greenhouse bioassay; Figure S1. Biosynthesis of silybins from taxifolin and coniferyl alcohol; Figure S2. Formation of coniferyl alcohol; Figure S3. Infrared spectrum of S. marianum extract (after lyophilization); Figure S4. GC–MS spectrum of *S. marianum* hydromethanolic extract; Figure S5. Chemical structures of some of the phytochemicals identified by GC-MS in the hydro-methanolic extract of *S. marianum.*

Author Contributions: Conceptualization, J.M.-G., P.M.-R. and V.G.-G.; methodology, J.M.-G., J.C.-G. and V.G.-G.; validation, J.C.-G., V.G.-G. and P.M.-R.; formal analysis, J.C.-G., V.G.-G. and P.M.-R.; investigation, L.B.-D., N.L.-L., V.G.-G., J.C.-G., J.M.-G., E.S.-H. and P.M.-R.; resources, J.M.-G. and P.M.-R.; data curation, J.C.-G.; writing—original draft preparation, L.B.-D., N.L.-L., V.G.-G., J.C.-G., J.M.-G., E.S.-H. and P.M.-R.; writing—review and editing, V.G.-G. and P.M.-R.; visualization, L.B.-D. and N.L-L.; supervision, V.G.-G. and P.M.-R.; project administration, V.G.-G., J.M.-G. and P.M.-R.; funding acquisition, J.M.-G. and P.M.-R. All authors have read and agreed to the published version of the manuscript.

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Coniferyl alcohol and ferulic acid esters in the hydro-methanolic extract of *Silybum marianum* capitula: activity against Botryosphaeriaceae fungi

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SUPPORTING INFORMATION

Table S1. Repetitions for each of the plant/treatment/pathogen combinations in the greenhouse bioassay. Each grafted plant was inoculated at two sites below grafting point.

Plant	Treatment	Pathogen	Number of replicates
'Tempranillo' (CL. 32 clone) on 775P rootstock	Stevioside–*S. marianum*	*N. parvum*	5
		D. viticola	5
		D. seriata	5
		None (negative control)	3
	None (positive control)	*N. parvum*	4
		D. viticola	4
		D. seriata	4
'Garnacha' (VCR3 clone) on 110R rootstock	Stevioside–*S. marianum*	*N. parvum*	5
		D. viticola	5
		D. seriata	5
		None (negative control)	3
	None (positive control)	*N. parvum*	4
		D. viticola	4
		D. seriata	4

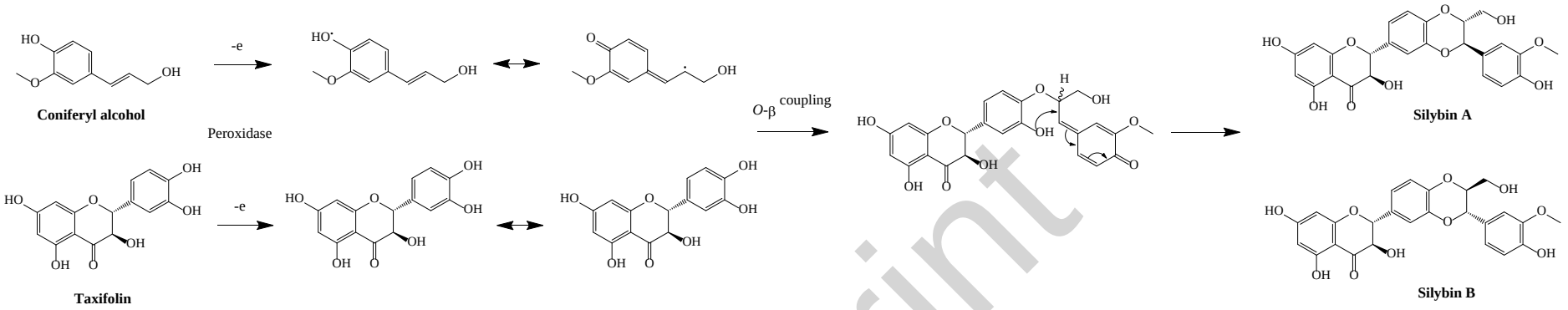

Figure S1. Biosynthesis of silybins from taxifolin and coniferyl alcohol.

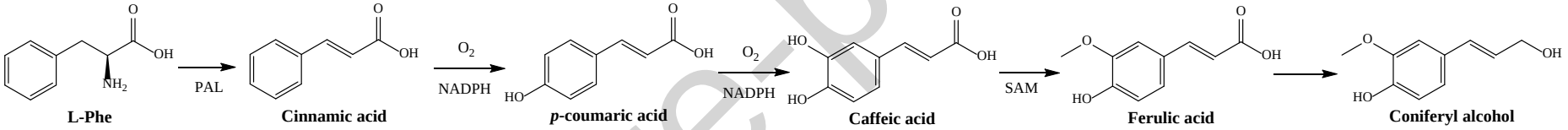

Figure S2. Formation of coniferyl alcohol.

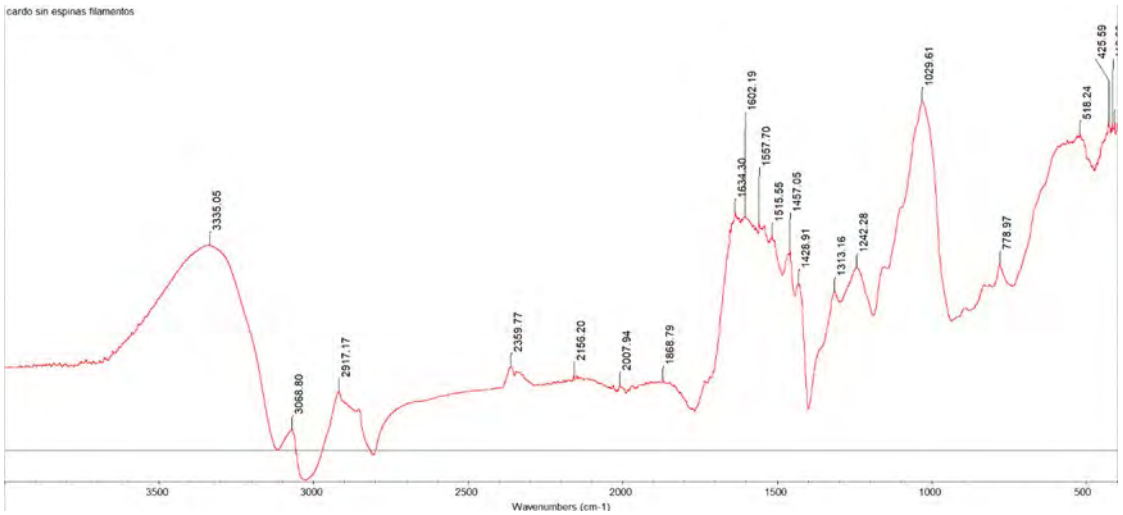

Figure S3. Infrared spectrum of *S. marianum* extract (after lyophilization).

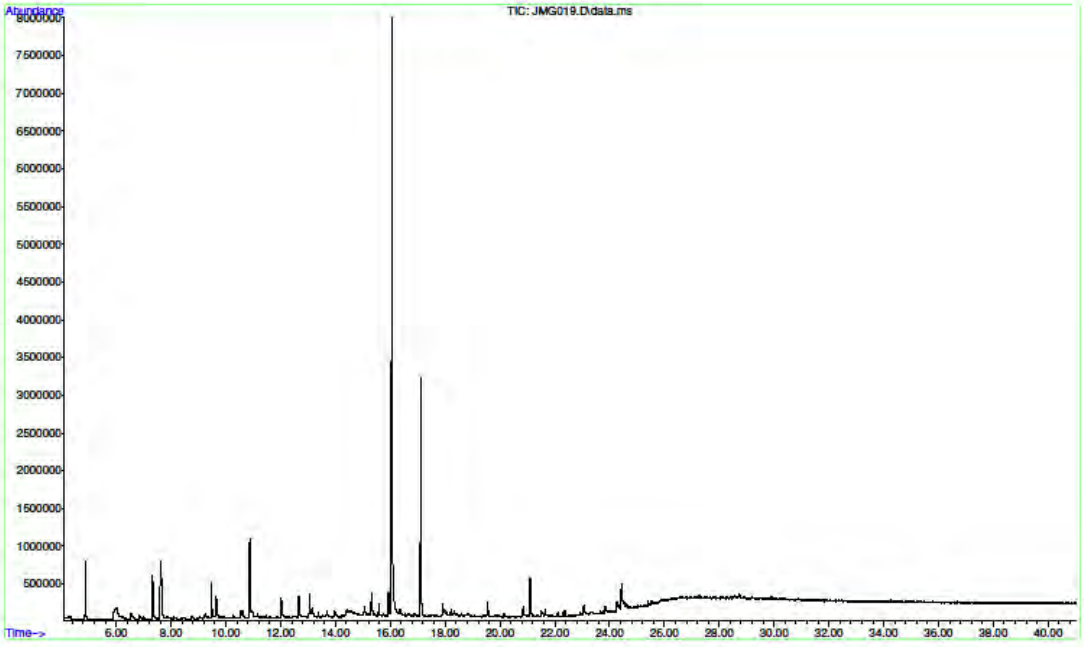

Figure S4. GC–MS spectrum of *S. marianum* hydromethanolic extract.

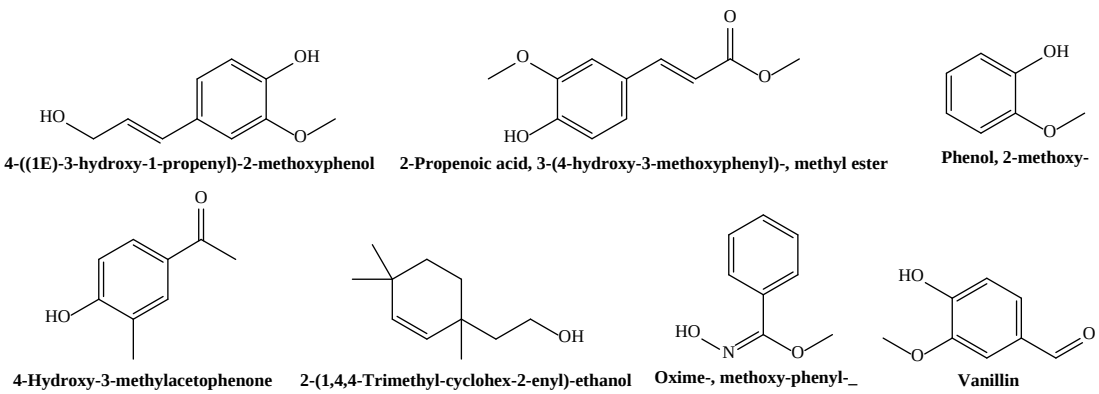

Figure S5. Chemical structures of some of the phytochemicals identified by GC-MS in the hydromethanolic extract of *S. marianum*.

PATENTES

Patente #1

Complejo de inclusión que comprende metabolitos antimicrobianos de *Streptomyces* y amino-oligosacáridos de quitosano, y biofertilizante que comprende este complejo y bacterias *Streptomyces*

Jesús Martín Gil; Celia Andrés Juan; Laura Buzón Durán; Eduardo Pérez Lebeña; Pablo Martín Ramos. P201930554. España. 18/06/2019. Universidad de Valladolid.

MINISTERIO
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Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	P201930554	
Fecha de recepción:	18 junio 2019, 12:17 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	2019-0004	
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Número de solicitantes:	1	
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Título:	COMPLEJO DE INCLUSION QUE COMPRENDE METABOLITOS ANTIMICROBIANOS DE STREPTOMYCES Y AMINO-OLIGOSACARIDOS DE QUITOSANO, Y BIOFERTILIZANTE QUE COMPRENDE ESTE COMPLEJO Y BACTERIAS STREPTOMYCES	
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La anotación en este tipo de autodenominados "registros" no despliega ningún tipo de eficacia jurídica ni tiene carácter oficial.

En estos casos le aconsejamos que se ponga en contacto con la Oficina Española de Patentes y Marcas en el correo electrónico informacion@oepm.es.

ADVERTENCIA: POR DISPOSICIÓN LEGAL LOS DATOS CONTENIDOS EN ESTA SOLICITUD PODRÁN SER PUBLICADOS EN EL BOLETÍN OFICIAL DE LA PROPIEDAD INDUSTRIAL E INSCRITOS EN EL REGISTRO DE PATENTES DE LA OEPM, SIENDO AMBAS BASES DE DATOS DE CARÁCTER PÚBLICO Y ACCESIBLES VÍA REDES MUNDIALES DE INFORMÁTICA.

Para cualquier aclaración puede contactar con la O.E.P.M.

/Madrid, Oficina Receptora/

MINISTERIO DE ENERGÍA, TURISMO Y AGENDA DIGITAL

Oficina Española de Patentes y Marcas

(1) MODALIDAD: **PATENTE DE INVENCIÓN** **MODELO DE UTILIDAD**	 [✓] [ ]
(2) FORMULARIO 5101. TIPO DE SOLICITUD: PRIMERA PRESENTACIÓN SOLICITUD DIVISIONAL CAMBIO DE MODALIDAD TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA PCT: ENTRADA FASE NACIONAL	 [✓] [ ] [ ] [ ] [ ]
(3) EXP. PRINCIPAL O DE ORIGEN: MODALIDAD: N.º SOLICITUD: FECHA SOLICITUD:	
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5-1) SOLICITANTE 1: DENOMINACIÓN SOCIAL: UNIVERSIDAD PÚBLICA NACIONALIDAD: CÓDIGO PAÍS: NIF/NIE/PASAPORTE: CNAE: PYME: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EMPRENDEDOR: PERSONA DE CONTACTO: MODO DE OBTENCIÓN DEL DERECHO: INVENCIÓN LABORAL: CONTRATO: SUCESIÓN: PORCENTAJE DE TITULARIDAD:	 Universidad de Valladolid [✓] España ES Q4718001C Ctt-Otri-Casa del Estudiante C/Real de Burgos, s/nº VALLADOLID 47 Valladolid 47001 España ES [ ] [✓] [ ] [ ] 100,00 %
(6-1) INVENTOR 1: APELLIDOS: NOMBRE: NACIONALIDAD: CÓDIGO PAÍS: NIF/NIE/PASAPORTE: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS:	 MARTIN GIL (20%) (DNI 12212221A) JESUS

TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-2) INVENTOR 2:	
APELLIDOS:	ANDRES JUAN (20%) (DNI 12709612L)
NOMBRE:	CELIA
NACIONALIDAD:	
CÓDIGO PAÍS:	
NIF/NIE/PASAPORTE:	
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-3) INVENTOR 3:	
APELLIDOS:	BUZON DURAN (20%) (DNI 71939510H)
NOMBRE:	LAURA
NACIONALIDAD:	
CÓDIGO PAÍS:	
NIF/NIE/PASAPORTE:	
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-4) INVENTOR 4:	
APELLIDOS:	PEREZ LEBEÑA (20%) (DNI 12369172W)
NOMBRE:	EDUARDO
NACIONALIDAD:	
CÓDIGO PAÍS:	
NIF/NIE/PASAPORTE:	
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-5) INVENTOR 5:	
APELLIDOS:	MARTIN RAMOS (20%) (DNI 71128357Y)
NOMBRE:	PABLO
NACIONALIDAD:	
CÓDIGO PAÍS:	
NIF/NIE/PASAPORTE:	
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	

PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EL INVENTOR RENUNCIA A SER MENCIONADO:	 [ ]
(7) TÍTULO DE LA INVENCIÓN:	COMPLEJO DE INCLUSION QUE COMPRENDE METABOLITOS ANTIMICROBIANOS DE STREPTOMYCES Y AMINO-OLIGOSACARIDOS DE QUITOSANO, Y BIOFERTILIZANTE QUE COMPRENDE ESTE COMPLEJO Y BACTERIAS STREPTOMYCES
(8) NÚMERO DE INFORME TECNOLÓGICO DE PATENTES (ITP):	
(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN SI NO	 [ ] [✓]
(10) EFECTUADO DEPÓSITO DE MATERÍA BIOLÓGICA: SI NO	 [ ] [✓]
(11) DEPÓSITO: REFERENCIA DE IDENTIFICACIÓN: INSTITUCIÓN DE DEPÓSITO: NÚMERO DE DEPÓSITO: ORÍGEN BIOLÓGICO:	
(12) RECURSO GENÉTICO: NÚMERO DE REGISTRO: NÚMERO DE CERTIFICADO DE ACCESO AL RECURSO: UTILIZACIÓN DEL RECURSO GENÉTICO: CONOCIMIENTO TRADICIONAL ASOCIADO A UN RECURSO GENÉTICO:	
(13) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS: LA LISTA DE SECUENCIAS NO VA MÁS ALLÁ DEL CONTENIDO DE LA SOLICITUD LA LISTA DE SECUENCIAS EN FORMATO PDF Y ASCII SON IDENTICOS	 [ ] [ ]
(14) EXPOSICIONES OFICIALES: NOMBRE: LUGAR: FECHA:	
(15) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(16) REMISIÓN A UNA SOLICITUD ANTERIOR: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(17) AGENTE DE PROPIEDAD INDUSTRIAL: APELLIDOS: NOMBRE: CÓDIGO DE AGENTE: NÚMERO DE PODER:	 UNGRIA LOPEZ JAVIER 0392/1 20084238
(18) DIRECCIÓN A EFECTOS DE COMUNICACIONES: DIRECCIÓN ASOCIADA AL PRIMER SOLICITANTE DOMICILIO:	

LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: MEDIO PREFERENTE DE COMUNICACIÓN	
(19) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN: DESCRIPCIÓN: REIVINDICACIONES: DIBUJOS: RESUMEN: FIGURA(S) A PUBLICAR CON EL RESUMEN: ARCHIVO DE PRECONVERSION: DOCUMENTO DE REPRESENTACIÓN: LISTA DE SECUENCIAS PDF: ARCHIVO PARA LA BUSQUEDA DE LS: OTROS (Aparecerán detallados):	 [✓] N.º de páginas: 20 [✓] N.º reivindicaciones: 15 [✓] N.º de dibujos: 2 [✓] N.º de páginas: 1 [✓] N.º de figura(s): [✓] [ ] N.º de páginas: [ ] N.º de páginas: [ ]
(20) EL SOLICITANTE SE ACOGE A LA REDUCCIÓN DE TASAS PARA EMPRENDEDORES PREVISTA EN EL ART. 186 DE LA LEY 24/2015 DE PATENTES Y, A TAL EFECTO, APORTA LA SIGUIENTE DOCUMENTACIÓN ADJUNTA:	[ ]
(21) NOTAS: 1) :	 LOS INVENTORES 1, 2 y 5 DEBEN FIGURAR COMO INVENCION LABORAL Y LOS INVENTORES 3 y 4 POR CONTRATO
(22) FIRMA: FIRMA DEL SOLICITANTE O REPRESENTANTE: LUGAR DE FIRMA: FECHA DE FIRMA:	 UNGRIA LOPEZ JAVIER - 05211582N MADRID 18 Junio 2019

RESUMEN

COMPLEJO DE INCLUSIÓN QUE COMPRENDE METABOLITOS ANTIMICROBIANOS DE *STREPTOMYCES* Y AMINO-OLIGOSACÁRIDOS DE QUITOSANO, Y BIOFERTILIZANTE QUE COMPRENDE ESTE COMPLEJO Y BACTERIAS *STREPTOMYCES*

Un complejo de inclusión formado por compuestos antimicrobianos y promotores del crecimiento de las plantas que pueden obtenerse por fermentación de cepas de bacterias del género *Streptomyces* y oligo-sacáridos hidrolizados de quitosano. Así como un biofertilizante que comprende este complejo de inclusión y bacterias del género *Streptomyc*es. Procedimientos de obtención de ambos productos y su utilización para el tratamiento de patógenos que afectan a la agricultura y, en general, a la agricultura y, simultáneamente estimular el crecimiento de las plantas.

Patente #2

Solución acuosa que comprende un complejo de inclusión, método de obtención y su uso para la aplicación en cultivos y la mejora de su rendimiento

Jesús Martín Gil; Celia Andrés Juan; Laura Buzón Durán; Eduardo Pérez Lebeña; Pablo Martín Ramos. P201931118. Solicitud Internacional PCT/ES2020/070797. 17/12/2019. Universidad de Valladolid

MINISTERIO
DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española
de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	P201931118	
Fecha de recepción:	17 diciembre 2019, 14:38 (CET)	
Oficina receptora:	OEPM Madrid	
Su referencia:	2019-0277	
Solicitante:	Universidad de Valladolid	
Número de solicitantes:	1	
País:	ES	
Título:	SOLUCIÓN ACUOSA QUE COMPRENDE UN COMPLEJO DE INCLUSIÓN, MÉTODO DE OBTENCIÓN Y SU USO PARA APLICACIÓN EN CULTIVOS Y LA MEJORA DE SU RENDIMIENTO	
Documentos enviados:	Descripcion.pdf (24 p.) Reivindicaciones.pdf (3 p.) Resumen.pdf (1 p.) Dibujos.pdf (3 p.) OLF-ARCHIVE.zip	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	CN=UNGRIA LOPEZ JAVIER - 05211582N,SN=UNGRIA LOPEZ,givenName=JAVIER,serialNumber=IDCES-05211582N,C=ES	
Fecha y hora de recepción:	17 diciembre 2019, 14:38 (CET)	
Codificación del envío:	32:15:68:D2:5B:50:CB:D2:D8:53:A2:2F:C7:39:4A:06:9B:AD:C7:7B	

AVISO IMPORTANTE

Las tasas pagaderas al solicitar y durante la tramitación de una patente o un modelo de utilidad son las que se recogen en el Apartado "Tasas y precios públicos" de la página web de la OEPM (http://www.oepm.es/es/propiedad_industrial/tasas/). Consecuentemente, si recibe una comunicación informándole de la necesidad de hacer un pago por la inscripción de su patente o su modelo de utilidad en un "registro central" o en un "registro de internet" posiblemente se trate de un fraude.

La anotación en este tipo de autodenominados "registros" no despliega ningún tipo de eficacia jurídica ni tiene carácter oficial.

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Para cualquier aclaración puede contactar con la O.E.P.M.

/Madrid, Oficina Receptora/

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DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española
de Patentes y Marcas

(1) MODALIDAD: **PATENTE DE INVENCIÓN** **MODELO DE UTILIDAD**	 [✓] [ ]
(2) FORMULARIO 5101. TIPO DE SOLICITUD: PRIMERA PRESENTACIÓN SOLICITUD DIVISIONAL CAMBIO DE MODALIDAD TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA PCT: ENTRADA FASE NACIONAL	 [✓] [ ] [ ] [ ] [ ]
(3) EXP. PRINCIPAL O DE ORIGEN: MODALIDAD: N.ºSOLICITUD: FECHA SOLICITUD:	
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5-1) SOLICITANTE 1: DENOMINACIÓN SOCIAL: UNIVERSIDAD PÚBLICA NACIONALIDAD: CÓDIGO PAÍS: NIF/NIE/PASAPORTE: CNAE: PYME: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EMPRENDEDOR: PERSONA DE CONTACTO: MODO DE OBTENCIÓN DEL DERECHO: INVENCIÓN LABORAL: CONTRATO: SUCESIÓN: OTROS: PORCENTAJE DE TITULARIDAD:	 Universidad de Valladolid [✓] España ES Q4718001C CTT-OTRI- CASA DEL ESTUDIANTE C/ REAL DE BURGOS, S/Nº VALLADOLID 47_Valladolid 47001 España ES [ ] [✓] [ ] [ ] [ ] 100,00 %
(6-1) INVENTOR 1: APELLIDOS: NOMBRE: NACIONALIDAD: CÓDIGO PAÍS: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS:	 MARTÍN GIL (20%) - DNI 12212221A JESÚS España ES AVDA. PALENCIA, 14 - 3ºC VALLADOLID 47_Valladolid 47011 España ES

TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-2) INVENTOR 2:	
APELLIDOS:	ANDRÉS JUAN (20%) - DNI 12709612L
NOMBRE:	CELIA
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	CERVANTES, 7-2ºB
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47005
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-3) INVENTOR 3:	
APELLIDOS:	BUZÓN DURÁN (20%) - DNI 71939510H
NOMBRE:	LAURA
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	AVDA. SIMÓN NIETO, 3-4º IZDA
LOCALIDAD:	PALENCIA
PROVINCIA:	34_Palencia
CÓDIGO POSTAL:	34005
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-4) INVENTOR 4:	
APELLIDOS:	PÈREZ LEBEÑA (20%) - DNI 12369172W
NOMBRE:	EDUARDO
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	ANTONIO ROYO VILLANOVA, 5-1ºC
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47014
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-5) INVENTOR 5:	
APELLIDOS:	MARTÍN RAMOS (20%) - DNI 71128357Y
NOMBRE:	PABLO
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	AVDA. VALLE ESGUEVA, 29 BJ
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47011
PAÍS RESIDENCIA:	España

CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EL INVENTOR RENUNCIA A SER MENCIONADO:	ES [ ]
(7) TÍTULO DE LA INVENCIÓN:	SOLUCIÓN ACUOSA QUE COMPRENDE UN COMPLEJO DE INCLUSIÓN, MÉTODO DE OBTENCIÓN Y SU USO PARA APLICACIÓN EN CULTIVOS Y LA MEJORA DE SU RENDIMIENTO
(8) NÚMERO DE INFORME TECNOLÓGICO DE PATENTES (ITP):	
(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN SI NO	 [ ] [✓]
(10) EFECTUADO DEPÓSITO DE MATERÍA BIOLÓGICA: SI NO	 [ ] [✓]
(11) DEPÓSITO: REFERENCIA DE IDENTIFICACIÓN: INSTITUCIÓN DE DEPÓSITO: NÚMERO DE DEPÓSITO: ORÍGEN BIOLÓGICO:	
(12) RECURSO GENÉTICO: NÚMERO DE REGISTRO: NÚMERO DE CERTIFICADO DE ACCESO AL RECURSO: UTILIZACIÓN DEL RECURSO GENÉTICO: CONOCIMIENTO TRADICIONAL ASOCIADO A UN RECURSO GENÉTICO:	
(13) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS: LA LISTA DE SECUENCIAS NO VA MÁS ALLÁ DEL CONTENIDO DE LA SOLICITUD LA LISTA DE SECUENCIAS EN FORMATO PDF Y ASCII SON IDENTICOS	 [ ] [ ]
(14) EXPOSICIONES OFICIALES: NOMBRE: LUGAR: FECHA:	
(15) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(16) REMISIÓN A UNA SOLICITUD ANTERIOR: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(17) AGENTE DE PROPIEDAD INDUSTRIAL: APELLIDOS: NOMBRE: CÓDIGO DE AGENTE: NÚMERO DE PODER:	 UNGRIA LÓPEZ JAVIER 0392/1 20084238
(18) DIRECCIÓN A EFECTOS DE COMUNICACIONES: DIRECCIÓN ASOCIADA AL PRIMER SOLICITANTE DOMICILIO: LOCALIDAD: CÓDIGO POSTAL:	

PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: MEDIO PREFERENTE DE COMUNICACIÓN	
(19) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN: DESCRIPCIÓN: REIVINDICACIONES: DIBUJOS: RESUMEN: FIGURA(S) A PUBLICAR CON EL RESUMEN: ARCHIVO DE PRECONVERSION: DOCUMENTO DE REPRESENTACIÓN: LISTA DE SECUENCIAS PDF: ARCHIVO PARA LA BUSQUEDA DE LS: OTROS (Aparecerán detallados):	 [✓] N.º de páginas: 24 [✓] N.º reivindicaciones: 22 [✓] N.º de dibujos: 5 [✓] N.º de páginas: 1 [✓] N.º de figura(s): [✓] [ ] N.º de páginas: [ ] N.º de páginas: [ ]
(20) EL SOLICITANTE SE ACOGE A LA REDUCCIÓN DE TASAS PARA EMPRENDEDORES PREVISTA EN EL ART. 186 DE LA LEY 24/2015 DE PATENTES Y, A TAL EFECTO, APORTA LA SIGUIENTE DOCUMENTACIÓN ADJUNTA:	[ ]
(21) NOTAS: 1) INVENTORES :	 LOS 1º, 2º Y 3º INVENTORES HAN CEDIDO SUS DERECHOS MEDIANTE INVENCIÓN LABORAL, LOS 4º Y 5º MEDIANTE CONTRATO A LA ENTIDAD SOLICITANTE
(22) FIRMA: FIRMA DEL SOLICITANTE O REPRESENTANTE: LUGAR DE FIRMA: FECHA DE FIRMA:	 UNGRIA LOPEZ JAVIER - 05211582N MADRID 17 Diciembre 2019

RESUMEN

SOLUCIÓN ACUOSA QUE COMPRENDE UN COMPLEJO DE INCLUSIÓN, MÉTODO DE OBTENCIÓN Y SU USO PARA APLICACIÓN EN CULTIVOS Y LA MEJORA DE SU RENDIMIENTO

La invención se refiere a una solución acuosa de un complejo de inclusión que comprende: un quitosano con peso molecular comprendido entre 100.000 a 300.000 g/mol como agente de vehiculización y al menos un compuesto biológicamente activo de aplicación en agricultura seleccionado dentro del grupo formado por: extracto de *Equisetum arvense* (Cola de caballo), extracto de *Urtica spp* (Ortiga), un aminoácido y cualquier combinación de los mismos. Asimismo, se refiere al método de obtención de la solución acuosa del complejo mediante sonicación de un gel previamente obtenido (también por sonicación) de un gel de quitosano con un ácido carboxílico con el CBA. Asimismo, se recoge el uso de la solución acuosa para potenciar el crecimiento de las plantas, así como para prevenir enfermedades patógenas en plantas, usos significativamente mejorados con respecto a los compuestos conocidos gracias al efecto sinérgico encontrado entre sus componentes.

MINISTERIO DE INDUSTRIA, ENERGÍA Y TURISMO

Oficina Española de Patentes y Marcas

TRATADO DE COOPERACIÓN EN MATERIA DE PATENTES
NOTIFICACIÓN DE LA RECEPCIÓN DE LOS DOCUMENTOS QUE CONSTITUYEN SUPUESTAMENTE UNA SOLICITUD INTERNACIONAL PRESENTADA DE FORMA ELECTRÓNICA.
(Instrucciones Administrativas del PCT, Parte Séptima)

1.-Se notifica al solicitante que la Oficina Receptora ha recibido en la fecha de recepción indicada más abajo, los documentos que supuestamente constituyen una solicitud internacional.
2.-Se llama la atención del solicitante sobre el hecho de que la Oficina Receptora no ha comprobado aún si estos documentos satisfacen las condiciones del art. 11.1, es decir, si cumple los requisitos para que le sea atribuida una fecha de presentación internacional. En cuanto la Oficina Receptora haya comprobado los documentos, avisará al solicitante.
3.-El número de la supuesta solicitud internacional indicado más abajo ha sido otorgado automáticamente a estos documentos. Se invita al solicitante a mencionar este número en toda la correspondencia con la Oficina Receptora.

Número de presentación	300391868	
Solicitud Número PCT	PCT/ES2020/070797	
Fecha de recepción	16 diciembre 2020	
Oficina Receptora	Oficina Española de Patentes y Marcas, Madrid	
Referencia del expediente del solicitante o mandatario	120200324	
Solicitante	UNIVERSIDAD DE VALLADOLID	
Número de solicitantes	1	
País	ES	
Título de la invención	SOLUCIÓN ACUOSA QUE COMPRENDE UN COMPLEJO DE INCLUSIÓN, MÉTODO DE OBTENCIÓN Y SU USO PARA APLICACIÓN EN CULTIVOS Y LA MEJORA DE SU RENDIMIENTO	
Documentos presentados	eolf-pkda.xml	eolf-requ.xml
	eolf-appb.xml	eolf-fees.xml
	eolf-vlog.xml	eolf-appb-P000001.pdf (24 p.)
	eolf-appb-P000002.pdf (3 p.)	eolf-appb-P000003.pdf (1 p.)

	eolf-appb-P000004.pdf (3 p.)	
Presentado por	CN=UNGRIA LOPEZ JAVIER - 05211582N,SN=UNGRIA LOPEZ,givenName=JAVIER,serialNumber=IDCES-05211582N,C=ES	
Método de Transmisión	Online	
Fecha y hora de expedición del recibo	16 diciembre 2020, 12:57 (CET)	
Información oficial condensada de la presentación	CF:A9:6E:07:66:52:05:41:FD:3A:D3:F6:4D:DE:5A:64:E5:B4:BC:24	

/Madrid, Oficina Receptora/

Patente #3

Composición nutracéutica que consiste en un polifenol en solución glicólica, método de obtención y su uso terapéutico

Jesús Martín Gil; Celia Andrés Juan; Laura Buzón Durán; Eduardo Pérez Lebeña; Pablo Martín Ramos. P202030007. España. 08/01/2020. Universidad de Valladolid

MINISTERIO
DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española
de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	P202030007	
Fecha de recepción:	08 enero 2020, 12:27 (CET)	
Oficina receptora:	OEPM Madrid	
Su referencia:	2019-0269	
Solicitante:	Universidad de Valladolid	
Número de solicitantes:	1	
País:	ES	
Título:	COMPOSICION NUTRACEUTICA QUE CONSISTE EN UN POLIFENOL EN SOLUCION GLICOLICA, METODO DE OBTENCION Y SU USO TERAPEUTICO	
Documentos enviados:	Descripcion.pdf (16 p.) Reivindicaciones.pdf (2 p.) Resumen.pdf (1 p.) OLF-ARCHIVE.zip	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	CN=UNGRIA LOPEZ JAVIER - 05211582N,SN=UNGRIA LOPEZ,givenName=JAVIER,serialNumber=IDCES-05211582N,C=ES	
Fecha y hora de recepción:	08 enero 2020, 12:27 (CET)	
Codificación del envío:	D8:7D:06:7D:B6:84:E6:5E:D8:16:30:70:BA:BD:5A:64:5D:B9:32:1A	

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La anotación en este tipo de autodenominados "registros" no despliega ningún tipo de eficacia jurídica ni tiene carácter oficial.

En estos casos le aconsejamos que se ponga en contacto con la Oficina Española de Patentes y Marcas en el correo electrónico informacion@oepm.es.

ADVERTENCIA: POR DISPOSICIÓN LEGAL LOS DATOS CONTENIDOS EN ESTA SOLICITUD PODRÁN SER PUBLICADOS EN EL BOLETÍN OFICIAL DE LA PROPIEDAD INDUSTRIAL E INSCRITOS EN EL REGISTRO DE PATENTES DE LA OEPM, SIENDO AMBAS BASES DE DATOS DE CARÁCTER PÚBLICO Y ACCESIBLES VÍA REDES MUNDIALES DE INFORMÁTICA.

Para cualquier aclaración puede contactar con la O.E.P.M.

/Madrid, Oficina Receptora/

MINISTERIO
DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española
de Patentes y Marcas

(1) MODALIDAD: **PATENTE DE INVENCIÓN** **MODELO DE UTILIDAD**	 [✓] [ ]
(2) FORMULARIO 5101. TIPO DE SOLICITUD: PRIMERA PRESENTACIÓN SOLICITUD DIVISIONAL CAMBIO DE MODALIDAD TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA PCT: ENTRADA FASE NACIONAL	 [✓] [ ] [ ] [ ] [ ]
(3) EXP. PRINCIPAL O DE ORIGEN: MODALIDAD: N.ºSOLICITUD: FECHA SOLICITUD:	
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5-1) SOLICITANTE 1: DENOMINACIÓN SOCIAL: UNIVERSIDAD PÚBLICA NACIONALIDAD: CÓDIGO PAÍS: NIF/NIE/PASAPORTE: CNAE: PYME: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EMPRENDEDOR: PERSONA DE CONTACTO: MODO DE OBTENCIÓN DEL DERECHO: INVENCIÓN LABORAL: CONTRATO: SUCESIÓN: OTROS: (INVENTORES 1, 2 Y 5 COMO INVENCION LABORAL E INVENTORES 3 Y 4 POR CONTRATO) PORCENTAJE DE TITULARIDAD:	 Universidad de Valladolid [✓] España ES Q4718001C Ctt-Otri-Casa del Estudiante C/Real de Burgos, s/nº VALLADOLID 47_Valladolid 47001 España ES [ ] [ ] [ ] [ ] [✓] 100,00 %
(6-1) INVENTOR 1: APELLIDOS: NOMBRE: NACIONALIDAD: CÓDIGO PAÍS: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA:	 MARTIN GIL (20%) DNI 12212221A JESUS España ES Ctt-Otri-Casa del Estudiante C/Real de Burgos s/nº VALLADOLID 47_Valladolid 47001 España

CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-2) INVENTOR 2:

APELLIDOS:	ANDRES JUAN (20%) DNI 12709612L
NOMBRE:	CELIA
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	Ctt-Otri-Casa del Estudiante C/Real de Burgos s/nº
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47001
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-3) INVENTOR 3:

APELLIDOS:	BUZON DURAN (20%) DNI 71939510H
NOMBRE:	LAURA
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	Avda. Simón Nieto 3-4º izda
LOCALIDAD:	PALENCIA
PROVINCIA:	34_Palencia
CÓDIGO POSTAL:	34005
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-4) INVENTOR 4:

APELLIDOS:	PEREZ LEBEÑA (20%) DNI 12369172W
NOMBRE:	EDUARDO
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	Antonio Royo Villanova 5-1ºC
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47014
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-5) INVENTOR 5:

APELLIDOS:	MARTIN RAMOS (20%) DNI 71128357Y
NOMBRE:	PABLO
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	Ctt-Otri-Casa del Estudiante C/Real de Burgos s/nº
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47001
PAÍS RESIDENCIA:	España

CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EL INVENTOR RENUNCIA A SER MENCIONADO:	ES [ ]
(7) TÍTULO DE LA INVENCIÓN:	COMPOSICION NUTRACEUTICA QUE CONSISTE EN UN POLIFENOL EN SOLUCION GLICOLICA, METODO DE OBTENCION Y SU USO TERAPEUTICO
(8) NÚMERO DE INFORME TECNOLÓGICO DE PATENTES (ITP):	
(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN SI NO	 [ ] [✓]
(10) EFECTUADO DEPÓSITO DE MATERÍA BIOLÓGICA: SI NO	 [ ] [✓]
(11) DEPÓSITO: REFERENCIA DE IDENTIFICACIÓN: INSTITUCIÓN DE DEPÓSITO: NÚMERO DE DEPÓSITO: ORÍGEN BIOLÓGICO:	
(12) RECURSO GENÉTICO: NÚMERO DE REGISTRO: NÚMERO DE CERTIFICADO DE ACCESO AL RECURSO: UTILIZACIÓN DEL RECURSO GENÉTICO: CONOCIMIENTO TRADICIONAL ASOCIADO A UN RECURSO GENÉTICO:	
(13) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS: LA LISTA DE SECUENCIAS NO VA MÁS ALLÁ DEL CONTENIDO DE LA SOLICITUD LA LISTA DE SECUENCIAS EN FORMATO PDF Y ASCII SON IDENTICOS	 [ ] [ ]
(14) EXPOSICIONES OFICIALES: NOMBRE: LUGAR: FECHA:	
(15) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(16) REMISIÓN A UNA SOLICITUD ANTERIOR: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(17) AGENTE DE PROPIEDAD INDUSTRIAL: APELLIDOS: NOMBRE: CÓDIGO DE AGENTE: NÚMERO DE PODER:	 UNGRIA LOPEZ JAVIER 0392/1 20084238
(18) DIRECCIÓN A EFECTOS DE COMUNICACIONES: DIRECCIÓN ASOCIADA AL PRIMER SOLICITANTE DOMICILIO: LOCALIDAD: CÓDIGO POSTAL: PAÍS RESIDENCIA:	

CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: MEDIO PREFERENTE DE COMUNICACIÓN	
(19) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN: DESCRIPCIÓN: REIVINDICACIONES: RESUMEN: FIGURA(S) A PUBLICAR CON EL RESUMEN: ARCHIVO DE PRECONVERSION: DOCUMENTO DE REPRESENTACIÓN: LISTA DE SECUENCIAS PDF: ARCHIVO PARA LA BUSQUEDA DE LS: OTROS (Aparecerán detallados):	 [✓] N.º de páginas: 16 [✓] N.º reivindicaciones: 12 [✓] N.º de páginas: 1 [✓] N.º de figura(s): [✓] [ ] N.º de páginas: [ ] N.º de páginas: [ ]
(20) EL SOLICITANTE SE ACOGE A LA REDUCCIÓN DE TASAS PARA EMPRENDEDORES PREVISTA EN EL ART. 186 DE LA LEY 24/2015 DE PATENTES Y, A TAL EFECTO, APORTA LA SIGUIENTE DOCUMENTACIÓN ADJUNTA:	[ ]
(21) NOTAS:	
(22) FIRMA: FIRMA DEL SOLICITANTE O REPRESENTANTE: LUGAR DE FIRMA: FECHA DE FIRMA:	 UNGRIA LOPEZ JAVIER - 05211582N MADRID 08 Enero 2020

RESUMEN

COMPOSICIÓN NUTRACÉUTICA QUE CONSISTE EN UN POLIFENOL EN SOLUCIÓN GLICÓLICA, MÉTODO DE OBTENCIÓN Y SU USO TERAPÉUTICO

La invención se refiere a un método de obtención de una composición nutracéutica que consiste en glicerina y un polifenol o un derivado del mismo, que comprende: a) calentar la glicerina a una temperatura comprendida entre 30ºC-35ºC; b) mezclar la glicerina calentada y el polifenol en una proporción de polifenol y glicerina comprendida entre 1:15 a 1:30 en peso; y c) dispersar la mezcla mediante cavitación inducida por ultrasonidos de 20 kHz, durante al menos 10 minutos, para formar interacciones intermoleculares débiles por enlaces de puente de hidrógeno entre los grupos hidroxilo de los componentes de la mezcla. Asimismo, es otro objeto de la invención la composición nutracéutica obtenible a partir del método descrito, así como su uso para el tratamiento de enfermedades como la diabetes, la colesterolemia y las enfermedades neurodegenerativas, siendo una de ellas el Alzheimer.

Patente #4

Composición farmacéutica que comprende una disolución de silimarina en un solvente eutéctico profundo de origen natural, métodos de obtención y uso en medicina

Jesús Martín Gil; Celia Andrés Juan; Laura Buzón Durán; Eduardo Pérez Lebeña; Pablo Martín Ramos P202030273. España. 03/04/2020. Universidad de Valladolid.

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DE INDUSTRIA, ENERGÍA
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Oficina Española
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Justificante de presentación electrónica de solicitud de patente

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Número de solicitud:	P202030273	
Fecha de recepción:	03 abril 2020, 13:19 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	2020-0067	
Solicitante:	Universidad de Valladolid	
Número de solicitantes:	1	
País:	ES	
Título:	COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE UNA DISOLUCIÓN DE SILIMARINA EN UN SOLVENTE EUTÉCTICO PROFUNDO DE ORIGEN NATURAL, MÉTODOS DE OBTENCIÓN Y USO EN MEDICINA	
Documentos enviados:	Descripcion.pdf (32 p.) Reivindicaciones.pdf (4 p.) Resumen.pdf (1 p.) OLF-ARCHIVE.zip	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	CN=UNGRIA LOPEZ JAVIER - 05211582N,SN=UNGRIA LOPEZ,givenName=JAVIER,serialNumber=IDCES-05211582N,C=ES	
Fecha y hora de recepción:	03 abril 2020, 13:19 (CEST)	
Codificación del envío:	1D:6A:0C:A2:01:C9:46:5D:E8:9F:A9:AF:AE:08:43:C1:67:F8:FA:5D	

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La anotación en este tipo de autodenominados "registros" no despliega ningún tipo de eficacia jurídica ni tiene carácter oficial.

En estos casos le aconsejamos que se ponga en contacto con la Oficina Española de Patentes y Marcas en el correo electrónico informacion@oepm.es.

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Y TURISMO

Oficina Española
de Patentes y Marcas

(1) MODALIDAD: **PATENTE DE INVENCIÓN** **MODELO DE UTILIDAD**	 [✓] [ ]
(2) FORMULARIO 5101. TIPO DE SOLICITUD: PRIMERA PRESENTACIÓN SOLICITUD DIVISIONAL CAMBIO DE MODALIDAD TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA PCT: ENTRADA FASE NACIONAL	 [✓] [ ] [ ] [ ] [ ]
(3) EXP. PRINCIPAL O DE ORIGEN: MODALIDAD: N.ºSOLICITUD: FECHA SOLICITUD:	
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5-1) SOLICITANTE 1: DENOMINACIÓN SOCIAL: UNIVERSIDAD PÚBLICA NACIONALIDAD: CÓDIGO PAÍS: NIF/NIE/PASAPORTE: CNAE: PYME: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EMPRENDEDOR: PERSONA DE CONTACTO: MODO DE OBTENCIÓN DEL DERECHO: INVENCIÓN LABORAL: CONTRATO: SUCESIÓN: OTROS: (LOS 1º, 2º Y 3º INVENTORES CEDEN SUS DERECHOS COMO INVENCIÓN LABORAL, LOS 4º Y 5º MEDIANTE CONTRATO) PORCENTAJE DE TITULARIDAD:	 Universidad de Valladolid [✓] España ES Q4718001C CTT-OTRI-CASA DEL ESTUDIANTE C/ REAL DE BURGOS, S/Nº VALLADOLID 47_Valladolid 47001 España ES [ ] [ ] [ ] [ ] [✓] 100,00 %
(6-1) INVENTOR 1: APELLIDOS: NOMBRE: NACIONALIDAD: CÓDIGO PAÍS: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL:	 MARTÍN GIL 20% - DNI 12212221A JESÚS España ES CTT-OTRI-CASA DEL ESTUDIANTE C/ REAL DE BURGOS, S/Nº VALLADOLID 47_Valladolid 47001

PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-2) INVENTOR 2:

APELLIDOS:	ANDRÉS JUAN 20% - DNI 12709612L
NOMBRE:	CELIA
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	CTT-OTRI-CASA DEL ESTUDIANTE C/ REAL DE BURGOS, S/Nº
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47001
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-3) INVENTOR 3:

APELLIDOS:	BUZÓN DURÁN 20% - DNI 71939510H
NOMBRE:	LAURA
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	CTT-OTRI-CASA DEL ESTUDIANTE C/ REAL DE BURGOS, S/Nº
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47001
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-4) INVENTOR 4:

APELLIDOS:	PÉREZ LEBEÑA 20% - DNI 12369172W
NOMBRE:	EDUARDO
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	CTT-OTRI-CASA DEL ESTUDIANTE C/ REAL DE BURGOS, S/Nº
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47001
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-5) INVENTOR 5:

APELLIDOS:	MARTÍN RAMOS 20% - DNI 71128357Y
NOMBRE:	PABLO
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
DOMICILIO:	CTT-OTRI-CASA DEL

	ESTUDIANTE C/ REAL DE BURGOS, S/Nº
LOCALIDAD:	VALLADOLID
PROVINCIA:	47_Valladolid
CÓDIGO POSTAL:	47001
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(7) TÍTULO DE LA INVENCIÓN:	COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE UNA DISOLUCIÓN DE SILIMARINA EN UN SOLVENTE EUTÉCTICO PROFUNDO DE ORIGEN NATURAL, MÉTODOS DE OBTENCIÓN Y USO EN MEDICINA
(8) NÚMERO DE INFORME TECNOLÓGICO DE PATENTES (ITP):	
(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN	
SI	[ ]
NO	[✓]
(10) EFECTUADO DEPÓSITO DE MATERÍA BIOLÓGICA:	
SI	[ ]
NO	[✓]
(11) DEPÓSITO:	
REFERENCIA DE IDENTIFICACIÓN:	
INSTITUCIÓN DE DEPÓSITO:	
NÚMERO DE DEPÓSITO:	
ORÍGEN BIOLÓGICO:	
(12) RECURSO GENÉTICO:	
NÚMERO DE REGISTRO:	
NÚMERO DE CERTIFICADO DE ACCESO AL RECURSO:	
UTILIZACIÓN DEL RECURSO GENÉTICO:	
CONOCIMIENTO TRADICIONAL ASOCIADO A UN RECURSO GENÉTICO:	
(13) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS:	
LA LISTA DE SECUENCIAS NO VA MÁS ALLÁ DEL CONTENIDO DE LA SOLICITUD	[ ]
LA LISTA DE SECUENCIAS EN FORMATO PDF Y ASCII SON IDENTICOS	[ ]
(14) EXPOSICIONES OFICIALES:	
NOMBRE:	
LUGAR:	
FECHA:	
(15) DECLARACIONES DE PRIORIDAD:	
PAÍS DE ORIGEN:	
CÓDIGO PAÍS:	
NÚMERO:	
FECHA:	
(16) REMISIÓN A UNA SOLICITUD ANTERIOR:	
PAÍS DE ORIGEN:	
CÓDIGO PAÍS:	
NÚMERO:	
FECHA:	
(17) AGENTE DE PROPIEDAD INDUSTRIAL:	
APELLIDOS:	UNGRIA LÓPEZ
NOMBRE:	JAVIER
CÓDIGO DE AGENTE:	0392/1
NÚMERO DE PODER:	20084238

(18) DIRECCIÓN A EFECTOS DE COMUNICACIONES: DIRECCIÓN ASOCIADA AL PRIMER SOLICITANTE DOMICILIO: LOCALIDAD: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: MEDIO PREFERENTE DE COMUNICACIÓN	
(19) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN: DESCRIPCIÓN: REIVINDICACIONES: RESUMEN: FIGURA(S) A PUBLICAR CON EL RESUMEN: ARCHIVO DE PRECONVERSION: DOCUMENTO DE REPRESENTACIÓN: LISTA DE SECUENCIAS PDF: ARCHIVO PARA LA BUSQUEDA DE LS: OTROS (Aparecerán detallados):	 [✓] N.º de páginas: 32 [✓] N.º reivindicaciones: 17 [✓] N.º de páginas: 1 [✓] N.º de figura(s): [✓] [ ] N.º de páginas: [ ] N.º de páginas: [ ]
(20) EL SOLICITANTE SE ACOGE A LA REDUCCIÓN DE TASAS PARA EMPRENDEDORES PREVISTA EN EL ART. 186 DE LA LEY 24/2015 DE PATENTES Y, A TAL EFECTO, APORTA LA SIGUIENTE DOCUMENTACIÓN ADJUNTA:	[ ]
(21) NOTAS:	
(22) FIRMA: FIRMA DEL SOLICITANTE O REPRESENTANTE: LUGAR DE FIRMA: FECHA DE FIRMA:	

RESUMEN

COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE UNA DISOLUCIÓN DE SILIMARINA EN UN SOLVENTE EUTÉCTICO PROFUNDO DE ORIGEN NATURAL, MÉTODOS DE OBTENCIÓN Y USO EN MEDICINA

La presente invención se refiere a una composición farmacéutica que consiste en una disolución de extracto de cardo mariano (silimarina) en un solvente eutéctico profundo de origen natural (*NADES*), que también es objeto de protección, y un método de obtención que comprende: preparar el NADES mezclando glicerol, previamente calentado a una temperatura inferior a 65ºC, con una determinada cantidad de agua y arginina, en agitación; dispersar la silimarina en el NADES en una proporción de 1:10-1:25 en peso; y agitar dicha mezcla en continuo, hasta conseguir la formación de complejos macromoleculares mediante interacciones intermoleculares débiles por enlaces de puente de hidrógeno entre el NADES (formados a su vez entre enlaces de los grupos hidroxilo de la glicerina, el agua y los grupos carboxilo y guanidino de la arginina) con los grupos hidroxilo fenólicos de la silimarina. Se contempla también el uso de la composición en medicina, concretamente su uso para el tratamiento de diabetes.

Universidad de Valladolid

D. Oscar Martínez Sacristán, Vicerrector de Investigación, Innovación y Transferencia de la Universidad de Valladolid,

DECLARA:

Que Doña Laura Buzón Durán con DNI 71939510H figura como inventora de las siguientes patentes, de las cuales se ha firmado respectivamente el oportuno acuerdo de licencia y explotación con la empresa Sistemas de Biotecnología y Recursos Naturales S.L., (SBioRN):

P201930554. Complejo de inclusión que comprende metabolitos antimicrobianos de Streptomyces y aminooligosacáridos de quitosano, y biofertilizante que comprende este complejo y bacterias Streptomyces, solicitada el 18 junio 2019.

P201930919. Método de despolimerización de biomasa lignocelulósica, solicitada el 17 octubre 2019.

P201931118. Solución acuosa que comprende un complejo de inclusión, método de obtención y su uso para aplicación en cultivos y la mejora de su rendimiento, solicitada el 17 diciembre 2019.

P202030007. Composición nutracéutica que consiste en un polifenol en solución glicólica, método de obtención y su uso terapéutico, solicitada el 8 enero 2020.

P202030273. Composición farmacéutica que comprende una disolución de silimarina en un solvente eutéctico profundo de origen natural, métodos de obtención y uso en medicina, solicitada el 3 abril 2020.

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Fdo.: Oscar Martínez Sacristán

CONCLUSIONES

Del trabajo experimental realizado en la presente Tesis Doctoral ("Nuevas formulaciones antifúngicas de origen natural con aplicación en Agricultura") se han obtenidos las siguientes conclusiones:

Primera: Entre la biomasa microbiana que coloniza los compost, el género *Streptomyces* resulta especialmente prometedor en tanto en cuanto produce varios antibióticos, polímeros orgánicos y nutrientes que pueden ser aprovechados como biofertilizantes o como bioproductos para proteger a la planta de los ataques de hongos y bacterias peligrosos[1].

Segunda: Los metabolitos secundarios de *Streptomyces rochei* y *S. lavendofoliae* no son capaces de inhibir el crecimiento de hongos relacionados con las EMV, probablemente por razones de hidrofobicidad. No obstante, la formación de complejos polielectrolíticos con oligómeros de quitosano y/o ε-polilisina permite alcanzar inhibiciones superiores al 80%.[2]

Tercera: El uso de oligómeros de quitosano también da lugar a un efecto sinérgico al formarse complejos conjugados con aminoácidos. Los ensayos de inhibición del crecimiento micelial contra patógenos responsables de las EMV y de la fusariosis han mostrado la mayor eficacia para el complejo conjugado COS-tirosina. Su viabilidad como tratamiento ha sido confirmada en ensayos de campo en ambos casos.[3]

Cuarta: Los extractos hidrometanólicos de las partes aéreas de *C. maritimum* y *D. carota*, ricos en apiol y acetato de geranilo, respectivamente, pueden ser también utilizados contra fitopatógenos de la vid (*X. ampelinus* y *D. seriata*) y de otros cultivos leñosos (*E. amylovora*). En los ensayos *in vitro*, se corroborado que la conjugación de los constituyentes bioactivos de los extractos vegetales con oligosacáridos de quitosano conduce a factores de sinergia en el rango de 3,9-5,1. El amplio espectro de acción de ambos extractos facilita que puedan ser propuestos como sustitutos de los tratamientos basados en fungicidas de síntesis química.[4]

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[1] Patente #1: "Complejo de inclusión que comprende metabolitos antimicrobianos de *Streptomyces* y aminooligosacáridos de quitosano, y biofertilizante que comprende este complejo y bacterias *Streptomyces*"; Capítulo de libro "Applications of *Streptomyces* spp. Enhanced Compost in Sustainable Agriculture"

[2] Artículo #1: "Antifungal Agents Based on Chitosan Oligomers, ε-polylysine and *Streptomyces spp.* Secondary Metabolites against Three Botryosphaeriaceae Species"

[3] Artículo #2: "On the Applicability of Chitosan Oligomers-Amino Acid Conjugate Complexes as Eco-Friendly Fungicides against Grapevine Trunk Pathogens"; Artículo #6: "Antifungal Activity of Chitosan Oligomers–Amino Acid Conjugate Complexes against *Fusarium culmorum* in Spelt (*Triticum spelta* L.)"

[4] Artículo #3: "Physicochemical Characterization of *Crithmum maritimum* L. and *Daucus carota* subsp. *gummifer* (Syme) Hook.fil. and Their Antimicrobial Activity against Apple Tree and Grapevine Phytopathogens"

Quinta: La modesta actividad antifúngica de los fitoquímicos identificados en los extractos de *E. arvense* y *U. dioica*, dos "sustancias básicas" conforme a normativa europea, también puede ser potenciada mediante conjugación con oligómeros de quitosano. El comportamiento sinérgico resultante ha sido evidenciado tanto *in vitro* -contra ocho hongos Botryosphaeriaceae, con valores de EC_{90} en el rango de 208-1000 $\mu g/mL^{-1}$, como *in vivo* -con diferencias estadísticamente significativas en las necrosis vasculares causadas por *N. parvum* y *D. seriata* en plantas de vid inoculadas artificialmente-. El uso combinado de estas sustancias básicas puede proponerse como un tratamiento prometedor contra las EMV.[5]

Sexta: Los efectos sinérgicos obtenidos a través de la conjugación de oligómeros de quitosano con sustancias bioactivas también pueden ser alcanzados –e incluso mejorados- mediante la utilización alternativa de glicósidos de esteviol. Este es el caso de la combinación de soluciones acuosas de esteviósido y soluciones alcohólicas de extractos de *Silybum marianum* (ricos en polifenoles), evidenciado contra hongos de las EMV y contra *F. culmorum*, tanto en ensayos *in vitro* como en ensayos de campo. También ha sido demostrada para flavonoides como la rutina, alcanzando total inhibición de la necrosis vascular en ensayos de campo contra hongos de la familia Botryosphaeriaceae. Obviamente, las combinaciones de oligomeros de quitosano y esteviósido con principios activos también proporcionan buenos resultados, confirmados *in vitro* contra *Phytophthora cinnamomi*.[6]

Séptima: Los productos desarrollados, respetuosos con el medio ambiente y aptos para su uso en producción integrada, son altamente prometedores para abordar el reto de disminuir el uso abusivo de tratamientos fitosanitarios de origen químico en Agricultura, cumpliendo con los requerimientos de la Directiva europea 2009/128/CE (Art. 14) y su transposición al RD 1311/2012.

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[5] Artículo #4: "Assessment of Conjugate Complexes of Chitosan and *Urtica dioica* or *Equisetum arvense* Extracts for the Control of Grapevine Trunk Pathogens"

[6] Artículo #5: "Antifungal Activity against *Fusarium culmorum* of Stevioside, *Silybum marianum* Seed Extracts, and Their Conjugate Complexes"; Artículo #7: "*In vitro* Antifungal Activity of Chitosan-Polyphenol Conjugates against *Phytophthora cinnamomi*"; Artículo #8: "Rutin-stevioside conjugates for the control of grapevine trunk diseases"; Artículo #9: "Coniferyl alcohol and ferulic acid esters in the hydro-methanolic extract of *Silybum marianum* capitula: activity against Botryosphaeriaceae fungi"; Patente #2: "Solución acuosa que comprende un complejo de inclusión, método de obtención y su uso para aplicación en cultivos y la mejora de su rendimiento"; Patente #3: "Composición nutracéutica que consiste en un polifenol en solución glicólica, método de obtención y su uso terapéutico"; Patente #4: "Composición farmacéutica que comprende una disolución de silimarina en un solvente eutéctico profundo de origen natural, métodos de obtención y uso en medicina"