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TESIS DOCTORAL:

Biocomposites antifúngicos de base quitosano para la protección de especies agrícolas, forestales y de madera

Presentada por Iosody Silva Castro
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No quiero convencer a nadie de nada…

Tratar de convencer a otra persona es indecoroso, es atentar contra su libertad de pensar o de creer o de hacer lo que le dé la gana. Yo quiero solo enseñar, dar a conocer, mostrar, no demostrar. Que cada uno llegue a la verdad por sus propios pasos, y que nadie le llame equivocado o limitado. (¿Quién es quién para decir esto es así, si la historia de la humanidad no es más que una historia de contradicciones y de tanteos y de búsquedas?)

Si a alguien he de convencer algún día, ese alguien he de ser yo mismo. Convencerme de que no vale la pena llorar, ni afligirse, ni pensar en la muerte. “La vejez, la enfermedad y la muerte”, de Buda, no son más que la muerte, y la muerte es inevitable. Tan inevitable como el nacimiento.

Lo bueno es vivir del mejor modo posible. Peleando, lastimando, acariciando, soñando. (¡pero siempre se vive del mejor modo posible!)

Mientras yo no pueda respirar bajo el agua, o volar (pero de verdad volar, yo solo, con mis brazos), tendrá que gustarme caminar sobre la tierra, y ser hombre, no pez ni ave.

No tengo ningún deseo que me digan que la luna es diferente a mis sueños.

Jaime Sabines

Poeta Mexicano

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¿Qué tienen que ver una gamba, una abeja y la cubertería de plata de la abuela?

En esta Tesis Doctoral lo cuento en más de 3 minutos, de hecho lo cuento profundamente, de la misma forma en que agradezco a todos; todos los que de múltiples formas aportaron sus granitos de arena y/o cáscaras de gambas para el desarrollo de este proyecto de investigación.

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Contenido

Resumen

El quitosano es uno de los polímeros de origen natural más interesantes de las últimas décadas. Es la forma desacetilada de la quitina, el segundo polisacárido natural más abundante en el planeta. Su actividad antimicrobiana, además de su naturaleza biodegradable, biocompatible y no tóxica le ha conferido un extenso número de aplicaciones en distintas áreas, incluida la agricultura. La versatilidad de su estructura, así como sus propiedades de sorción de macromoléculas y quelación de partículas metálicas, gracias a su carácter catiónico derivado del grupo amino, le otorgan un mayor interés y la facilidad de generar nuevos materiales que potencien su actividad. No obstante, las extensas aportaciones de la investigación sobre los productos derivados de quitosano, en sus formas, síntesis, aplicaciones, incluso a nivel teórico en la descripción de su comportamiento químico y de los mecanismos de acción que ejercen, no cesan, por el contrario su desarrollo continúa en auge. En la presente tesis doctoral se realiza en primer lugar una revisión del estado del arte de la síntesis de nuevos compuestos basados en quitosano, que incluyen desde extractos naturales hasta nanopartículas metálicas, mediante la utilización de nuevas tecnologías. Se revisan también sus aplicaciones como antimicrobiano para diversos materiales que deban mantenerse y conservarse de microorganismos y patógenos. Posteriormente, se realiza por primera vez un análisis molecular de la N-acetil-D-glucosamina (la molécula constituyente de la quitina y el quitosano) por combinación de técnicas de espectroscopía de THz-TDS y FTIR con estudios teóricos basados en métodos semiempíricos de química cuántica. Se propuso el diseño y la síntesis de un nuevo material compuesto a partir de oligómeros de quitosano, óxido de grafeno reducido y nanopartículas de plata, mediante una técnica asistida por microondas; consiguiendo un material funcionalizado y estable, como evidencia la caracterización realizada por UV-Vis, FTIR, XRD y TEM. Se prepararon también nuevos materiales compuestos a partir de quitosano, oligómeros de quitosano, propóleo y nanoplata. Estos composites fueron caracterizados por FTIR y se llevaron a cabo estudios *in vitro* e *in vivo* para evaluar su efectividad contra hongos fitopatógenos de interés agrícola, forestal y de la industria de la madera. Se consiguió controlar con éxito hongos devastadores como *Hemileia vastatrix* causante de grandes pérdidas económicas por la enfermedad de la roya de las hojas del café, *Fusarium circinatum* causante del chancro resinoso de los pinos, el hongo más dañino de bosques de coníferas a nivel mundial y *Trametes versicolor* causante de la pudrición blanca de la madera, entre otros. Los análisis *in vivo* han demostrado la potencialidad del quitosano y productos derivados como una herramienta -más respetuosa con el medio ambiente- ideal para ser incluida en los programas de manejo integrado para el control de las enfermedades el ámbito agroforestal.

Palabras Clave: copolímeros, oligómeros de quitosano, propóleo, nanoplata, fitopatógenos

Abstract

Chitosan is one of the most interesting natural polymers of recent decades. It is the acetylated form of chitin, the second most abundant natural polysaccharide on the planet. Its antimicrobial activity, in addition to its biodegradable, biocompatible and non-toxicity nature, has conferred an extensive number of applications in different areas, including agriculture. The versatility of its structure, as well as its sorption properties of macromolecules and chelation of metallic particles, due to its cationic character derived from the amino group, give it a greater interest and the ease of generating new materials that enhance its activity. However, the extensive contributions of research on chitosan-derived products, in their form, synthesis, applications, even at the theoretical level in the description of their chemical behavior and the mechanisms of action they exert, do not stop, on the contrary its development continues to rise. In the present doctoral thesis, a review of the state of the art of the synthesis of new compounds based on chitosan, ranging from natural extracts to metal nanoparticles, is made through the use of new technologies. Its applications as an antimicrobial are also reviewed for various materials that must be maintained and conserved from microorganisms and pathogens. Subsequently, a molecular analysis of N-acetyl-D-glucosamine (the constituent molecule of chitin and chitosan) is performed for the first time by combining THz-TDS and FTIR spectroscopy techniques with theoretical studies based on semiempirical quantum chemistry methods. The design and synthesis of a new composite material was proposed from chitosan oligomers, reduced graphene oxide and silver nanoparticles, by means of a microwave-assisted technique; obtaining a functionalized and stable material, as evidenced by the characterization made by UV-Vis, FTIR, XRD and TEM. New composite materials were also prepared from chitosan, chitosan oligomers, propolis and nanosilver. These composites were characterized by FTIR and *in vitro* and *in vivo* studies were carried out to evaluate their effectiveness against phytopathogenic fungi of agricultural, forest and wood industry species. Achieving successful control of devastating fungi such as *Hemileia vastatrix*, which causes coffee leaf rust (CLR), the worst disease of coffee worldwide; *Fusarium circinatum*, that causes pine pitch canker (PPC), the most harmful fungus of coniferous forests worldwide; and *Trametes versicolor* which causes white rot decay of the wood, among others. The *in vivo* analyzes have demonstrated the potential of chitosan and derived products as a tool -more environmentally friendly- ideal to be included in an integrated management approach for the control of diseases in the agriculture and forestry fields.

Keywords: copolymers, chitosan oligomers, propolis, nanosilver, phytopathogens

1. INTRODUCCIÓN GENERAL

1.1 Quitosano, un polímero natural

El quitosano es un polisacárido lineal compuesto por cadenas de N-glucosamina, se deriva de la deacetilización de la quitina, el segundo polímero natural más abundante en el planeta, después de la celulosa (Shahidi et al. 1999) y con una gran similitud entre sí. La quitina es el componente estructural de las paredes celulares de hongos, del exoesqueleto de artrópodos y de órganos de otros organismos invertebrados (Se-Kwon Kim 2011; Zakrzewski et al. 2014). El quitosano y sus derivados han sido de los polímeros de origen natural de mayor interés en las últimas décadas, especialmente por su actividad antimicrobiana y sus características de biocompatibilidad, no toxicidad y biodegradabilidad (Ma et al. 2017).

1.1.1 Origen

La quitina, fue descrita por primera vez por Henri Braconnot en 1811, quien logró aislarla de hongos superiores llamándole fungina. Posteriormente, Auguste Odier en 1823 descubrió una sustancia similar al tejido de las plantas en la cutícula de los insectos, llamándola "quitina" del griego χιτών (túnica, envoltura). El quitosano, por su parte, fue descubierto en 1859 por Rouget, cuando trató quitina -insoluble en agua- con una solución muy concentrada de hidróxido de potasio a altas temperaturas y esta se disolvió en ácidos orgánicos. No obstante, fue hasta 1894 que Hoppe-Seyler le llamó quitosano y hasta 1950, que fue claramente descrito como un polímero compuesto por glucosamina, del mismo modo que la quitina por N-acetilglucosamina. A partir de ese año el interés por su uso en aplicaciones químicas e industriales, creció rápidamente (Muzzarelli et al. 2012).

El quitosano también puede encontrarse naturalmente en algunos organismos, sin embargo, su fuente de producción a escala industrial deriva de la N-desacetilación termoalcalina de quitina aislada de deshechos de crustáceos (Goycoolea et al. 2009). Aunque se ha estimado que 1×10^{10} t de quitina se encuentran constantemente en la biósfera (Hamed et al. 2016), las dos especies principales de crustáceos marinos aprovechadas como fuente de quitina para la producción de quitosano son los camarones y cangrejos (Rinaudo 2006).

El exoesqueleto de estos invertebrados consiste en un tejido fibroso mineralizado a base de quitina, con diferentes niveles estructurales (Figura 1). A nivel molecular, presenta una alineación antiparalela formando cristales de α-quitina (Fig 1a), que se encuentran en una disposición de 18 a 25 moléculas en forma de unidades cristalinas estrechas y largas (Fig 1b), envueltas por proteínas que forman nanofibras de 2 a 5 nm de diámetro y 300 nm de longitud (Fig 1c). Estas nanofibras se agrupan en fibras más largas de quitina-proteína, de

50 a 300 nm de diámetro (Fig 1d), formando una red plana tejida y periódicamente ramificada (Fig 1e). El espacio entre las fibras se rellena con proteínas y biominerales de tamaño nanoscópico y microscópico, principalmente $CaCO_3$ cristalino, aunque también pueden aparecer partículas amorfas según la especie de crustáceo. En el siguiente nivel, se forma una capa por el apilamiento helicoidal de las capas de proteína en planos que giran gradualmente alrededor de su eje (Fig 1f), creando así complejas estructuras como la endocutícula, la exocutícula y la epicutícula (Fig 1g), las cuales se caracterizan por ser fuertes estructuras mineralizadas, diferenciadas jerárquicamente de menor a mayor rigidez (Raabe et al. 2005).

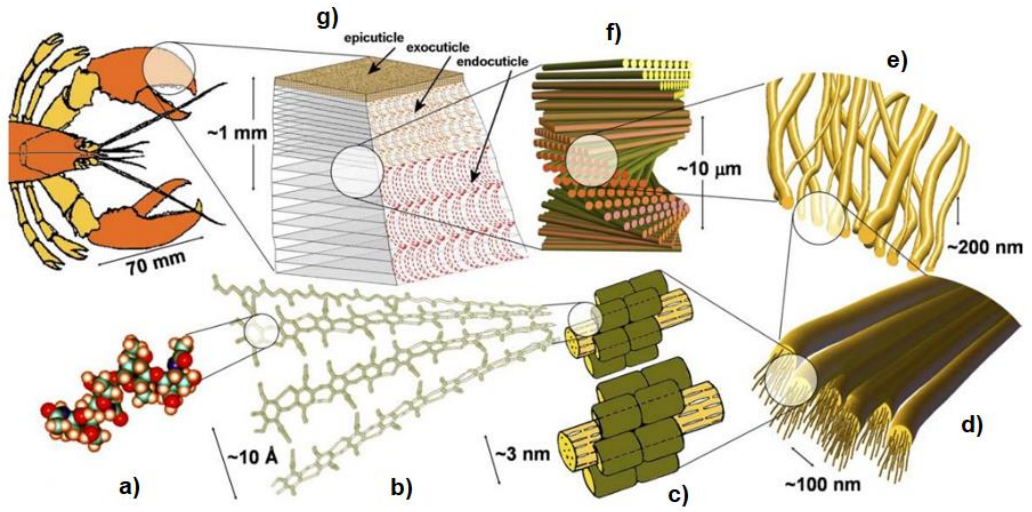


Figura 1. Niveles estructurales de la composición del exoesqueleto de los crustáceos formado a base de quitina (ejemplo: *Homarus americanus*).

1.1.2 Estructura química

El quitosano, producto de la quitina desacetilada, presenta una estructura química similar, parecida también a la celulosa (Figura 2). La quitina a diferencia de la celulosa, posee un grupo acetilamida (-$NHCOCH_3$) enlazado al carbono 2 (C-2) del anillo de piranosa. El quitosano, por su parte, difiere de la quitina por la ausencia del grupo acetil, de tal forma que en el C-2 contiene un grupo amino (NH_2). Sin embargo, el quitosano en realidad está compuesto de cadenas lineales distribuidas aleatoriamente de β-(1-4)-2-amino-2-desoxi-D-glucopiranosa y β-(1-4)-2-acetamido-2-desoxi-D-glucopiranosa, unidades desacetiladas y acetiladas, respectivamente, en proporciones variantes a partir de 80:20 según el grado de pureza o desacetilación (Zou et al. 2016).

La presencia del NH_2 le confiere un carácter catiónico, siendo el único polímero natural con esta característica (Kean & Thanou 2010). El grupo amino en el quitosano tiene un valor de pK_a de 6,5 (Thakur & Thakur 2014); por lo que es un polímero sensible a los valores del pH;

esto significa que el carácter catiónico de la molécula se presenta a valores neutro-ácidos de pH.

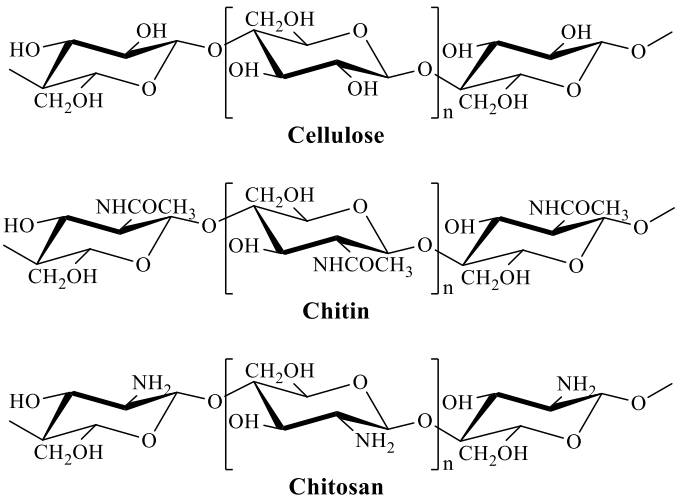


Figura 2. Estructura de las cadenas poliméricas de celulosa (*arriba*), quitina (*centro*) y quitosano (*abajo*).

La principal ventaja del quitosano en comparación a la quitina y a la celulosa es su versatilidad, porque contiene tres tipos de grupos funcionales reactivos en cada unidad monomérica, además del grupo amino del C-2 derivado de la desacetilación, el C-3 y C-6 contienen grupos hidroxilos en ambas unidades, acetiladas y desacetiladas (Xia et al. 2011). Esta disponibilidad de sitios reactivos permite la unión a otros grupos funcionales, por lo tanto, posee mayor actividad química y biológica.

Además de la estructura, el tamaño de las moléculas en un polímero como el quitosano es determinante de sus propiedades fisicoquímicas. Por lo tanto, el peso molecular (PM) es una variable muy importante que depende del grado de desacetilación (GD), así como del grado de polimerización (GP). En el quitosano se reconocen 4 tipos distintos respecto al peso molecular: alto (> 300 kDa), medio (de 190 a 300 kDa), bajo (de 50 a 190 kDa) y los oligómeros (< 16 kDa), aunque no hay un acuerdo universal sobre los límites del PM entre cada tipo, la clasificación anterior ha sido presentada recientemente por Verlee et al. 2017.

Otra ventaja del quitosano frente a la celulosa y a la quitina, es que presenta una fácil conversión a oligómeros por diversos métodos (Dash et al. 2011). La degradación de los enlaces O-glucosídico en la cadena polimérica del quitosano genera oligómeros con dos grupos hidroxilo más en los extremos, que junto a los grupos amino libres, hacen más reactivos a los oligómeros (Figura 3). No obstante, el tamaño de los oligómeros es determinante para su actividad, de tal forma que los heptámeros, octámeros, dodecámeros y demás oligómeros por debajo de 10 kDa, pueden tener propiedades específicas (Hadwiger 1994).

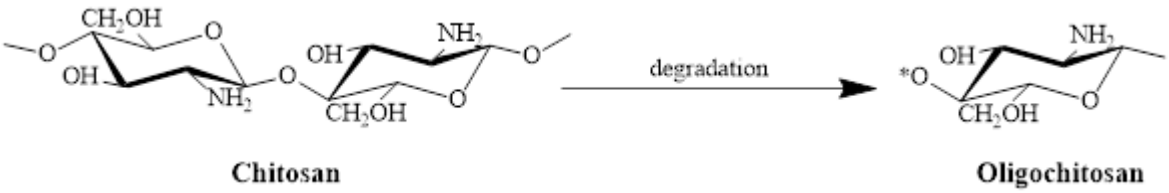


Figura 3. Formación de olígomeros más reactivos que la cadena polimérica del quitosano por la degradación del enlaces O-glucosídicos.

Por otra parte, la caracterización de la unidad monomérica es fundamental en un polímero. La N-acetilglucosamina ($C_8H_{15}O_6N$), ha sido caracterizada por Johnson desde 1966, quien reportó que posee una estructura cristalina monoclínica, con un grupo espacial $P2_1$ y dimensiones de *a*= 11,25 Å, *b*= 4,82 Å, *c*= 9,72 Å, y β= 113,7°. El conocimiento de las características de la estructura cristalina de las moléculas, es necesaria para entender su actividad, propiedades y funcionamiento.

1.1.3 Propiedades

La estructura química del quitosano y sus oligómeros, por su alcalinidad y alta reactividad, le confiere diversas propiedades físicas, químicas y biológicas, que destacan sobre otros polímeros. Entre las características físicas se encuentra la facilidad de formación de geles, hidrogeles, películas, esferas, membranas, nanofibras, nanopartículas, etc. (Shukla et al. 2013). Entre las propiedades químicas destacan la quelación de metales y sorción de moléculas, por copolimerización, formación de complejos, agente floculante, formación de sales con ácidos orgánicos e inorgánicos, entrecruzamiento y formación de enlaces por puentes de hidrógeno (Thakur and Thakur 2014). Respecto a sus ventajas en la actividad biológica el quitosano ha sido ampliamente estudiado por ser biocompatible con células humanas, biodegradable, no tóxico en mamíferos, antioxidante, antiviral, antibacteriano, y antifúngico, por nombrar algunas (Dash et al. 2011; Ngo et al. 2015).

Por tanto, es de gran interés para ser aplicado en sectores desde medicina, p. ej. como control de colesterol (Zou et al. 2016); en farmacia, como matriz de liberación controlada de medicamentos (Elgadir et al. 2015); en la industria alimentaria, como empaque antibacteriano de alimentos (Aider 2010); en medio ambiente, como floculante-coagulante para tratamiento de aguas residuales (Meraz et al. 2016); en preservación del patrimonio, como protector de artefactos antiguos de madera (El-Gamal et al. 2016); y en agricultura, como inductor de crecimiento y resistencia de semillas y plantas, así como antifúngico contra hongos fitopatógenos (Kumaraswamy et al. 2018) entre los más destacados.

Quitosano, un material antifúngico

Las propiedades antifúngicas del quitosano y sus derivados les han merecido ser de gran interés, como alternativa para combatir microorganismos fitopatógenos. Su actividad contra hongos y bacterias, se atribuye a la presencia del grupo amino (NH_3^+) que le confiere

un carácter catiónico, aunque otras propiedades inherentes al quitosano como el valor del pKa, el peso molecular, el grado de desacetilación y factores externos como el pH del medio y la fuerza iónica o el tipo de organismo y la especie, son variables importantes de las que depende esta actividad (Aider 2010).

Los mecanismos de acción más aceptados son dos principalmente: (i) Interacción extracelular con la pared y membrana celular e (ii) Interacción intracelular con las proteínas y ácidos nucleicos. Por una parte, el grupo amino puede interactuar electrostáticamente con la superficie de la membrana celular de los microorganismos, cargada negativamente por la presencia de lipopolisacáridos, ácido teicoico, peptidoglucanos, otras proteínas e iones metálicos; esta interacción incrementa la permeabilidad de la membrana, con la consecuente fuga de sustancias, desestabilizando completamente sus funciones y generando una lisis celular (Figura 4a). Por otra parte, como consecuencia de la presencia del quitosano dentro de la célula pueden generase respuestas intracelulares como la inactivación o bloqueo de actividad enzimática, la inhibición de la síntesis de proteínas y del ARN, así como de la transcripción y traducción del ADN (Figura 4b) (Verlee et al. 2017; Kumaraswamy et al. 2018).

A pesar de que la actividad antimicrobiana del quitosano no se asume como completamente dilucidada (Palma-Guerrero et al. 2008), estos dos procesos han sido estudiados y observados sobre diferentes bacterias como *Escherichia coli* y *Staphylococcus aureus* (Kong et al. 2010) y hongos como *Phytophthora capsici* (Xu et al. 2007), *Sphaeropsis sapinea* y *Trichoderma harzianum* (Singh et al. 2008), entre otros.

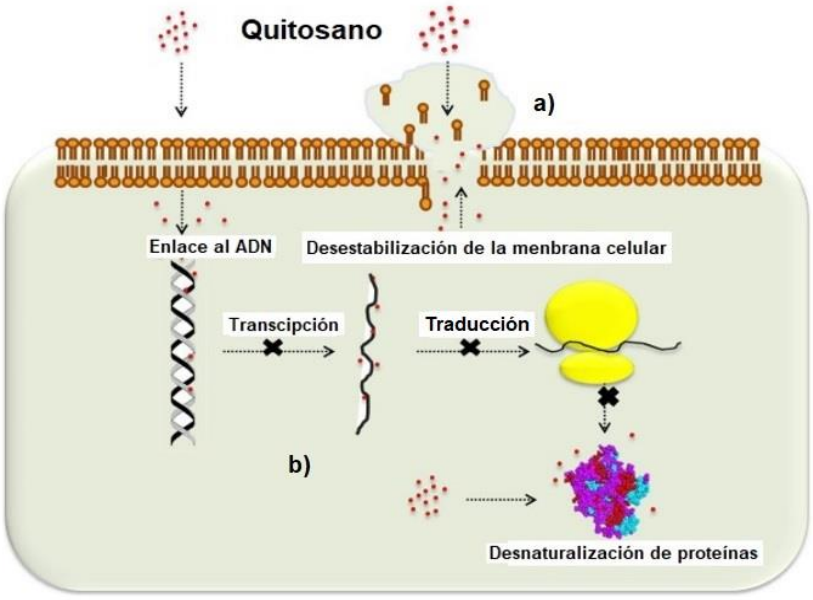


Figura 4. Modo de acción antifúngico del quitosano y sus derivados, a través de dos vías: a) desestabilización de la membrana celular y b) interrupción de la síntesis de proteínas (Kumaraswamy et al. 2018).

1.2 Propóleo y nanoplata, otros materiales antifúngicos

La creciente necesidad de alternativas para sustituir productos químicos convencionales en aplicaciones agrícolas, ha llevado a los investigadores del mundo entero a echar un vistazo al pasado y retomar los conocimientos ancestrales para el desarrollo de nuevas tecnologías. Desde el uso de materiales de fuentes renovables, hasta la aplicación de los mismos en otros formatos. Dos materiales que han despertado gran interés por su actividad antifúngica son el propóleo, como un producto de origen natural y la nanopartículas de plata, más efectivas que la plata en bloque.

1.2.1 Propóleo

El propóleo es un material resinoso compuesto por un diverso conjunto de sustancias químicas producido por las abejas a partir de brotes de árboles, savia, resinas, mucílagos y exudados propios, que emplean como protección de su colmena, tanto de agente sellador como de higienización (Marghitas et al. 2013). La palabra "propóleo" deriva del griego πρόπολη *própolis*, donde pro significa "a la entrada de" y polis "ciudad", indicando que se trata de una sustancia para la defensa de la colmena (Burdock 1998).

El propóleo es hidrofóbico, duro y quebradizo en estado sólido pero flexible y pegajoso en estado líquido (Zabaiou et al. 2017). Sus características como aroma y color (entre verde, amarillo, marrón y rojizo) pueden variar según su heterogénea composición química. Debido a su origen botánico, esta composición varía en relación a las especies de plantas existentes en la zona que las abejas habiten, entre ellas se encuentran principalmente árboles como álamos, abedul, pinos, alisos, sauces y palmeras (Toreti et al. 2013). No obstante, también varía según la especie de abejas colectoras, la época del año, u otros factores geográficos como altura e iluminación (Oryan et al. 2018). En general, el propóleo puede contener un promedio de 50% de resina y bálsamo, 30% de cera, 10% de aceites esenciales y aromáticos, 5% de polen y 5% de impurezas, compuestos por más de 300 especies químicas diferentes (Huang et al. 2014). Los principales grupos de sustancias identificadas son: alcoholes, aldehídos, ácidos y ésteres alifáticos, aminoácidos, ácidos aromáticos, ésteres aromáticos, flavonoides como chalconas, flavonas, flavonoles; cetonas, terpenoides, asteroides, polisacáridos, entre otros (Marcucci 1994).

En los últimos años, el interés por los productos renovables y de origen natural ha renovado la atención de los científicos hacia el propóleo, que actualmente se ha posicionado como uno de los materiales naturales más estudiados en medicina (Silva-Carvalho et al. 2015), farmacia (Franca et al. 2014), alimentación (Rollini et al. 2017) y agricultura (Ali et al. 2013), entre otros sectores, debido a sus múltiples propiedades.

El propóleo tiene una alta actividad biológica, el efecto antimicrobiano es uno de los más destacados, pues ha sido utilizado como un producto desinfectante y antiséptico en la medicina tradicional (Marcucci 1994). Debido al contenido de compuestos fenólicos y

flavonoides se ha observado que es capaz de alterar la permeabilidad de la membrana celular e inhibir la síntesis de proteínas en los microorganismos (Mirzoeva et al. 1997; Oryan et al. 2018). Su actividad antibacteriana de amplio espectro ha sido bien documentada (Torlak and Sert 2013; Mascheroni et al. 2014). Sin embargo, la actividad antifúngica ha sido demostrada con un menor número de hongos fitopatógenos (Pastor et al. 2010; Ali et al. 2013), a pesar de que tiene un prometedor potencial para el control de enfermedades agrícolas y forestales.

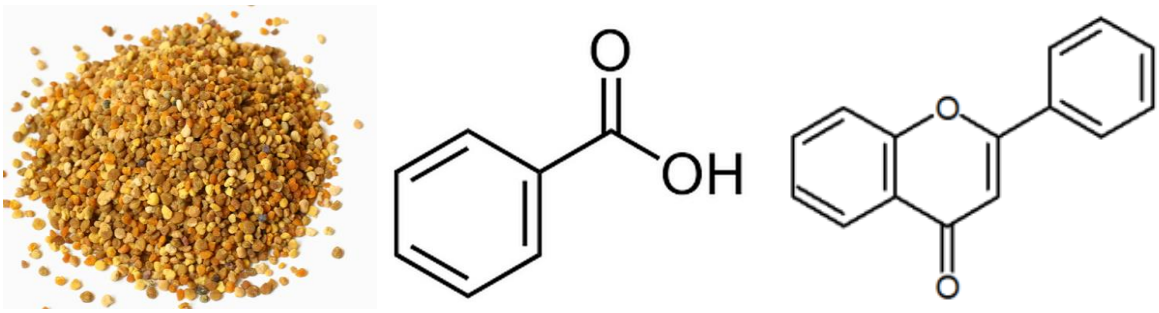


Figura 6. Aspecto físico del propóleo y estructura química de sus principales grupos funcionales: fenoles y flavonoides.

1.2.2 Nanopartículas de plata

La nanotecnología ha sido una de las áreas de mayor desarrollo en las últimas décadas, debido a las propiedades fisicoquímicas únicas que presentan las nanopartículas en comparación con sus contrapartes de mayor tamaño (Savage et al. 2007). Las nanopartículas son aquellas partículas con un tamaño comprendido entre 1 y 100 nanómetros. Existen de manera natural nanopartículas como proteínas, polisacáridos u otras moléculas orgánicas producidas por microorganismos o en diferentes procesos biológicos (Heiligtag and Niederberger 2013); así como nanopartículas de compuestos inorgánicos como hidróxidos de hierro, aluminosilicatos u óxidos metálicos, producidos por la acción del clima, en erupciones volcánicas, o incendios forestales (Méndez-Rojas et al. 2014).

Aunque actualmente la nanotecnología es un campo de conocido desarrollo tecnológico, la fabricación y uso de las nanopartículas data de mucho tiempo atrás. Nanopartículas metálicas como las de plata y cobre han sido empleadas para pigmentar y dar brillo a las vidrieras de catedrales góticas (Colomban 2009). Hoy en día, los nanometales son materiales versátiles que pueden ser aplicados en la industria química, en remediación ambiental, en cosmética, en medicina y en alimentación (Cañas-Carrell et al. 2014; Edebali et al. 2018). En estas últimas áreas, las nanopartículas de plata (nAg) se aplican debido a su

actividad antimicrobiana, la cual ha sido ampliamente estudiada y utilizada en productos comerciales (Ávalos et al. 2013).

El interés por el uso de nanoplata como bactericida, recae principalmente en la resistencia generada por múltiples bacterias a los antibióticos convencionales (Morones et al. 2005). La nanoplata posee una actividad antibacteriana de muy amplio espectro, bastante superior a la plata en bruto debido a que presenta una mayor proporción superficie/volumen. Este efecto se ha comprobado en un extenso número de bacterias (Carbone et al. 2016), aunque se ha observado que las Gram positivas son menos susceptibles que las Gram negativas, debido a que las pared celulares son más gruesas (Shrivastava et al. 2007). Se sabe que las nanopartículas de plata tienen la habilidad de destruir las paredes celulares e interferir con los procesos de síntesis de proteína y replicación del ADN bacteriano (Yun and Lee 2017). No obstante, su actividad depende del tamaño y forma, las nanopartículas que presentan un tamaño inferior a 10 nm exhiben una mayor capacidad de perturbar drásticamente la superficie de la membrana celular (Morones et al. 2005). En cuanto a la forma, las nanopartículas triangulares truncadas presentan una actividad más fuerte que las nanoesferas, nanobarras, nanoplacas o nanocables (Sun and Xia 2002).

El gran potencial de las nanopartículas de plata como antifúngico en aplicaciones contra enfermedades causadas por hongos fitopatógenos ha sido menos estudiado (Narayanan and Park 2014; Kim et al. 2018). Quizá porque existen desventajas que limitan su uso, principalmente la facilidad de agregación, la liberación incontrolada de iones de plata y su potencial citotoxicidad (Savage et al. 2007). Una de las alternativas para controlar estos inconvenientes es la quelación de las nanopartículas por una matriz polimérica. El quitosano, por ejemplo, ha sido combinado con la nanoplata por dos razones básicas: la estabilización de las nanopartículas a través de un proceso de quelación, debido a que el grupo amino primario y el grupo hidroxilo del quitosano presentan una alta afinidad a los iones metálicos, como los de la plata (Chowdappa et al. 2014); y por la síntesis en un solo paso, puesto que el quitosano puede actuar como agente reductor del nitrato de plata ($AgNO_3$) y después de estabilizador de las nonapartículas (Venkatesham et al. 2012). Además, se ha comprobado que esta combinación incrementa el efecto antimicrobiano de ambos (Wang et al. 2015), evita el uso de agentes reductores comunes que son tóxicos en el medio ambiente (Wei et al. 2009) y reduce el riesgo citotóxico en mamíferos del uso de nanoplata (Jena et al. 2012).

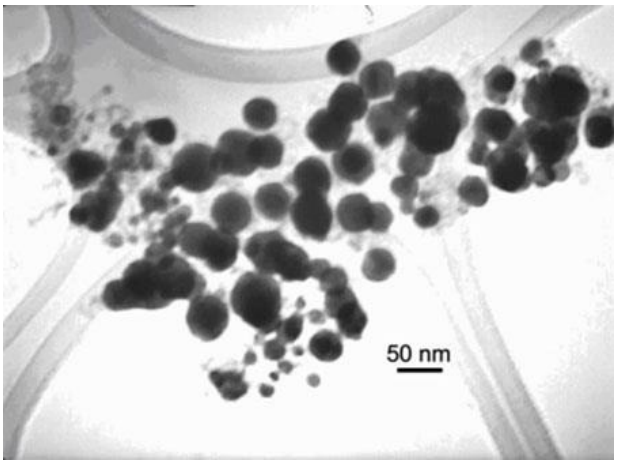


Figura 7. Micrografía electrónica de nanopartículas de plata en una matriz de quitosano.

1.3 Composites de base quitosano

En la creciente importancia del uso de biopolímeros, el quitosano ha sido uno de los más utilizado en las últimas décadas para la formación de materiales compuestos. Como se mencionó anteriormente, una de la grandes ventajas del quitosano son la capacidad de sorción y quelación, conferidas por el grupo amino y la funcionalidad del alcohol primario (OH) (Velmurugan et al. 2009), generados en la desacetilación de la quitina y en la degradación de la cadena polimérica para la generación de oligómeros. La formación de los nuevos enlaces no se limita a un grupo de especies químicas, pues se han reportado composites de quitosano con macromoléculas orgánicas como con nanopartículas metálicas (Saharan et al. 2015; Vieira et al. 2016). Las técnicas de síntesis desde la producción de quitosano a partir de quitina, la formación de oligómeros y la composición de nuevos materiales involucran diversos procesos físicos, químicos y biológicos. Por otra parte, la caracterización fisicoquímica de estos nuevos materiales es muy importante, no solo como control de los procesos de síntesis, también para evaluar su actividad y sus potenciales aplicaciones.

1.3.1 Técnicas de síntesis

De quitina a quitosano

Las fuentes de quitina para la producción comercial de quitosano son principalmente las cáscaras de crustáceos marinos. Una vez procesados estos residuos, mediante el lavado y la molienda de los mismos, el siguiente paso es someterlos a un proceso químico que consiste en 3 pasos fundamentales: desmineralización ácida, desproteinización alcalina y desacetilación alcalina como se representa en la Figura 8 (Leceta et al. 2013).

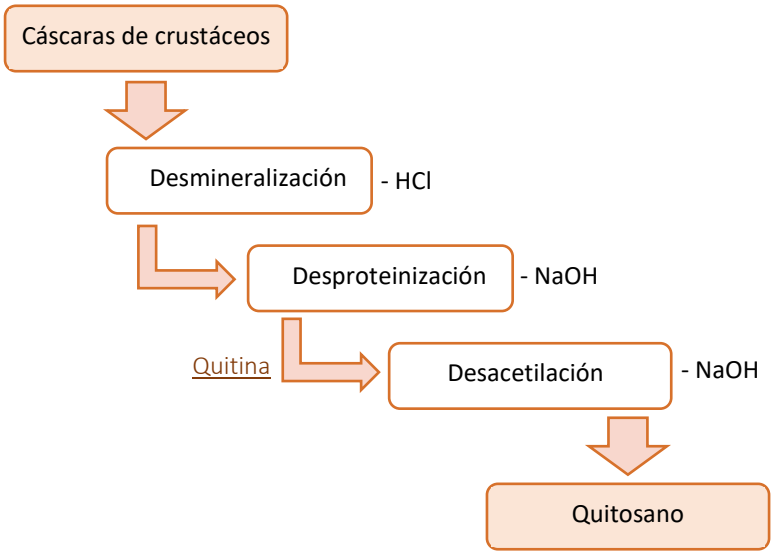


Figura 8. Proceso esquemático de la producción de quitosano

El primer paso, la disolución de los minerales, se realiza con ácido clorhídrico (HCl) concentrado (1N) para disolver el carbonato cálcico ($CaCO_3$) presente de 30 a 50% en la composición de estos residuos. El segundo paso, la degradación de las proteínas, se lleva a cabo con hidróxido de sodio (NaOH) diluido; pues del 30 al 40% del exoesqueleto de los crustáceos está compuesto por proteínas. El resultado de este segundo paso es la quitina aislada, generalmente α-quitina (más abundante). Después puede realizarse una decoloración con hipoclorito de sodio (NaOCl) diluido, para remover pigmentos de carotenoides como la astaxantina, que se encuentran en el 20 o 30% de peso seco de los residuos. El último paso es la desacetilación de la quitina; esta es la remoción de los grupos acetilos ($COCH_3$) y puede llevarse a cabo a través de un tratamiento térmico acompañado de un proceso químico de hidrólisis alcalina con NaOH concentrado o biológico vía enzimática con algunos hongos y bacterias, durante tiempos prolongados de reacción. Posteriormente se realiza un lavado y secado del material, como resultado obtenemos partículas sólidas de quitosano (Aranaz et al. 2009; Pillai et al. 2011; Benhabiles et al. 2012).

De quitosano a oligómeros

La aplicación de los oligómeros de quitosano presenta mayor interés debido a que mejoran algunas desventajas del quitosano con más alto GP, como su alta viscosidad y baja solubilidad en agua (Benhabiles et al. 2012; Zou et al. 2016). Las técnicas de degradación de las cadenas poliméricas consisten en el rompimiento de los enlaces glucósidos β-(1-4) entre cada molécula de glucosamina (como se mostró en la figura 3), para generar cadenas más pequeñas u oligómeros de aproximadamente 2 a 10 monómeros (Verlee et al. 2017). Algunos de los métodos físicoquímicos y biológicos más utilizados en la despolimerización del quitosano son la degradación oxidativa (Sun et al. 2007; Xia et al. 2013), hidrólisis ácida

(Tsao et al. 2011), hidrólisis enzimática (Kim & Rajapakse 2005), irradiación (Duy et al. 2011) y ultrasonidos (Savitri et al. 2014), entre otras donde se combinan dos o más de estas reacciones.

El mecanismo de degradación oxidativa, puede llevarse a cabo con peróxido de hidrógeno (H_2O_2) como material oxidante, adicionándolo a una solución de quitosano diluido en medio ácido (ácido acético, láctico, etc.) para que una vez distribuidas las cadenas poliméricas en el medio, entren en contacto con los radicales libres formados por la disociación del H_2O_2, estos tienen la capacidad de atacar los enlaces glucosídicos de los polisacáridos (Sun et al. 2007).

Composites orgánicos e inorgánicos

Los métodos de síntesis para generar materiales compuestos entre moléculas de quitosano y macromoléculas orgánicas se basan en la capacidad de sorción de este polímero. El mecanismo de sorción, puede establecerse a través de distintas vías: la interacción con el N de la amina, con el O del hidroxilo o ambos (Verlee et al. 2017). Tal es el caso del material compuesto por quitosano-propóleo, se ha reportado que los polifenoles del propóleo pueden formar enlaces de hidrógeno y enlaces covalentes con los grupos funcionales de la glucosamina del quitosano o sus oligómeros (Siripatrawan & Vitchayakitti 2016). Una de las ventajas de esta unión es que ambos compuestos presentan un mayor poder antimicrobiano respecto a la aplicación de los mismos individualmente (Torlak & Sert 2013; Rollini et al. 2017).

Por otra parte, los mecanismos de síntesis para la producción y estabilización de nanopartículas metálicas, se basan en la propiedad de quelación de iones metálicos del quitosano. Se sabe que los grupos amino tienden a formar complejos, por ejemplo, con los iones Ag^+ de la nanoplata, con los que forma un enlace coordinado (Wei et al. 2009). Además de una síntesis efectiva de las nanopartículas con el uso del quitosano como reductor y la estabilización de las mismas por la quelación, se han encontrado mejores propiedades antibacterianas y antifúngicas con el uso de este compuesto (Saharan et al. 2013; Chowdappa et al. 2014; Wang et al. 2015).

También existe el caso donde se requieren ambos mecanismo de síntesis, para producir composites ternarios, entre quitosano, especies orgánicas y especies inorgánicas. Por ejemplo, en un material de oligómeros de quitosano, con óxido de grafeno reducido (rGO) y nanopartículas de plata, los oligómeros se unen al óxido de grafeno a través de la sorción, creando un copolímero. Posteriormente, las nanopartículas de plata se enlazan a la matriz polimérica, principalmente a las hojas del rGO que brindan mayor área superficial para la deposición de las nanopartículas, que junto a los oligómeros se obtiene un material estable y funcionalizado para diversas aplicaciones (Marta et al. 2015). Entre otros como se muestran en la tabla 1.

Tabla 1. Materiales compuestos de base quitosano, con macromoléculas orgánicas y nanopartículas metálicas.

Naturaleza del material	Composite	Síntesis	Aplicación	Referencia
Macromoléculas orgánicas	Quitosano–almidón	Método de fundición	Recubrimiento antifúngico de papayas	Escamilla-García et al. 2018
	Quitosano–*Aloe vera*	Método de fundición	Recubrimiento antifúngico de moras	Vieira et al. 2016
	Quitosano–cinamaldehido	Enlace por base de Shiff	Empaque antifúngico de alimentos	Demitri et al. 2016
	Quitosano–saponina	Gelificación iónica	Protección antifúngico de cultivos	Saharan et al. 2013
	Quitosano–aceites esenciales	Método de fundición	Empaque antifúngico de alimentos	Avila-Sosa et al. 2012
Nanopartículas metálicas	Quitosano–nanocobre	Gelificación iónica	Inductor de crecimiento y antifúngico en tomate	Saharan et al. 2015
	Quitosano–nanoplata	Un solo paso – tratamiento químico	Antifúngico	Wang et al. 2015
	Quitosano–nanoplata	Un solo paso – tratamiento térmico	Antifúngico – contra antracnosis del mango	Chowdappa et al. 2014
	Quitosano–nanocobre	Gelificación iónica	Antifúngico - Protección de cultivos	Saharan et al. 2013
Ambos (orgánico e inorgánico)	Quitosano–aceite de neem–nanopartículas de óxido de zinc	Método de fundición	Antibacteriano – empaques de alimentos	Sanuja et al. 2015

1.3.2 Técnicas de caracterización

El desarrollo de nuevos materiales involucra el desarrollo de nuevas técnicas, o por lo menos, de técnicas más sensibles para su análisis. Entre las técnicas más utilizadas para el estudio y caracterización de polímeros como el quitosano, sus derivados y materiales compuestos basados a partir de su estructura tenemos la espectroscopía y la microscopía, como dos grandes áreas con muchas herramientas disponibles, entre otras técnicas características destacadas en la tabla 2.

Técnicas espectroscópicas

UV-vis. La espectroscopía o espectrofotometría UV-visible, consiste en la emisión de fotones, o radiación electromagnética, en las regiones del espectro visible, ultravioleta cercano (UV) e infrarrojo cercano (IR), por tanto abarca longitudes de onda desde 380 nm a 780 nm. A través de esta técnica es posible cuantificar las transiciones electrónicas en las moléculas causadas por la absorción de la radiación. Suele emplearse para determinar la composición química de las sustancias identificando grupos funcionales e iones metálicos. En el caso del quitosano, se emplea para evaluar el grado de desacetilación (Czechowska-biskup et al. 2012), o para determinar el contenido de moléculas orgánicas (Esmaeili & Asgari 2015) o nanopartículas metálicas (Moharram et al. 2014) en un composite de base quitosano.

FT-IR. La espectroscopía infrarroja (IR), consiste en la cuantificación de las oscilaciones de los átomos de una molécula tras la absorción de energía emitida en un rango de frecuencia de 12 800 a 4 000 cm^{-1} en el IR cercano, de 4 000 a 400 cm^{-1}, en el IR medio (más utilizado) o 400 a 50 cm^{-1} en el IR lejano. La energía no absorbida por la muestra se capta en un colector y la transformada de Fourier posibilita la formación un espectro interpretable. El espectro vibracional de una molécula se considera una propiedad física única, por tanto es una "huella dactilar" que la identifica. A través de FTIR se ha podido comprobar la interacción entre el quitosano y los polifenoles del propóleo, que se unen a través de enlaces de hidrógeno (Siripatrawan & Vitchayakitti 2016).

ATR – FTIR. La reflectancia total atenuada (ATR por sus siglas en inglés) es una técnica acoplada a un espectrofotómetro FTIR, para un análisis rápido de las muestras en estado sólido, gel o líquido. Consiste en la emisión de un haz de IR que se desplaza por un cristal ópticamente denso y con alto índice de refracción, en un ángulo determinado; la reflectancia interna en el cristal crea una onda evanescente que se extiende hasta hacer contacto con la muestra ubicada en la superficie. En las regiones del espectro IR donde la muestra absorbe energía, la onda evanescente se atenúa, el haz atenuado vuelve al cristal, sale a continuación por el extremo opuesto a la emisión y se dirige al detector para generar un espectro de IR. Esta técnica ha sido utilizada para evaluar el rol de los grupos funcionales del quitosano (OH y NH) en la formación de enlaces de hidrógeno, para la preparación de un film con nanoarcillas (Branca et al. 2016).

THz – TDS. La radiación de terahercios medida tiempo-dominio (THz–TDS por sus siglas en inglés) es una técnica de espectroscopia en la cual las propiedades de la muestra son estudiadas por la irradiación de ondas desde 100 µm a 1 mm de longitud. Esta provee más información que la espectroscopía por transformada de Fourier, que es solo sensible a la amplitud. Debido a que se conoce el tiempo-dominio, la frecuencia-dominio de la señal de THz está disponible, así el efecto de distorsión de la difracción se puede mitigar y la resolución de las imágenes de THz puede ser mejorada (Ahi 2018).

DFT y GPU. La química cuántica computacional es una herramienta muy valiosa para la interpretación de espectros Far FT-IR debido a la complejidad de los modos de vibración asociados. Los métodos *Ab initio* y la teoría funcional de la densidad (DFT) son muy precisos en la predicción de vibraciones moleculares (Scott & Radom 1996). Sin embargo, los cálculos de estado sólido sobre grandes sistemas supramoleculares generalmente son necesarios debido a la relevancia de las interacciones de largo alcance, lo que limita el uso de estos métodos computacionalmente costosos. Por otro lado, los métodos semiempíricos (Coolidge et al. 1991), especialmente con las implementaciones paralelas desarrolladas recientemente para multiprocesadores de memoria compartida y arquitecturas de unidades de procesamiento de gráficos GPU (Maia et al. 2012), permiten el estudio de las vibraciones en grandes cristales moleculares en tiempos de cálculo reducidos.

XRD. La difracción de rayos X (XRD por sus siglas en inglés) se utiliza para determinar la disposición de los átomos en un cristal, consiste en la emisión e incidencia de un rayo X sobre el cristal, el cual se difracta en muchas direcciones específicas. Desde estos haces difractados, se puede producir una imagen tridimensional de la densidad de los electrones dentro del cristal. Después, a partir de la densidad electrónica, se pueden determinar las posiciones medias de los átomos, así como otra información estructural. En el caso de polímeros como el quitosano, por sus características anisotrópicas un patrón de cristalinidad es difícilmente obtenido, por tanto se deduce que es un material amorfo (Kim 2014). El método de Debye-Scherrer en XRD es utilizado para estudiar la estructura de sustancias finamente cristalinas, por ejemplo, la formación de nanopartículas de plata utilizando quitosano en el proceso de síntesis (Wang et al. 2015).

Técnicas microscópicas

SEM. La microscopía electrónica de barrido (SEM por sus siglas en inglés) es una tecnología aplicada a la caracterización morfológica de las muestras capaz de producir imágenes de alta resolución. Consiste en la interacción electrón-materia, un haz de electrones acelerados en un campo eléctrico es emitido y enfocado a través de lentes hacia la muestra, cuando el haz incide sobre ella se producen interacciones entre los electrones del haz y los átomos de la muestra; por ejemplo, electrones secundarios desprendidos de la muestra pueden salir disparados tras la colisión, siendo estos los electrones detectados generalmente para la formación de las imágenes. Las micrografías de SEM se utilizan frecuentemente en los estudios de síntesis de materiales compuestos a partir de quitosano para determinar la micromorfología de los mismos (Torabi et al. 2016; Fortunati et al. 2017).

TEM. Microscopio electrónico de transmisión (TEM por sus siglas en inglés). Este tipo de microscopio aunque aplica el mismo principio que un SEM (un haz de electrones acelerados disparados hacia la muestra) tiene mejores prestaciones, pues tiene la capacidad de aumentar hasta 1 millón de veces el tamaño de la muestra, debido a que la imagen se

genera directamente de los electrones que la atraviesan, por lo cual esta debe tener de un espesor muy fino. La morfología de la superficie y la forma de varios nanomateriales pueden obtenerse de esta forma (Gu et al. 2014; Saharan et al. 2015).

Tabla 2. Técnicas principales utilizadas para la caracterización de materiales compuestos basados en quitosano.

Compuesto	FTIR	UV-Vis	TEM	SEM	XRD	Referencia
Clorhidrato de quitosano	x			x		Fortunati et al. 2017
Quitosano-piridina	x	x		x		Jia et al. 2016
Quitosano-sílica	x	x		x		Dabóczi et al. 2016
Quitosano-acrílico	x			x		Torabi et al. 2016
Quitosano - nanopartículas de vidrio bioactivo	x		x	x	x	Pourhaghgouy et al. 2016
Quitosano-óxido de grafeno-trientina	x			x	x	Ge and Ma 2015
Quitosano-alginato-aceites esenciales	x			x		Natrajan et al. 2015
Quitosano-Vainillina	x			x	x	Stroescu et al. 2015
Quitosano-Cu NPs	x		x	x		Saharan et al. 2015
Quitosano-aceite de neem-óxido de zinc	x			x	x	Sanuja et al. 2015
Quitosano-Ag NPs	x	x	x			Moharram et al. 2014
Quitosano-policaprolactona-Ag NPs	x	x	x		x	Gu et al. 2014
Quitosano-isicianato	x					Gallego et al. 2013
Quitosano-cloroquinolina	x			x	x	Kumar et al. 2011

1.4 Composites de quitosano para la sanidad agroforestal

El quitosano y sus derivados han sido estudiados con el objetivo de ser aplicados potencialmente en el área agrícola, a través de dos usos generales: como antimicrobiano y como estimulante vegetal; ambas propiedades, son conferidas por el carácter catiónico de sus moléculas y la reactividad de sus grupos funcionales. Como se mencionó anteriormente, la capacidad antimicrobiana del quitosano se ha utilizado para el control de enfermedades causadas por hongos fitopatógenos. Los formas de ser aplicado varían según el objetivo de la aplicación, de tal forma que puede encontrarse como recubrimiento de semillas, tratamiento foliar, recubrimiento de frutos post cosecha, enmienda de suelos, etc.

1.4.1 Aplicaciones de quitosano

Estimulante vegetal

El quitosano puede ser usado en las plantas como estimulante, elicitor o inductor de crecimiento y resistencia a enfermedades causadas por agentes bióticos o abióticos. La aplicación del quitosano favorece la germinación de las semillas, el vigor de las plantas y el rendimiento productivo (Ramirez et al. 2010). Se ha comprobado que al interior de las plantas, el quitosano y sus derivados pueden activar enzimas encargadas de la degradación y movilización de nutrientes, incluso pueden activar hormonas de crecimiento como la auxina y la citoquinina, entre otros mecanismos involucrados en el crecimiento (Kumaraswamy et al. 2018). Además puede regular el sistema inmune de las plantas, incrementando la producción de enzimas antioxidantes tales como la superóxido dismutasa, catalasa y peroxidasa, las cuales atacan a las especies reactivas de oxígeno, mejorando la tolerancia a los factores de estrés (Zeng and Luo 2012).

La aplicación de quitosano en plantas de interés agrícola como arroz, soja, tomate y lechugas, se ha evaluado en las primeras etapas de crecimiento y se han encontrado incrementos en el tamaño y peso de las hojas, al aplicarse en solución a la zona foliar (Chibu and Shibayama 2003). En pruebas de campo se ha comprobado que plántulas de trigo, germinadas de semillas recubiertas con quitosano, han presentado mejores características físicas (tasa de germinación, contenido de biomasa, actividad radicular, rendimiento), así como fisiológicas (contenidos estables de enzimas y sales) bajo condiciones de sequía (Zeng and Luo 2012). También se han realizado aplicaciones de quitosano en especies forestales como pino silvestre en vivero, la dosificación en spray de quitosano soluble en agua, a pesar de que no incrementó la germinación, sí mejoró la longitud de las plántulas y el contenido de biomasa en la parte aérea y raíz (Aleksandrowicz-trzcińska et al. 2015).

Como reportan Khan et al. 2003, también se ha comprobado el aumento de la actividad enzimática en diferentes cultivos agrícolas como la soja, con la aplicación de oligómeros de quitosano de distintos tamaños, donde la fenilalanina amonio liasa y la tirosina amonio liasa incrementaron su actividad, estas dos son enzimas precursoras de metabolitos secundarios como la lignina, flavonoides y fitoalexinas, que desempeñan un papel clave en la interacción planta-patógeno.

Control antifúngico

El quitosano posee por sí mismo propiedades para combatir un amplio espectro de organismos fitopatógenos (Katiyar et al. 2014). El contacto directo actúa en contra de la superficie celular de los hongos ejerciendo alguno de los mecanismo de acción descritos en el apartado 1.1.3, la lisis de la membrana plasmática o la interrupción de síntesis de proteína al interior de las células.

En evaluaciones *in vitro* adicionando diferentes tipos de quitosano en el medio de cultivo se ha demostrado un efecto fungicida o inhibidor del crecimiento de micelio de hongos que causan pudrición de vegetales, como *Aspergillus niger*, *Alternaria alternata*, *Rhizopus oryzae* (Ziani et al. 2009); pudrición de semillas, como *Aspergillus flavus*, *Rhizoctonia solani* (Kaur et al. 2012); o pudrición de madera, como *Leptographium procerum* y *Sphaeropsis sapinea* (Torr et al. 2005), entre muchos otros, para los cuales se han reportado las concentraciones mínimas de inhibición de crecimiento (MIC).

Por otra parte los estudios *in vivo*, demuestran cómo la protección de semillas o frutos con recubrimientos semipermeables de materiales de base quitosano, son capaces de aislar al producto del ataque de los hongos extendiendo su tiempo de almacenaje. Por ejemplo, en semillas de *Jatropha curcas* se ha probado un recubrimiento de quitosano de bajo PM, encontrando que más de un 60% de semillas germinadas fueron protegidas del ataque del hongo (Pabón-Baquero et al. 2015). Un recubrimiento de *aloe vera* con quitosano extendió la vida post cosecha de mora azul más de 5 días, el recubrimiento consiguió aislar a la fruta del ataque de *Botrytis cinerea* (Vieira et al. 2016). También se han encontrado resultados efectivos en *Carica papaya* al aplicar un film de quitosano y almidón, tras 15 días de almacenamiento a temperatura ambiente, la calidad en la piel y pulpa de la papaya se mantuvieron intactas, mientras que sin el film solo se mantienen 5 días (Escamilla-García et al. 2018). Más pruebas en mango (Chowdappa et al. 2014), kiwi y lechuga (Fortunati et al. 2017), zanahorias, manzanas, fresas, frambuesas, tomates (Gutiérrez-Martínez et al. 2016) y otros cultivos agrícolas tratados con recubrimientos basados en quitosano han registrado efectos exitosos preservándolas de los efectos negativos causados por hogos fitopatógenos.

1.4.2 Enfermedades agrícolas y forestales

Las enfermedades causadas por hongos fitopatógenos son las principales causantes de grandes pérdidas económicas asociadas a la producción agrícola y forestal. En la tabla 3 se describen algunos de los hongos más devastadores de diferentes cultivos de interés económico y ecológico a nivel mundial como el café, la madera de chopo y diversidad de coníferas.

Tabla 3. Algunos de los hongos fitopatógenos de especies agrícolas y forestales más importantes a nivel mundial por los daños que causan

Especie/División	Localización	Hospedero	Patología	Referencia
Fusarium circinatum Ascomiceto	Nivel mundial	Coníferas. Más de 60 especies de *Pinus* susceptibles	Chancro de coníferas. La más grande amenaza de bosques de pinos a nivel mundial	Martínez-Álvarez 2015
Diplodia pinea Ascomiceto	Nivel mundial	Coníferas.	Chancro de coníferas. Causa chancros en el tronco de los árboles, desecación	Martínez-Álvarez 2015

Especie/División	Localización	Hospedero	Patología	Referencia
		Pinus radiata la especie más susceptible	en los brotes, inhibición de la germinación	
Gremmeniella abietina Ascomiceto	Europa, Norte América y Japón	Coníferas. Como picea, abeto, alerce, pino y enebro	Acículas secas, defoliación de la copa, distorsión de ramillos terminales y eventualmente la muerte de algunos pies	Romeralo 2015
Cryphonectria parasítica Ascomiceto	Asia, Europa y Norte América	Especies del género *Castanea*	Chancro del castaño. Chancros en el tronco, ramas o brotes que obstruyen la circulación de savia, desecando los brotes o ramas encima de la lesión	Zamora et al. 2012
Heterobasidium annosum Basidiomiceto	Hemisferio norte	Coníferas	Pudrición de las raíces y el pie de coníferas. Presenta crecimiento anormal de las acículas, corteza amarilla y pálida o marchitez general	Prieto-Recio et al. 2014
Hemileia vastatrix Basidiomiceto	Nivel mundial	Especies del género *Coffea*. Principalemente cultivos de *C. arabica*	Roya de las hojas del café. Manchas color naranja intenso sobre las hojas, que causan su pudrición.	Cristancho et al. 2012
Trametes versicolor Basidiomiceto	Nivel mundial	Madera estructural. Diferentes especies (duras y blandas)	Pudrición blanca de la madera, la degradación más importante y extendida	Spavento 2015
Phytophthora ×alni Oomiceto	Europa	Alisos	Muerte del aliso común. Desecación del árbol, hojas amarillentas en la copa, exudados enmohecidos a la altura del cuello y parte baja del tronco	Érsek and Nagy 2008
Phytophthora camvibora Oomiceto	Europa	Castaño, hayas y otras maderas duras	Tinta del castaño. Coloniza las raíces más pequeñas e infecta el tronco consiguiendo matar al árbol rápidamente	Jung et al. 2005
Phytophthora plurivora Oomiceto	Austria, Alemania, Rumania y España	Alisos	Chancro y pudrición del tronco. Afecta la parte aérea del árbol, cuello y parte baja del tronco, aunque se encuentra en el suelo, en la zona radicular	Haque et al. 2014

2. Marco de referencia y estructura de la tesis

El control con productos agroquímicos ha sido la estrategia principal para prevenir pérdidas debidas a las enfermedades de los cultivos desde los años de la posguerra (1960); esto ha dado lugar a un gran crecimiento de la agricultura y ha contribuido al desarrollo económico, reduciendo las enfermedades endémicas de los cultivos y protegiendo o restaurando plantaciones, bosques y productos de madera cosechados (Orzali et al. 2017). Sin embargo, el uso excesivo de estos productos, también ha traído grandes inconvenientes, como el impacto negativo en el medio ambiente y la salud humana, entre otros específicos como la resistencia de los microorganismos a estos productos.

Existen en el mundo más de 1 000 productos plaguicidas, utilizados en las distintas etapas de la producción de alimentos, para evitar pérdidas causadas por microorganismos, insectos o hierbas (OMS 2018). Se estima que solo en el año 2016, la utilización de estos productos a nivel global alcanzó más de 4 116 832 toneladas, de las cuales 357 578 corresponden a la aplicación de fungicidas y bactericidas (FAO 2016). En Europa, los países de mayor consumo de productos fungicidas y bactericidas durante el 2016 fueron España (38 919 t), Italia (36 791 t), Francia (31 900 t), Turquía (18 325 t) y Alemania (12 140 t), seguidos de Portugal y otros países con menos de 10 000 t (Eurostat 2016).

En España, de las 38 919 t consumidas en productos fungicidas y bactericidas, el 84,4% (equivalente a 32 843 t) corresponden a productos inorgánicos; únicamente el 0,036% (14 t) está representado por productos de origen microbiológico o botánicos (MAPAMA 2017). El uso extendido de los productos agroquímicos, como son los fungicidas inorgánicos, se debe en gran parte a los relativos precios bajos en el mercado, su fácil aplicación, así como al amplio espectro de acción que cubren (Orzali et al. 2017).

No obstante, el control químico presenta desventajas directamente relacionadas con su aplicación, como la resistencia de los organismos y que las regulaciones de su uso son cada vez más restrictivas (Hirooka & Ishii 2013). Por lo tanto, los programas integrados de manejo de plagas deben enfocarse cada vez más a la implementación de productos de origen microbiológico o botánico, productos de menor riesgo como los basados en quitosano.

El creciente interés por agentes de control biológico o productos de origen botánico que actúen como antifúngicos para el control de enfermedades, para evitar los impactos ambientales y en la salud humana de los productos convencionales, ha llevado al desarrollo de una serie de alternativas diversas. Aunque su producción y consumo aún representan una proporción minúscula, porque presentan algunos inconvenientes principalmente a nivel de producción, como la producción continua, el control de la calidad y los costes de producción.

En el caso del quitosano, todos los estudios muestran diferentes resultados sobre su actividad contra hongos, por lo que debería evaluarse su efecto en cada uno de ellos para determinar las dosis y formas de aplicación ideales. Incluso, no se ha dilucidado el mecanismo de acción específico por el que ejerce su actividad en cada caso y es necesario continuar investigando a nivel biológico para poder hacer más eficiente su aplicación. Por otra parte, respecto a los costes de producción, aunque es un producto comercializado a nivel mundial con precios asequibles según su calidad, los métodos de síntesis para la obtención de productos óptimos que puedan ser aplicados directamente en campo aún se encuentran es fases experimentales. No obstante, la perspectiva de uso en las próximas décadas es muy prometedora.

La presente tesis doctoral se compone de la compilación de 7 artículos. El primero es una revisión sobre el estado del arte de la síntesis de nuevos compuestos basados en quitosano, que incluyen extractos naturales y nanopartículas metálicas, entre otros, sintetizados mediante la utilización de nuevas tecnologías; se revisan también sus aplicaciones como antimicrobiano de diversos materiales que requieren ser preservados del ataque de microorganismos patógenos. Posteriormente se incluyen 6 artículos de resultados experimentales. En el artículo 1, se realizó por primera vez un análisis molecular de la N-acetil-D-glucosamina (el monómero constituyente de la quitina y el quitosano) por combinación de técnicas de espectroscopía de THz-TDS y FTIR con estudios teóricos basados en métodos semiempíricos de química cuántica. En el artículo 2, se propuso el diseño y la síntesis de un nuevo material compuesto a partir de oligómeros de quitosano, óxido de grafeno reducido y nanopartículas de plata, a través de una técnica asistida por microondas; consiguiendo un material funcionalizado y estable, como evidencia la caracterización realizada por UV-Vis, FTIR, XRD y TEM. En los artículos 3 y 6 se prepararon nuevos materiales compuestos a partir de quitosano, oligómeros de quitosano, propóleo y nanoplata. Estos composites fueron caracterizados por FTIR y se llevaron a cabo estudios *in vitro* e *in vivo* para evaluar su efectividad contra hongos fitopatógenos de interés agrícola, forestal y de la industria de la madera. En el artículo 4, se consiguió controlar con éxito al hongo *Hemileia vastatrix* causante de grandes pérdidas económicas por la enfermedad de la roya de las hojas del café; en el artículo 5 se controló a *Fusarium circinatum* causante del chancro resinoso de los pinos, el hongo más dañino de bosques de coníferas a nivel mundial; y en el artículo 6 a *Trametes versicolor* causante de la pudrición blanca de la madera.

Lista de Artículos Originales

Revisión: Eco-friendly nanocomposites of chitosan with natural extracts, antimicrobial agents and nanometals

Artículo 1: A Study of the Far Infrared Spectrum of N-Acetyl-D-Glucosamine Using THz-TDS, FTIR, and Semiempirical Quantum Chemistry Methods

Artículo 2: Silver Nanoaggregates on Chitosan Functionalized Reduced Graphene Oxide using Microwaves Radiation

Artículo 3: Potential control of forest diseases by solutions of chitosan oligomers, propolis and nanosilver

Artículo 4: Control of coffee leaf rust by chitosan oligomers and propolis

Artículo 5: Application of Bioactive Coatings Based on Chitosan and Propolis for *Pinus* spp. Protection against *Fusarium circinatum*

Artículo 6: Chitosan-based coatings to prevent the decay of *Populus* spp. wood caused by *Trametes versicolor*

3. OBJETIVOS

El quitosano es un material prometedor para ser usado como agente de control de enfermedades causadas por hongos fitopatógenos, con un amplio espectro de aplicaciones, en su uso individual, a través de modificaciones en su estructura o formando materiales compuestos con otras especies químicas. Sin embargo, aún existen inconvenientes que limitan su uso comercial en superficies agrícolas, forestales y en madera estructural. Por lo cual, el objetivo principal de este trabajo es potenciar la actividad antifúngica del quitosano adicionando propóleo y nanoplata, evaluar técnicas de síntesis más rápidas y eficientes, y determinar la concentración y forma de aplicación más efectiva para cada patógeno estudiado.

Los objetivos específicos de este trabajo son:

I. Determinar el estado del arte sobre la síntesis y características del quitosano en el desarrollo de nuevos nanomateriales sostenibles, para aplicaciones avanzadas basadas en el ensamblaje del quitosano con extractos naturales, agentes antimicrobianos y nanometales. [**Revisión**]

II. Caracterizar una de las moléculas constituyente de las cadenas polímericas del quitosano, la N-acetil-D-glucosamina, a través de espectroscopía infrarroja lejana (Far-FTIR), por terahercios (THz–TDS) y cálculos semiempíricos de química cuántica. [**Art. 1**]

III. Sintetizar un material compuesto de nanopartículas de plata a base de oligómeros de quitosano y óxido de grafeno reducido a través de técnicas de microondas. [**Art. 2**]

IV. Sintetizar un material compuesto a base de oligómeros de quitosano, extracto etanólico de propóleo y nanopartículas de plata a través de técnicas de ultrasonidos. [**Art. 3**]

V. Determinar la actividad antifúngica y las concentraciones mínimas inhibitorias (MIC) de quitosano de medio PM, oligómeros de quitosano, extracto etanólico de propóleo y nanopartículas de plata, individualmente y de los materiales compuestos en mezclas binarias y ternaria, contra el hongo causante de la pudrición blanca de la madera *Trametes versicolor*. [**Art. 6**]

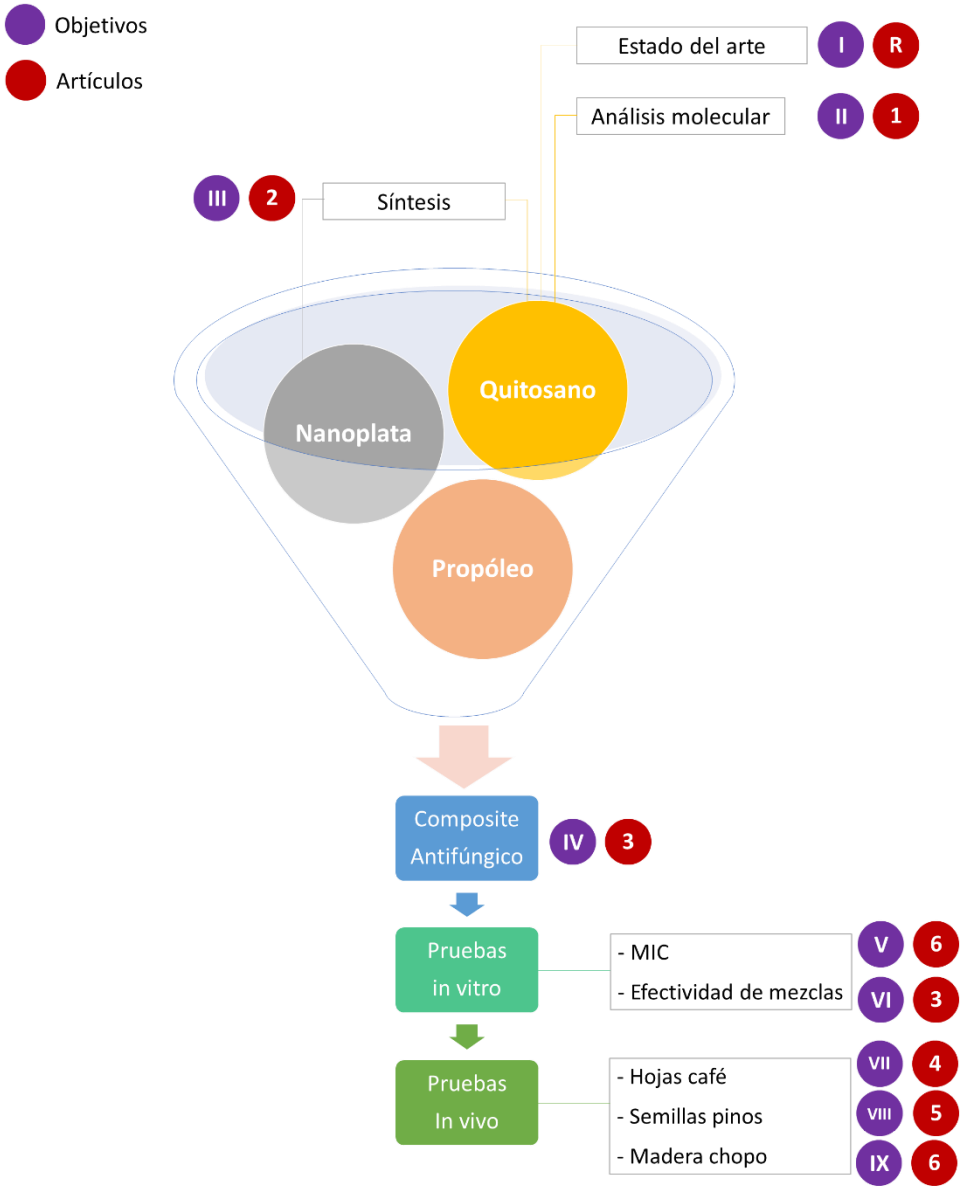
VI. Evaluar la actividad antifúngica y antioomicetos de los composites de oligómeros de quitosano, extracto etanólico de propóleo y nanopartículas de plata, en sus combinaciones binarias y ternaria, así como individualmente frente a ocho patógenos forestales. [**Art. 3**]

VII. Evaluar la capacidad de las soluciones de oligómeros de quitosano, incorporando o no, extracto etanólico de propóleo, para el control de la enfermedad de la roya de café causada por *Hemileia vastatrix*. [**Art. 4**]

VIII. Evaluar la protección conferida por recubrimientos bioactivos basados en quitosano de medio y bajo PM y en extracto etanólico de propóleo, a semillas de *Pinus radiata* y *Pinus silvestrys* frente a *Fusarium circinatum*. [**Art. 5**]

IX. Evaluar el efecto del quitosano de medio PM, de los oligómeros de quitosano y su combinación con el extracto etanólico de propóleo y las nanopartículas de plata, como protector superficial para prevenir la degradación de madera de *Populus* spp, frente a *Trametes versicolor*. [**Art. 6**]

Representación esquemática de los objetivos de la tesis y los artículos del compendio



4. MATERIALES Y MÉTODOS

El conjunto de materiales, equipos y softwares, así como las técnicas de síntesis, caracterización, ensayos y análisis estadísticos utilizados para llevar a cabo los estudios presentados en los diferentes artículos del compendio que compone la presente Tesis se muestran a modo de resumen en esta sección.

4.1 Reactivos, material biológico, equipos y softwares

Reactivos:

- N-acetil-D-glucosamina (NAG) (Art. 1)
- Quitosano de peso molecular medio (60-130 y 140-300 kDa) (Art. 2, 3, 4, 5 y 6)
- Óxido de grafeno reducido (rGO) (Art. 2)
- Propóleo de la Cuenca del Duero (Burgos, España) (Art. 3, 4, 5 y 6)
- Nitrato de plata (No. CAS 7761-88-8) (Art. 2, 3 y 6).
- Todos los demás reactivos utilizados para las síntesis fueron de grado analítico.

Hongos:

- Aislados de *Fusarium circinatum*, *Diplodia pinea*, *Gremmeniella abietina*, *Phytophthora ×alni* y *Phytophthora plurivora* provistos por el Laboratorio de Patología Forestal de la Universidad de Valladolid (España) (Art. 3).
- Aislados de *Cryphonectria parasítica*, *Heterobasidion annosum*, *Phytophthora cambivora* provistos por el Centro de Salud Forestal de Calabazanos - JCyL (España)
- Urenidiosporas de *Hemileia vastatrix* provistas por el Laboratorio de Micología de Universidad de Federal de Viçosa (Brasil) (Art. 4).
- Aislado de *Trametes versicolor* provisto por la Colección Española de Cultivos Tipo (Valencia, España) (Art. 6).

Material vegetal:

- Hojas de *Coffea arabica* (var: Caturra) provistas por el Laboratorio de Micología de Universidad de Federal de Viçosa (Brasil) (Art. 4).
- Semillas de *Pinus sylvestris* provistas por el Vivero El Serranillo del Ministerio de Agricultura y Medio Ambiente (España) (Art. 5)
- Semillas de *Pinus radiata* provistas por la Conserjería del Medio Rural de la Xunta de Galicia (España) (Art. 5).
- Probetas de madera de *Populus euroamericana* clon I-214 por el Laboratorio de Tecnología de la Madera de la Universidad de Valladolid (España) (Art. 6).

Equipos:

- Espectrómetro THz-TDS Tera K15 de Menlo para el análisis de NAG (Art. 1)
- Espectrómetro FTIR Nicolet iS50 de Thermo Scientific equipado con un sistema de Reflexión Total Atenuada (ATR) de diamante, para análisis de estructura química de la NAG, composites y degradación de la madera (Art. 1, 2, 3 y 6)
- Espectrómetro UV-Vis Shimazdu UV-2450 para el análisis de la nanoplata (Art. 2)
- Difractómetro de Rayos X Bruker D8 Advance Bragg-Brentano para caracterización de composite (Art. 2)
- Microscopio Electrónico de Transmisión (TEM) JEOL JEM-FS2200 HRP equipado con una sonda INCA Energy TEM 250 EDS de Oxford Instruments para caracterización de composite (Art. 2)
- Microondas Milestone Ethos-One para síntesis de composites (Art. 2)
- Sonicador model CSA 20-S500 para síntesis de composites (Art. 2, 3, 5 y 6)
- Espectrómetro UV-Vis Multiscan Go Microplate de Thermo Scientific para el análisis de TPC y RSA en plántulas (Art. 5)
- Microscopio óptico de transmisión Leica DMLM para análisis de degradación de la madera (Art. 6)

Softwares:

- PM6 software para análisis molecular de NAG (Art. 1)
- R software, para análisis estadísticos (Art. 3, 4, 5 y 6)

4.2 Síntesis de composites

Materiales individuales:

- Quitosano de medio peso molecular (QMPM) disuelto en ácido acético (1%) (Art. 2, 3, 4, 5 y 6)
- Oligómeros de quitosano (OQ) obtenidos por degradación oxidativa con H_2O_2 (Art. 2, 3, 4, 5 y 6)
- Extracto etanólico de propóleo (EEP) en solución hidroalcohólica 7:3 v/v (Art. 3, 4, 5 y 6)
- Nanopartículas de plata (nAg) por reducción con citrato de sodio y $NaBH_4$ con sonicación (Art. 2)
- Nanopartículas de plata con quitosano como agente reductor y estabilizante en autoclave (Art. 3)
- Nanopartículas de plata por reducción con citrato de sodio en tratamiento térmico (Art. 6).

Composites:

- Composite de OQ-rGO-nAg por microondas a 60 y 120 °C (Art. 2)
- Mezclas binarias y ternaria de OQ, P y nAg por cortos ciclos de sonicación, mezclando las soluciones individuales a las concentraciones deseadas (Art. 3, 4, 5 y 6)

4.3 Pruebas *in vitro*

- Concentración mínima inhibitoria (MIC) de QMPM, OQ, EEP, nAg, y mezclas obtenidas por pruebas de difusión en agar para *T. versicolor* (Art. 6)
- Efectividad de OQ, EPP, nAg y sus combinaciones contra los ocho patógenos forestales, evaluada por difusión en agar a las MIC de la prueba anterior (Art. 3)
- Efecto del OQ, EPP y OQ-EPP en la germinación de las urenidiosporas de *H. vastatrix* evaluado en medio líquido (Art. 4).

4.4 Pruebas *in vivo*

- Efecto del OQ, EEP y OQ-EPP, en el desarrollo de *H. vastatrix* evaluado sobre discos de hojas de café en tres distintas aplicaciones (Art. 4)
- Efecto de QMPM, OQ, EEP, QMPM-EPP y OQ-EPP evaluado como recubrimientos bioactivos de semillas de *P. silvestrys* y *P. radiata* (Art. 5)
- Efecto del QMPM, OQ, OQ-EEP y OQ-EEP-nAg evaluado como protector superficial de madera de *Populus* spp (Art. 6)

4.5 Análisis estadístico

- Análisis de varianza (ANOVAs) y de comparación múltiple (Art. 3, 4, 5 y 6)
- Procedimientos generalizados de Welch (Art. 3, 6)
- Pruebas de Kruskal-Wallis (Art. 4)
- Prueba Chi cuadrado (χ^2), Kaplan-Meier y de Tukey (Art. 5)
- Prueba de Shapiro-Wilks, de Bartlett y Bootstrapping (Art. 6)

5. RESULTADOS PRINCIPALES

En este apartado se presentan los resultados más destacados de la revisión y de cada uno de los artículos que componen el compendio de publicaciones, organizados por el tipo de información que aportan: estudios teóricos y estudios prácticos. Por ejemplo, el estudio de la estructura y formación de nuevos nanocompuestos a partir de quitosano, el análisis molecular del monómero constituyente de sus cadenas poliméricas, la NAG; la síntesis y caracterización de composites basados en oligómeros de quitosano, ya sea con rGO, EEP o nAg; así como los datos descubiertos en las aplicaciones *in vitro*: las MIC para *T. versicolor*, la efectividad de las combinaciones de los materiales antifúngicos individuales para ocho patógenos forestales y la efectividad de OQ y EEP -solos y en mezcla- en la germinación de esporas de *H. vastatrix*. Finalmente, se presenta una de las aportaciones más importantes de esta Tesis en el ámbito de sus aplicaciones directas en la ingeniería agrícola y forestal: el efecto de los productos contra enfermedades como la roya de las hojas del café, el chancro de los pinos o la pudrición blanca de la madera evaluados *in vivo*.

5.1 Análisis molecular, síntesis y caracterización de composites

Nanomateriales basados en quitosano (Review)

El carácter alcalino del quitosano, debido a la presencia de grupos amino, especialmente en los oligómeros, brinda una mayor tendencia para formar enlaces con biomoléculas como la nisina o liposomas; con extractos naturales como propóleo, tomillo, canela o vainillina; incluso con minerales, metales o productos de síntesis como sílice, nanoplata o polivinilpirrolidona (PVA). Estos nuevos materiales pueden sintetizarse a través métodos bilógicos como hidrólisis enzimática; físicos como microondas, ultrasonidos o radiación; y químicos como copolimerización, hidrólisis ácida, catálisis, etc. Todos estos materiales pueden ser de gran utilidad en aplicaciones para biomedicina, alimentos, medio ambiente, preservación del patrimonio y agricultura, principalmente.

N-acetil-D-glucosamina (Art. 1)

El espectro infrarrojo lejano de la N-acetil-D-glucosamina (NAG) generado por Far-FTIR y THz-TDS muestra un pico dominante a 60 cm^{-1} y dos más, a 45 y 72 cm^{-1} aunque de mucha menor intensidad en la zona de los THz. En la zona de FTIR se encontraron resonancias más definidas e intensas, las cuales se corresponden cercanamente a las frecuencias teóricas encontradas a partir del método PM6, un método semiempírico de química cuántica. El espectro vibracional obtenido muestra bandas adicionales no registradas en la literatura.

Síntesis y caracterización de rGO-OQ-nAg (Art. 2)

El material compuesto de óxido de grafeno reducido (rGO), oligómeros de quitosano (OQ) y nanopartículas de plata (nAg), obtenido exitosamente por un sencillo proceso de síntesis asistido por microondas a 60°C y 120 °C, fue caracterizado por FTIR, UV-vis, XRD y TEM.

A través de cambios en las bandas de absorción del espectro FTIR de los OQ se observa la presencia del rGO y de la nAg en el material compuesto. Por ejemplo, un pico intenso a 1636 cm^{-1} y otro más débil 1412cm^{-1}, denotan enlaces C=O y C-O de grupos carboxilo o alcoxi y la amida. La espectroscopía UV-vis revela más claramente la formación del material compuesto, debido a que el plasmón de la nanoplata, registrado a 420 nm, permanece en los composites, aunque en menor intensidad. El estudio por XRD muestra un espectro donde se identifican los tres componentes del material, un banda a $2\theta \approx 21°$ pude asignarse a los OQ, a 24° al rGO y otra bastante intensa a 38° corresponde a la nAg de acuerdo a la comparación con un patrón de referencia. En las micrografías por TEM se pudo observar que las Ag NPs son esféricas, tienen un diámetro entre 10 y 30 nm y se observan incrustadas en la matriz de hojas de rGO y OQ. La temperatura no muestra diferencias significativas en las características de los composites, por tanto 60°C son suficiente para el proceso de síntesis.

Caracterización de OQ, OQ-EEP, OQ-EEP-nAg (Art. 3)

La interacción de los oligómero de quitosano con los grupos funcionales del propóleo y la quelación de las nanopartículas de plata, fue evaluada a través de FTIR, comparando el espectro individual de OQ, el de la mezcla binaria OQ-EEP y la ternaria OQ-EPP-nAg. El primer espectro (OQ) mostró las bandas características de absorción del quitosano (a 3256 cm^{-1} las bandas sobrelapadas del O-H y N-H, entre otras variadas vibraciones entre C y O en la zona de la huella dactilar de 1600 cm^{-1} a 800 cm^{-1}). Los enlacen entre OQ y EPP se evidenciaron con picos más altos en la huella dactilar y un desplazamiento de 1257 cm^{-1} a 1263 cm-1 asociado al $\nu(C_{\Phi}\text{-}O)$ debido a enlaces por puentes de hidrógeno. Respecto a la nAg se encontraron ligeros decrecimientos en algunas bandas, sin embargo, no se observó un esperado desplazamiento en un pico asociado a la amina del OQ, lo que hubiera indicado un fuerte enlace, por el contrario se evidencia una débil interacción.

5.2 MIC y efectividad *in vitro*

Concentración mínima inhibitoria (Art. 6)

El trabajo experimental de esta tesis respecto el efecto antifúngico inició con la evaluación de la concentración mínima inhibitoria (MIC), es decir, la concentración que alcanza el 100% de inhibición del crecimiento del micelio, de quitosano de mediano PM (QMPM), oligómeros de quitosano (OQ), extracto etanólico de propóleo (EEP) y nanopartículas de plata (nAg), individualmente y en mezclas binarias y ternarias contra el hongo *T. versicolor*.

La MIC para OQ fue de 3 mg·mL^{-1}, para EPP de 0,5 mg·mL^{-1} y para nAg de 3 µg·mL^{-1}. En el caso de QMPM la concentración más alta aplicada (10 mg·mL^{-1}) inhibió el 95,5 % del crecimiento del micelio. Las mezclas binarias de OQ, EPP y nAg mostraron un efecto mejorado respecto a los productos individuales, pues se alcanzó el 100% de inhibición con concentraciones de 2,0 y 0,2 mg·mL^{-1} para OQ-EEP (respectivamente) y 2 µg·mL^{-1} para nAg en la mezcla ternaria.

Efecto antifúngico y antioomicetos contra patógenos forestales (Art. 3)

Se evaluaron *in vitro* soluciones de OQ (1 mg·mL^{-1}), EEP (0,1 mg·mL^{-1}) y nAg (1 µg·mL^{-1}), individualmente y en mezclas, para controlar el crecimiento de diferentes hongos y oomicetos fitopatógenos de árboles de interés forestal. Estas dosis fueron elegidas a partir del análisis de la MIC del estudio anterior.

La solución ternaria no presentó un efecto mejorado sobre las mezclas binarias OQ-EEP y OQ-nAg, las cuales tuvieron el mayor efecto antifúngico contra los hongos *Fusarium circinatum* (con 82% de inhibición) y *Diplodia pinea* (con 77% de inhibición), respectivamente. La solución de OQ por sí misma también resultó muy efectiva contra los hongos *Gremmeniella abietina*, *Cryphonectria parasitica* y *Heterobasidion annosum*, causando una inhibición del 78%, 86% y 93% respectivamente, además de un 100% en el oomiceto *Phytophthora cambivora*. Por otro lado, el EEP inhibió totalmente (100%) el crecimiento de *Phytophthora ×alni* y *Phytophthora plurivora*. Por su parte, la aplicación de nAg individualmente redujo el crecimiento micelial de *F. circinatum* y *D. pinea*, sin embargo, las mezclas binarias y ternaria con nAg, tuvieron un efecto contraproducente en la actividad antifúngica y anti-oomiceto sobre algunos patógenos.

Efecto sobre la germinación de esporas de *H. vastratrix* (Art. 4)

Soluciones acuosas de OQ y EEP, individualmente o como mezclas binarias, se probaron contra *Hemileia vastatrix*, el hongo que causa la roya de las hojas de café, la peor enfermedad del café a nivel mundial. En este estudio fueron aplicados únicamente los OQ y EEP, debido a los mejores resultados observados en el análisis anterior. Los resultados muestran una excelente actividad antifúngica de los OQ a 2 mg·mL^{-1}, pues la germinación de esporas de *H. vastatrix* fue inhibida en un 99,5%. En el caso del EEP, con una dosis 10 veces menor (0,2 mg·mL^{-1}) el resultado fue la mitad de efectivo que los OQ, con 54,4% de inhibición. De tal forma que en la mezcla de OQ-EPP se observa una ligera reducción del efecto de los oligómeros solos, con 96,6% de inhibición.

5.3 Control de enfermedades de especies agrícolas, forestales y madera

Aplicación en hojas de café (Art. 4)

La efectividad de las soluciones de OQ, EEP y su mezcla para evitar los daños que causa la enfermedad de la roya del café (CLR) fue evaluada sobre discos de hojas con ensayos en

tres diferentes tratamientos. En el primer tratamiento (inoculación del patógeno y 24 horas después aplicación de soluciones) el patógeno causó daños sobre un 12% del disco y no se observaron diferencias significativas con los tratamientos. En el segundo tratamiento (aplicación de soluciones y 24 horas después inoculación) se encontraron excelentes resultados, las soluciones de OQ, EEP y OQ-EEP presentan una protección total de los discos, pues los daños causados por el hongo 30 días después de la inoculación representan solo 0,2%. En el tercer tratamiento (aplicación de soluciones a la vez que la inoculación) también se encontraron resultados positivos con la aplicación de las soluciones respecto al control, el cual registra la afectación más alta (20% del disco). En este caso, el EEP fue menos efectivo que los OQ y la mezcla.

Recubrimientos bioactivos (Art. 5)

En este estudio, semillas de *P. sylvestris* y *P. radiata* fueron recubiertas con tratamientos de QMPM, OQ, EEP y las mezclas de ambos quitosanos con EPP, para evaluar su potencial como protectores de estas semillas ante la presencia de *F. circinatum* en el sustrato de cultivo. En una evaluación previa, en sustrato no inoculado, se encontró que los recubrimientos no afectan la germinación de las semillas, excepto por los recubrimientos de QMPM en *P. sylvestris* y de OQ-EEP en *P. radiata*, que la reducen ligeramente. Todos los recubrimientos presentaron un impacto positivo en *P. sylvestris*, especialmente QMPM, OQ y QMPM-EEP con más de 50% de sobrevivencia de plántulas germinadas de semillas recubiertas respecto a las no protegidas, las cuales murieron a los 40 días después de la inoculación. Este efecto se comprobó en el contenido total de polifenoles (TPC) y en la actividad antioxidante (RSA) de las plántulas, las cuales presentaron tasas similares a plántulas sanas (de semillas no inoculadas). En el caso de *P. radiata* la eficacia de los tratamientos fue limitada, aunque se sabe que es la especie más susceptible a este patógeno.

Protección de madera (Art. 6)

Los compuestos de QMPM, OQ, OQ-EEP y OQ-EEP-nAg se probaron en pequeños bloques de madera de chopo como protectores superficiales durante 30 días de exposición a *T. versicolor*, el hongo causante de la pudrición blanca. Con una toma de muestra cada 5 días, se fue registrando la pérdida de peso de los bloques, que alcanzaron un 42,3% en la madera no protegida. Por el contrario, todos los tratamientos exhibieron una protección debido a que la pérdida de peso fue menor, 11,4% en QMPM, 26,9% en OQ, 32,8% en OQ-P y 39,9% en OQ-EEP-nAg, de tal forma que el composite ternario es el único que no presenta diferencias significativas. El seguimiento de la degradación de la madera por microscopía óptica y espectroscopía FTIR, también evidenció que el QMPM es el mejor agente protector, probablemente debido a su mayor viscosidad y propiedades de adhesión.

6. DISCUSIÓN GENERAL

El quitosano se considera uno de los polímeros de origen natural más prometedores en la preparación de nuevos materiales nanocompuestos. Como se ha reportado en la revisión bibliográfica, el quitosano y sus oligómeros pueden enlazarse a diversas especies de origen natural o de síntesis; como propóleo (Matei et al. 2015), vainillina (Stroescu et al. 2015), aceites esenciales de canela (Ojagh et al. 2010) etc., o nanoplata (Venkatesham et al. 2012), cobre (Xue and Wilson 2016), sílice (Dabóczi et al. 2016) o PVA (Tahtat et al. 2011), entre otros. Además, la versatilidad de los materiales derivados del quitosano les permite ser procesados en geles (Jun et al. 2010), membranas (Santos et al. 2013), nanofibras (Pillai et al. 2009), esferas (Benamer et al. 2011), micropartículas, nanopartículas y bloques (Shukla et al. 2013) a través de varios métodos de síntesis que involucran procesos biológicos, químicos y físicos, por ejemplo, hidrólisis enzimática (Zou et al. 2016), co-polimerización (Thakur and Thakur 2014), irradiación gama (Taşkin et al. 2014), microondas (Ge and Ma 2015) o técnicas de ultrasonidos (Ho et al. 2016). Lo anterior, denota que actualmente existe un interés sustancial en la investigación para el desarrollo de nuevos nanomateriales de origen renovable, como los basados en quitosano.

El análisis molecular de la NAG reportado en el Artículo 1, ha sido realizado por primera vez por un método experimental a partir del espectro infrarrojo lejano (obtenido por Far-FTIR y THz-TDS), así como por un método teórico semiempírico de química cuántica (PM6), el cual mostró bandas adicionales en el espectro vibracional, no registradas en un estudio similar realizado por Kovacs et al. (2008). En comparación con los estudios previos basados en *ab inition* y la teoría funcional de la densidad (DFT), el enfoque semiempírico elegido en este trabajo, adecuado para la aceleración de la unidad de procesamiento gráfico (GPU) y de múltiples núcleos en paralelo, permite un estudio completo utilizando condiciones de frontera periódicas y no solo aproximaciones dentro de un tiempo de cálculo limitado. El conocimiento de las características vibracionales de esta molécula puede ayudar a predecir modos de acción antifúngicos específicos en estudios de estructura-actividad del quitosano.

La síntesis del material compuesto por rGO-OQ-nAg reportada en el Artículo 2, asistida por microondas y su caracterización, mostraron un método sencillo y adecuado para el auto-ensamblaje del rGO en la matriz polimérica de los OQ y la estabilización de la nAg, pues nanoagrados de este metal se observaron anclados uniformemente en las hojas de rGO funcionalizadas con OQ. Además, la principal ventaja encontrada en este composite es que presenta una forma sólida cristalina de textura grafénica y no de hidrogel, lo cual facilitaría y ampliaría el rango de sus aplicaciones (Jiao et al. 2015; Marta et al. 2015).

La caracterización por FTIR reportada en el artículo 3 de los composites OQ, OQ-EEP y OQ-EEP-nAg reveló la interacción entre los grupos funcionales del OQ y el EEP como se observa en estudios similares (Siripatrawan & Vitchayakitti 2016), no obstante, mostró una débil

interacción con la nAg, a diferencia de otros trabajos (Wang et al. 2015), probablemente debido a las bajas dosis de nAg utilizadas en este estudio.

La MIC reportada en el artículo 6 para *T. versicolor* muestra una mayor actividad antifúngica *in vitro* de OQ vs QMPM, así como un mejor desempeño de las mezclas binarias en comparación con los productos individuales (la inhibición completa del crecimiento del micelio se logró a concentraciones de 2 $mg \cdot mL^{-1}$ para OQ, 0.2 $mg \cdot mL^{-1}$ para EEP y 2 $\mu g \cdot mL^{-1}$ para nAg para cualquiera de las mezclas binarias). No obstante, no se encontraron mejoras significativas asociadas con el compuesto ternario sobre sus contrapartes binarias para este hongo; probablemente debido a que las nanopartículas metálicas tienden a asociarse fuertemente a moléculas orgánicas impidiendo su difusión al medio. Las MIC determinadas en este estudio se encuentran dentro de un rango de concentraciones semejantes a las dosis aplicadas en otros trabajos sobre la actividad antifúngica del quitosano (Badawy & Rabea 2009; Younes et al. 2014), el propóleo (Pastor et al. 2010; Ali et al. 2013) y la nanoplata (Wang et al. 2015; Kim et al. 2018), como de sus mezclas. Lo que denota la necesidad de evaluar la dosis óptima de cada producto según la especie.

La actividad contra microorganismos patógenos forestales reportada en el artículo 3, muestra que todas las soluciones individuales y las mezclas demostraron capacidad de inhibición del crecimiento micelial contra alguno de los microorganismos estudiados. Sin embargo, esta actividad antifúngica y anti-oomicetos mostró una dependencia del tipo particular de microorganismo. El tratamiento con la mayor actividad registrada fueron los OQ, que por sí solos presentaron tasas de inhibición del 78% al 100% contra 4 de los microorganismos, este efecto ha sido estudiado en otros hongos (Ahmed et al. 2001; Avelelas et al. 2014; Qiu et al. 2014; Cobos et al. 2015), no obstante, las dosis aplicadas pueden ser superiores a 1 $mg \cdot mL^{-1}$ utilizada en este estudio. La aplicación del EEP fue exitosa contra dos oomicetos (*P. ×alni* y *P. plurivora*), inhibió el 100% del crecimiento con solo 0,1 $mg \cdot mL^{-1}$; la actividad antibacteriana del propóleo es bien conocida en medicina tradicional, sin embargo, como antifúngico se ha estudiado contra pocas especies (Iturritxa et al. 2013). Por otra parte la nanoplata sí ha sido estudiada como antifúngica contra diversos hongos (Narayanan and Park 2014; Mahdizadeh et al. 2015), aunque en el presente estudio sólo presentó efectos significativos contra *F. circinatum* y *D. pinea*.

La capacidad antifúngica y anti-oomicetos de los OQ fue incrementada por las mezclas binarias en algunos casos, como OQ-EPP en *F. circinatum* y OQ-nAg en *D. pinea*, que alcanzaron tasas de inhibición alrededor de 80%. Sin embargo, la adición de un tercer componente no presentó un efecto mejorado sobre estas; contrario a la actividad contra *Diplodia seriata* (Matei et al. 2015) o *Bipolaris Oryzae* (Araujo-Rufino et al. 2016) donde el mejor efecto antifúngico fue observado al combinar OQ, EPP y nAg, aunque a dosis superiores a las aplicadas en este trabajo.

La evaluación *in vitro* del artículo 4, donde la capacidad de los OQ para inhibir la germinación de esporas de hongos fitopatógenos, se evidencia una vez más en este estudio

contra *H. vastatrix*, donde se ha encontrado una excelente opción para el tratamiento de la enfermedad causada por este hongo en las hojas de café. En un estudio llevado a cabo por Rahman et al. (2015), se ha observado una hinchazón de las esporas además de inclusiones granulares en el citoplasma de las células tras el contacto con oligómeros de quitosano, lo cual genera una interrupción del desarrollo del tubo germinativo, es decir, la inhibición de la germinación. El propóleo, otro producto de origen natural, también mostró una alta actividad antifúngica considerando la baja concentración utilizada, como en el caso de otros estudios (Silva-Carvalho et al. 2015) su efecto podría mejorarse aumentando la dosis de aplicación. La combinación de ambos productos (OQ-EPP) no presentó mejores resultados que el uso de OQ individualmente.

En el artículo 4 también se evalúa, la aplicación de las soluciones de OQ, EEP y su mezcla después de la inoculación del patógeno sobre los discos de hojas del café donde no causó un efecto positivo, debido a que la infección inicial del hongo puede efectuarse durante las primeras 24 horas de contacto con las hojas por procesos de adhesión y germinación (Cristancho et al. 2012), por lo tanto, al aplicar los tratamientos ya había empezado el proceso de infección. En el segundo tratamiento, cuando se aplican las soluciones de manera preventiva, se consiguió un excelente resultado con las 3 soluciones, lo cual sugiere que la aplicación de los productos genera un capa protectora sobre la hoja que bloquea la adhesión de las esporas e inhibe la germinación y por tanto evita el proceso de infección. Este resultado representa una herramienta de fácil acceso para ser implementada en los programas de manejo integrado, a la vez que se desarrollan especies más resistentes al patógeno y se investiga las características genéticas de la enfermedad (Toniutti et al. 2017; Silva et al. 2018). Finalmente, en el tercer tratamiento, cuando se aplican las soluciones al mismo tiempo que el inóculo, se observa una inhibición de la germinación similar al tratamiento *in vitro*, lo que se traduce en porcentajes de daño cercanos a 0% registrados para OQ y la mezcla ya sea por inhibición de la germinación de las esporas y/o por bloqueo de la adhesión de éstas en tejido vegetal. Este efecto de los oligómeros de quitosano fue reportado en ensayos similares contra patógenos de fresas (Rahman et al. 2015). Aunque con la aplicación de EEP la efectividad se redujo, su aplicación es efectiva respecto al control.

Artículo 5. Los recubrimientos de semillas a base de quitosano (de diferente PM) y propóleo, fueron aplicados para comprobar si estos productos bioactivos eran capaces de conferir resistencia contra *F. circinatum* a dos especies de pinos (*P. sylvestris* y *P. radiata*). Aunque no se encontró un efecto positivo en la germinación como se ha observado en otras plantas (Zeng et al. 2012; Peña-Datoli et al. 2016), los recubrimientos de quitosano y propóleo redujeron significativamente la mortalidad de las plántulas, principalmente en *P. sylvestris*, con tasas de sobrevivencia superiores al 50%. Demostrado también en los análisis del TPC y RSA, pues se ha reportado que el quitosano y el propóleo tienen la capacidad de inducir mecanismos de defensa a las plantas, como la producción de fenoles y compuestos antioxidantes (Liu et al. 2007; Badawy & Rabea 2009). Es este estudio, como recubrimientos

de semillas, no se observó que el quitosano de bajo PM tuviera mejores efectos que el quitosano de medio PM, como se sugiere en la mayoría de las aplicaciones (Kananont et al. 2010), excepto en el análisis de TPC, donde los OQ presentaron mejores resultados que el QMPM. Todo lo anterior sugiere que los recubrimientos propuestos son prometedores para la protección de plántulas de *P. sylvestris* contra PPC.

Artículo 6. La actividad del QMPM, los OQ, y los composites OQ-EEP y OQ-EEP-nAg, se evaluó en la protección superficial de madera, en una prueba rápida de degradación llevada a cabo durante 30 días de exposición al hongo *T. versicolor*, el hongo causante de la pudrición blanca. Esta prueba, mostró una tasa de degradación mayor en *Populus euroamericana* que en *Fagus sylvatica* expuesta durante el mismo tiempo al ataque del hongo, lo que denota una mayor susceptibilidad como lo reportan Jia-qi et al. (2005) y Spavento (2015). Por otra parte, la mejor actividad protectora del quitosano de mayor PM frente al de menor y sus composites, coincide con otros estudios sobre madera de *P. sylvestris* (Eikenes et al. 2005), aunque evidentemente este efecto es altamente dependiente de la concentración del producto aplicado (El-Gamal et al. 2016). Respecto a la desintegración de la madera causada por el hongo monitorizada mediante microscopía y espectroscopía vibracional, se evidencian las limitaciones de los recubrimientos a base de OQ en comparación con MMWC, que tiene una mayor viscosidad y mejores propiedades de adhesión. Por tanto el uso de MMWC es prometedor para la protección de la madera de chopo, con posibles aplicaciones industriales.

7. CONCLUSIONES

Los resultados obtenidos derivan en las siguientes conclusiones generales presentadas en el orden de los objetivos planteados según el desarrollo de la investigación.

Análisis molecular, síntesis y caracterización de composites

1. El quitosano puede considerarse como el polímero natural más prometedor para la preparación de nuevos materiales nanocompuestos. El estado del arte sobre su síntesis y características evidencia un interés sustancial en la investigación para el desarrollo de nuevos nanomateriales sostenibles, para aplicaciones avanzadas basadas en el ensamblaje, especialmente, de los oligómeros de quitosano con extractos naturales, agentes antimicrobianos y nanometales.

2. El análisis molecular de la NAG realizado, combinando por primera vez técnicas de espectroscopía infrarroja lejana (THz-TDS y FTIR) con estudios teóricos basados en métodos semiempíricos de química cuántica, presenta un pico dominante a 60 cm^{-1} en el espectro de terahercios y el espectro de alta resolución ATR-FTIR permitió la identificación de algunas bandas adicionales no reportadas en la literatura. Por su parte, los cálculos semiempíricos hamiltonianos de PM6, permitieron estudiar las vibraciones moleculares en un tiempo de cálculo reducido obteniendo una buena calidad y concordancia semicuantitativa entre los resultados calculados y los experimentos.

3. La síntesis de un material compuesto a base de OQ-rGO-nAg asistida por microondas, muestra un método novedoso, de fácil diseño y eficaz. Gracias a las caracterizaciones morfológicas de los compuestos obtenidos se observa la formación de nanoagregados de plata en las láminas del rGO funcionalizadas con OQ mediante un proceso de autoensamblaje. Las nAg aparecieron ancladas uniformemente en el rGO, lo cual reitera que el desarrollo de nuevos nanocompuestos multifuncionales sobre la base de los nanomateriales existentes es posible de una forma sencilla y precisa.

4. La síntesis y caracterización por FTIR de los composites sintetizados a partir de OQ con EEP y nAg, en mezclas binarias y ternaria, reveló la interacción entre los grupos funcionales de los oligómeros de quitosano y los polifenoles del propóleo en el compuesto binario OQ-EEP. Sin embargo, en la mezcla ternaria (OQ-EEP-nAg) se observó una débil interacción con la nAg, probablemente debida la bajas dosis de nAg utilizadas en este estudio.

Pruebas *in vitro*

5. Los estudios *in vitro* para determinar la actividad antifúngica y la MIC de QMPM, OQ, EEP y nAg individualmente, las mezclas binarias (OQ-EEP, OQ-nAg, EEP-nAg) y la ternaria (OQ-EEP-nAg) contra *T. versicolor*, revelaron una mayor actividad de los OQ respecto al QMPM, así como un mejor desempeño de las mezclas binarias en comparación con los productos

individuales. No obstante, no se encontraron mejoras significativas asociadas con el compuesto ternario.

6. Los resultados *in vitro* de los experimentos de la actividad antifúngica y anti-oomiceto de mezclas individuales, binarias y ternarias de OQ, EEP y nAg, analizadas contra ocho patógenos de especies forestales demuestran que la actividad inhibidora de los OQ contra hongos y oomicetos fue significativamente alta (alcanzando reducciones en la tasa de crecimiento desde 78% hasta 100% contra algunos patógenos); la actividad de los OQ se ve incrementada por la asociación con EEP (contra *F. circinatum*) y con nAg (contra *D. pinea*); y que el complejo ternario OQ-EEP-nAg no mejoró la actividad antifúngica y anti-oomicetos en comparación con las soluciones binarias.

7. Los OQ en solución (a 2 $mg \cdot mL^{-1}$) muestran una excelente capacidad para inhibir la germinación de las urenidiosporas *H. vastatrix* causante de CLR. Este efecto se ha comprobado también con el EEP (a 0,2 $mg \cdot mL^{-1}$), aunque presentó un porcentaje de inhibición menor, debido a la menor concentración aplicada. Al combinar ambas soluciones se encontró una reducción en la capacidad inhibitoria del quitosano.

Pruebas *in vivo*

8. La aplicación de OQ, EEP y su mezcla contra el desarrollo de CLR en discos de hojas de café mostraron un excelente efecto de inhibición en la germinación y crecimiento de *H. vastatrix*. Por lo tanto, representan una prometedora alternativa para el control de la enfermedad, principalmente cuando se aplican de forma preventiva, antes de la inoculación del patógeno, de lo contrario, cuando el hongo ha infectado las hojas, no se observa algún efecto.

9. La aplicación de recubrimientos bioactivos a base de quitosano y propóleo para proteger las semillas de especies de *Pinus* contra *F. circinatum* podría ser incluida en los programas de sanidad de viveros forestales. A pesar de que su eficacia contra el chancro del pino fue limitada en el caso de *P. radiata*, ésta ha sido reportada como la especie más susceptible a este patógeno. Por el contrario los recubrimientos redujeron significativamente la mortalidad de las plántulas de *P. sylvestris*, lo que dio como resultado tasas de supervivencia superiores al 50%, respecto a las plántulas de semillas no recubiertas. Los recubrimientos de las semillas también tuvieron una influencia positiva en el contenido fenólico total, lo que llevó a valores similares a los encontrados en semillas no inoculadas, y ayudó a preservar la actividad antioxidante de las plántulas.

10. El efecto protector de los compuestos basados quitosano (QMPM, OQ, OQ-EEP y OQ-EPP-nAg) como recubrimientos de madera de chopo, demostraron una posible aplicación comercial, principalmente el QMPM que debido a su mayor viscosidad y propiedades de adhesión, causó una mayor protección contra el hongo de pudrición blanca de la madera *T. versicolor*.

7.1 Conclusions

The general conclusions are presented in the same order of the proposed objectives, according to the development of the investigation.

Molecular analysis, synthesis and characterization of composites

1. Chitosan can be considered as one of the most promising natural polymer for the preparation of new nanocomposite materials. The state of the art on its preparation and chemical and physical characteristics clearly indicates a substantial interest of several research groups for the development of new sustainable nanomaterials for advanced applications based on the assembly, in particular of chitosan oligomers with natural extracts, antimicrobial agents and nanometals.

2. The molecular analysis performed for the nAg composites, combining for the first time techniques of far infrared spectroscopy (THz-TDS and FTIR) with theoretical studies based on semiempirical methods of quantum chemistry, showed the presence of a dominant peak at 60 cm^{-1} in the spectrum of terahertz, which correlates with the high-resolution spectrum obtained by ATR-FTIR; both spectra allowed for the identification of some additional bands not reported in the literature before. On the other hand, the semi-empirical Hamiltonian calculations using the semi-empirical method PM6, allowed to study the molecular vibrations in shorter time of calculation obtaining a good quality and semiquantitative agreement between the calculated results and the experiments.

3. The synthesis by a microwave-assisted method of a COs-rGO-nAg based composite material seem to be a novel, easy-to-design and efficient preparation technique. The morphological characterization of the obtained compounds showed the formation of silver nanoaggregates in the rGO sheets functionalized with COs through a self-assembly process. The nAg appeared uniformly anchored in the rGO, which reiterates that development of new multifunctional nanocomposites based on existing nanomaterials is possible in a simple and precise way.

4. The synthesis and characterization by FTIR of the composites synthesized from COs with PEE and nAg, in binary and ternary mixtures, revealed the interaction between the functional groups of the chitosan oligomers and the polyphenols of propolis in the binary compound COs-PEE. However, in the ternary mixture (COs-PEE-nAg) a weak interaction with nAg was observed, probably due to the low doses of nAg used in this study.

***In vitro* assays**

5. *In vitro* studies were carried out to determine the antifungal activity and MIC of MMWC, COs, PEE and nAg individually, as well as for the binary mixtures: COs-PEE, COs-nAg, PEE-nAg and the ternary systems: COs-PEE-nAg, against *T. versicolor*. These studies revealed a greater activity of the COs with respect to the MMWC, as well as a better performance of

the binary mixtures in comparison with the individual products. However, no significant improvements were found associated with the ternary systems.

6. The *in vitro* results of the antifungal and anti-oomycete activity of individual, binary and ternary mixtures of COs, PEE and nAg, assayed against eight pathogens of forest species, demonstrate that the inhibitory activity of COs against fungi and oomycetes was significantly high (reaching reductions in the growth rate from 78% to 100% against some pathogens); the activity of COs is increased by the association with PEE (against *F. circinatum*) and with nAg (against *D. pinea*); and the ternary complex COs-PEE-nAg did not improve the antifungal and anti-oomycete activity compared to the binary solutions.

7. COs in solution (at 2 $mg \cdot mL^{-1}$) show an excellent ability to inhibit the germination of *H. vastatrix* urenidiospores that causes CLR. This effect has also been demonstrated with the PEE (at 0.2 $mg \cdot mL^{-1}$), although it had a lower inhibition percentage, due to the lower concentration applied. By combining both solutions, a reduction in the inhibitory capacity of chitosan was found.

***In vivo* assays**

8. The application of COs, PEE and its mixture against the development of CLR on detached coffee leaf discs showed an excellent effect on germination inhibition and on growth of *H. vastatrix*. Therefore, they represent a promising alternative for the control of the disease, mainly when they are applied preventively, before the inoculation of the pathogen; otherwise, when the fungus has infected the leaves, no effect is observed.

9. The application of bioactive coatings based on chitosan and propolis to protect seeds of *Pinus* species against *F. circinatum* could be included in the health programs of forest nurseries. Although, its effectiveness against PPC was limited in the case of *P. radiata*, it has been reported as the species most susceptible to this pathogen. On the contrary, the coatings significantly reduced the mortality of the seedlings of *P. sylvestris*, which resulted in survival rates higher than 50%, compared to seedlings of uncoated seeds. The coatings of the seeds also had a positive influence on the total phenolic content, which led to values similar to those found in non-inoculated seeds, and helped to preserve the antioxidant activity of the seedlings.

10. The protective effect of the chitosan-based compounds (MMWC, COs, COs-PEE and COs-PEE-nAg) as poplar wood coatings, demonstrated a possible commercial application. Mainly the MMWC, which due to its higher viscosity and properties of adhesion caused greater protection against *T. versicolor*, the white rot fungus of wood.

8. Perspectivas de la investigación

Derivado del análisis de resultados del presente trabajo de Tesis doctoral, se han identificado tres principales líneas de investigación futuras. Las áreas de oportunidad en las que sería recomendable continuar haciendo investigaciones derivadas de este proyecto doctoral describen a continuación:

- *Evaluación contra nuevos patógenos*

De acuerdo al estado del arte de la actividad antimicrobiana del quitosano, el propóleo y las nanopartículas de plata, es bien sabido que estos productos actúan eficazmente contra una amplia cantidad de microorganismos patógenos. Además, en el presente trabajo, se demuestra el efecto antifúngico de los productos mencionados más sus combinaciones contra 10 distintos hongos fitopatógenos. Debido a los resultados positivos encontrados contra los hongos, durante el desarrollo de esta investigación se han realizado pruebas preliminares sobre los efectos del quitosano, el propóleo y la nanoplata, individualmente y en mezclas, sobre bacterias patógenas como *Bacillus subtilis*, *Escherichia coli*, *Enterobacter* sp, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* y *Staphylococcus aureus*; sobre las cuales se han observado efectos importantes (datos no presentados).

Continuar evaluando los efectos *in vitro* e *in vivo* de estos materiales contra otros patógenos (no estudiados previamente) es importante para seguir ampliando las posibilidades de uso industrial de estos productos y avanzar hacia el desarrollo de herramientas que permitan una agricultura más sostenible.

- *Pruebas en invernadero y en campo*

Debido a que el tema de la Tesis es de alta relevancia para la solución de problemas de sanidad en la agricultura, los bosques y la industria de la madera, además de las pruebas realizadas sobre material vivo, es importante continuar con evaluaciones de la actividad de los composites en ambientes naturales, invernaderos o cultivos.

En el caso de la aplicación de los productos como protectores de madera, es importante también seguir analizando otras técnicas de impregnación como los tratamientos a presión en autoclave, evaluaciones preliminares se han realizado con madera de *Populus* encontrando muy buenos resultados (datos no mostrados).

- *Análisis de ecotoxicidad*

El alto potencial de aplicación a escala industrial que presentan los materiales compuestos estudiados en este trabajo, implica la necesidad de evaluar previamente los posibles efectos que los productos presentarían en el ecosistema donde se apliquen. Realizar específicamente análisis sobre sus efectos en la microbiota del suelo, ayudaría a medir el grado de ecotoxicidad que podría representar su uso.

En análisis preliminares que se han realizado en este trabajo de investigación (datos no mostrados) sobre microorganismos de suelos agrícolas y forestales, se encontró que la aplicación de los productos causa un decrecimiento de las colonias presentes en el suelo, sin embargo, este efecto se pierde con el paso del tiempo.

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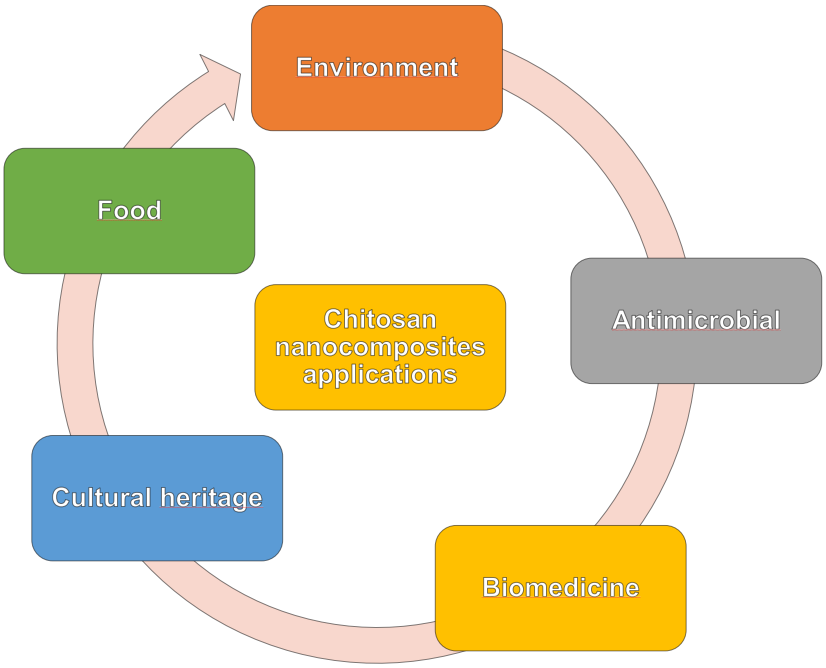
Artículos originales

Revisión

Nanocompuestos eco-amigables de quitosano con extractos naturales, agentes antimicrobianos y nanometales



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Chapter 3: Eco-friendly nanocomposites of chitosan with natural extracts, antimicrobial agents and nanometals

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Abstract

Chitosan is a natural polymer, the second most abundant after cellulose, that has antimicrobial and antifungal properties. Its activity and resistance can be increased by oligomer formation, by adhesion of biological products or liposomes, natural extracts rich in natural oils as polyphenols and phenolic acids, etc. or by synthetic methods such as the doping with silver nanoparticles. The state of the art of the synthesis of novel composites and the obtaining of biofilms that incorporate chitosan and natural drugs together with nanometals -using nanotechnologies; microwave, sonochemical and radiation techniques and enzymatic methods- is presented. In addition, their applications as adhesive substances and films for food protection, biomedicine, nanopesticides in agriculture, cultural heritage preservation and, in general, for any material that needs to be maintained and preserved from microorganisms and pathogens, are reviewed. The aim of the present perspective is to describe the recent advances in the novel synthesis methods of chitosan with particular emphasis on copolymerization with natural extracts (oils and vanillin) and natural antimicrobial agents (nisin) by synthetic methods such as the formation of hydrogels.

Keywords: antimicrobial; antifungal; biofilms; chitosan; composites; graft copolymerization; natural extracts; synthesis.

3.1. Introduction

Since 1990, in view of the depletion of oil resources, traditional synthetic polymers are being replaced by natural polymers, mainly cellulose derivatives, because of their biodegradability, eco-friendliness, easy availability and light weight (Thakur et al., 2014a; Thakur et al., 2011; Thakur & Thakur, 2015; Thakur et al., 2014b, 2013b, c).

The applications of these natural polymers in composites have attracted the attention of both researchers and manufacturers, since they pose an alternative to the exploitation of non-renewable resources and the mismanagement of agro-industrial wastes, thus avoiding environmental and ecological imbalances (Pappu et al., 2015).

Amongst the various renewable polymers, chitosan is one of the most important from a commercial perspective, provided that it features exciting properties that recommend it for a great variety of applications (Kim, 2010; Samal & Dubruel, 2014; Thakur & Thakur, 2014). Chitosan is the deacetylated form of chitin, which is the second most abundant polysaccharide in natural macromolecules, after cellulose, and that is a long-chain polymer comprising N-acetylglucosamine

(see Figure 3.1). It can be obtained from naturally occurring sources as the exoskeletons of insects, arthropods, beaks of cephalopods, including the shells of crustaceans, shrimp, prawns, lobsters, crabs, as well as cell walls of fungi and yeast (Jayakumar et al., 2010). However, the biggest obstacle to the application of chitin is its low water solubility and poor biodegradation performance (Pillai et al., 2009).

Chitin differs structurally from cellulose by the presence of acetamide groups (-$NHCOCH_3$) inserted in the C-2 (see Figure 3.1). Conversely, chitosan is composed of less than 20% of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranose and more than 80% of β-(1,4)-2-deoxy-2-amino-D-glucopyranose (Figure 3.1, *bottom*).

The main advantage of chitosan in comparison to cellulose and chitin is its versatility, since the deacetylation process releases amine groups (NH_2), providing chitosan with a highly alkaline character. In chitosan, the availability of reactive sites allows collateral bonding and the formation of aldimines and ketimines with aldehydes and ketones. For example, chitosan and aldehydes under hydrogenation originate N-alkyl chitosan, leading to the formation of films. Due to its biodegradability, biocompatibility and non-toxic properties, chitosan has been deemed a *biomaterial* (Kean & Thanou, 2010).

Moreover, in comparison to cellulose, chitosan has the advantage of its easy conversion into chitosan oligomers by applying a relatively mild reaction conditions (Dash et al., 2011). The degraded products have a smaller molecular weight and are readily soluble in aqueous solutions, affording oligosaccharides with valuable biological activity at the cellular or molecular level (Xia et al., 2013). Both chitosan and chitosan oligomers are also particularly interesting because of their antioxidant activity (Sashiwa & Aiba, 2004; Sun et al., 2007).

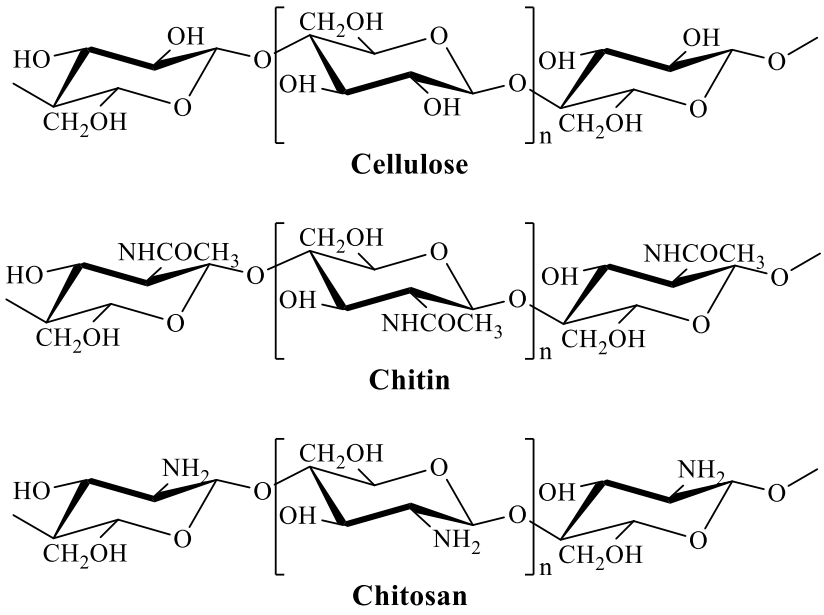

Figure 3.1. Structure of the polymer chains of cellulose (*top*), chitin (*center*) and chitosan (*bottom*).

To further improve chitosan properties, some studies have focused on the incorporation of natural or synthetic species to the chitosan polymer matrix or to the chitosan oligomers (López-Mata

et al., 2013; Mascheroni et al., 2014; Natrajan et al., 2015). For example, chitosan -either alone or blended with other natural polymers- is broadly used as an antimicrobial agent (Kong et al., 2010).

Chitosan can also be easily processed into gels, beads, membranes, nanofibers (NFs), nanoparticles (NPs) and scaffolds (Shukla et al., 2013) through several synthesis methods that involve biological, chemical and physical processes, such as enzymatic hydrolysis (Pan et al., 2016), graft polymerization (Thakur & Thakur, 2014) or gamma irradiation (Taşkın et al., 2014), to name a few.

Nanotechnologies have also allowed to boost the development of novel products or bio-nanomaterials based on chitosan, that -due to their enhanced properties- have found potential uses in the biomedical, pharmaceutical, food, agricultural, conservation and environmental fields (Zou et al., 2016). Among these, biomedical applications have been the most widely studied, for instance in tissue engineering, drug-delivery, gene-delivery, wound healing and biosensors (Davis, 2011).

In this chapter, we discuss the state of the art of the synthesis of novel composites that use a chitosan polymer matrix assembled with natural extracts or synthesis compounds so as to enhance its inherent properties, paving the way for the development of novel potential applications. Emphasis is placed on the graft copolymerization of tailored natural polymers with other suitable natural materials as the most suitable approach (Thakur & Kessler, 2015; Thakur et al., 2012; Thakur et al., 2013a) to incorporate the desired functionalities into the polysaccharidic backbone and improve the existing physicochemical properties of the start materials.

3.2. Properties and formation of chitosan oligosaccharides

Although –as noted above– chitosan is a source of potentially bioactive material, it has several shortcomings that prevent its direct use in biological applications, namely a low solubility under physiological conditions and a high viscosity in dilute acidic solutions (Kim & Rajapakse, 2005). In contrast, the hydrolyzed products of chitosan, such as its oligomers, feature better solubility and lower viscosity, due to their shorter chain lengths and the presence of aforementioned free amino groups in the D-glucosamine units. Whereas low molecular weight (MW) chitosan has a weight-average MW in the 10,000 Da to 100,000 Da range, oligochitosan generally has a MW lower than 10,000 Da (Duy et al., 2011). The activity of chitosan is narrowly correlated with its structure and physicochemical properties, such as its cationic nature, its degree of polymerization or its degree of deacetylation, which are improved in the oligomers (Xia et al., 2011).

The degradation of the O-glycosidic bonds of chitosan by different methods results in different numbers and sequences of glucosamine, as well as different degrees of polymerization. Several technological approaches have been assessed for the preparation of the oligomers, including acid hydrolysis, enzymatic methods, ultrasonic degradation, irradiation and oxidative degradation (Figure 3.2). Regardless of the chosen procedure, the resulting molecular weight is decreased after the rupture of the O-glycosidic bonds (Zou et al., 2016).

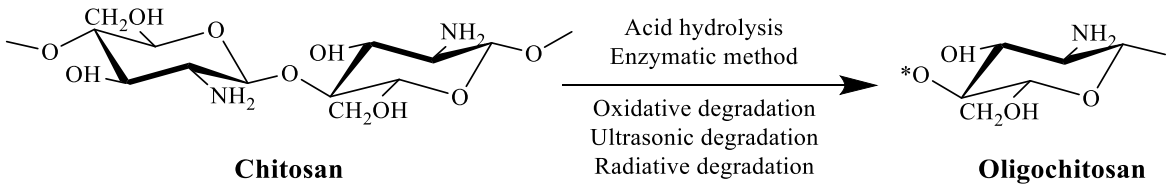

Figure 3.2. Preparation of chitosan oligomers from the chitosan polymeric chain by different degradation methods.

Chemical hydrolysis is the most frequently used in the industrial-scale production, in spite of the fact that it has some disadvantages, such as lower production yields, the generation of toxic compounds and a higher risk associated with the environmental pollution (Kim & Rajapakse, 2005).

On the other hand, enzymatic methods have significant advantages in terms of ease of control and safety. Apart from chitosanases, many other nonspecific enzymes (e.g, cellulases, lipases and proteases) have been successfully used to prepare chitosan oligomers (Xia et al., 2011).

The oxidative degradation method consists in adding a strong oxidant to a chitosan solution in order to oxide the β-(1,4)-glycosidic linkage and thus degrade chitosan into chitosan oligomers with different molecular weights in acidic, neutral, basic reaction systems. This process can be improved with the addition of other oxidant elements, such as hydrogen peroxide, and by resorting to microwave radiation, which is also useful in degrading polysaccharides (Sun et al., 2007).

Sonication has also received great interest as an alternative for the degradation of chitosan into low-MW chitosan, chitosan oligomers and glucosamine. The NH_2 groups on the C-2 of chitosan facilitate the site-specific fragmentation of the glycosidic bond during β-cleavage after the sonication treatment, thus decreasing the degree of deacetylation (Savitri et al., 2014).

Synergistic degradation of chitosan in solution by γ-irradiation in the presence of a small amount H_2O_2 has also been explored. The efficiency of the process was assessed by gel permeation chromatography (GPC) analysis, showing that oligochitosan with MW ranging from 5000 to 10,000 Da could be efficiently prepared by this procedure at low dose (less than 10kGy). This approach would be remarkably efficient for the scale-up manufacturing of oligochitosan (Duy et al., 2011).

The resulting chitooligomers from aforementioned procedures have received much attention, as they have found application in agricultural, environmental, pharmaceutical, biomedical and food and industries because of their biodegradability, their biocompatibility and their non-toxic and non-allergenic nature. Furthermore, chitosan and its derivatives have been shown to feature other various biological activities, including anti-hypertensive, anti-diabetic, anti-obesity, anti-cancer, anti-allergic, anti-coagulant, anti-inflammatory, anti-microbial, antioxidant, neuroprotective and matrix metalloproteinases inhibitory effects (Ngo et al., 2015).

3.3. Nanomaterials from renewable materials

Polymer-based materials, ranging from synthetic to renewable polymers, have attracted strong interest during the last few decades in industries related to energy storage in batteries, super capacitors, structural composites and biomedicine, among others (Thakur et al., 2014c). An intense research activity is focused on the usage of renewable resource-based materials as one of the components in the resulting final products.

In this regard, chitosan can be deemed as a promising material, since it contains three types of reactive functional groups: an amino/acetamide group in addition to primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions. The amino content is key to the differences in structural and physicochemical properties, which in turn influence the chelation, flocculation and biological functions (Xia et al., 2011). Chitosan is a cationic polysaccharide due to the presence of the amino group, which confers more reactivity and the ability to bind functional groups that appear in natural or synthetic extracts, resulting in the formation of novel compounds (Figure 3.3).

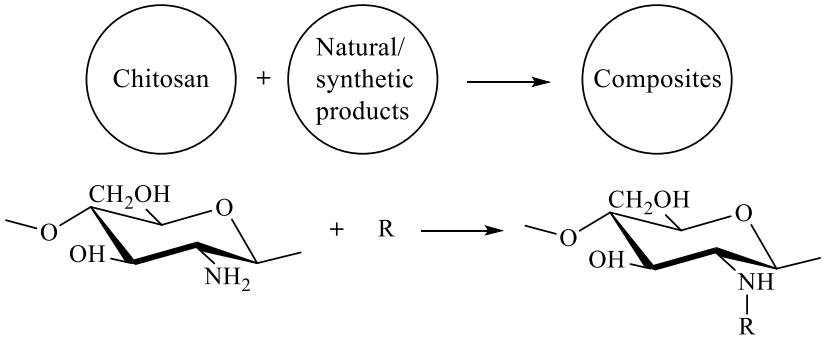

Figure 3.3. Composite formation from the D-glucosamine structure by addition of a functional group.

Chitosan carries free amine functionalities on the deacetylated units and hydroxyl groups on both the acetylated and deacetylated units. So as to increase its solubility at neutral and alkaline pH without affecting its cationic character, chitosan can be modified by introduction of small functional groups, such as alkyl or carboxymethyl groups. Thus, chitosan can be grafted with other molecules through covalent binding. The amino groups can be used for acetylation, reactions with aldehydes and ketones, chelation of metals, quaternization, etc., while the hydroxyl groups can lend to o-acetylation, H-bonding with polar atoms, etc. (Kumar Tiwary et al., 2011). For example, composites in Table 3.1 are materials based on chitosan which have found a wide range of uses in diverse scientific disciplines.

Table 3.1. Composites based on chitosan from materials with different origins and their applications.

Mixtures	Nature of materials	Composites	Applications	Reference
Binary	Biological	Chitosan / liposomes	Food	(Tan et al., 2016)
		Chitosan / acetylcholinesterase	Environmental	(Warner & Andreescu, 2016)
	Natural extracts	Chitosan nanoparticles / *Carum copticum* essential oil	Antimicrobial	(Esmaeili & Asgari, 2015)
		Chitosan / thyme oil	Biomedical	(Altiok et al., 2010)
		Chitosan / cinnamon essential oils	Food packaging	(Ojagh et al., 2010)
		Chitosan / vanillin	Food packaging	(Stroescu et al., 2015)
		N-vanillyl chitosan / 4-hydroxibenzyl chitosan	Food packaging	(Jagadish et al., 2012)
		Chitosan / propolis	Antimicrobial	(Matei et al., 2015; Torlak & Sert, 2013)
		Chitosan / propolis	Biomedical	(Franca et al., 2014)
	Synthetic	Chitosan / chloroquinoline	Biomedical	(Kumar et al., 2011)
		Chitosan / PVA	Antimicrobial	(Tahtat et al., 2011)
		Chitosan / silica xerogel	Biomedical	(Jun et al., 2010)
		Chitosan / silica	Biomedical	(Dabóczi et al., 2016)

		Chitosan / nanosilica	Multiple applications	(Podust et al., 2014)
		Chitosan / gelatin / essential oils	Antimicrobial	(Gómez-Estaca et al., 2010)
		Chitosan / gelatin	Food packaging	(Palma et al., 2016)
Ternary (or more)	Natural extracts	Chitosan films / lauric arginate / cinnamon oil / ethylenediaminetetra acetate	Antimicrobial	(Ma et al., 2016)
	Natural extracts / Synthetic	Chitosan / collagen / bioactive glass NPs	Biomedical	(Moreira et al., 2016)
	Natural extracts / Metals	Chitosan oligomers / propolis / silver NPs	Fungicide	(Matei et al., 2015)
		Chitosan / neem oil / zinc oxide	Fungicide	(Sanuja et al., 2015)
	Synthetic	Chitosan / tween 20 / span 60	Fungicide	(Ziani et al., 2009)
	Synthetic / Metals	Chitosan / glutaraldehyde / copper	Biomedical	(Xue & Wilson, 2016)

3.3.1. *Chitosan combined with biomaterials*

Composites based on chitosan binary mixtures encompass the addition of species of biological nature and of synthetic chemistry species in order to obtain the desired composite design. For the food industry, biopolymer-coated liposomes by electrostatic adsorption of chitosan have been prepared. It was demonstrated by fluorescence polarization that chitosan can flatly adsorb onto the membrane surface through electrostatic attraction, inducing charge inversion but maintaining the spherical shape of liposomes. These results may contribute to the development of *chitosomes* as potential candidates for an efficient delivery of bioactive compounds in nutraceutical and functional foods (Tan et al., 2016).

A biosensor based on chitosan for pesticides detection with enhanced solvent resistance was recently manufactured from the acetycholinesterase enzyme. Solvent tolerance of immobilized enzymes is important for many biosensing and biotechnological applications, and these findings can enable future selection of the immobilization matrix and solvent type for the development of organic phase enzyme-based systems (Warner & Andreescu, 2016).

3.3.2. *Chitosan cross-linked with natural extracts*

In recent years, the unparalleled and functional properties of essential oils have been widely studied, but their sensitivity to environmental factors and their poor aqueous solubility have limited their applications in industries.

Carum copticum essential oil was combined with chitosan nanoparticles by an emulsion ionic gelation, using pantasodium tripolyphosphate and sodium hexametaphosphte as cross-linkers. The biological properties of *Carum copticum* essential oil, before and after the encapsulation process, were determined by FTIR and thermal analysis, concluding that the essential oil had been encapsulated into the chitosan nanoparticles without any chemical reaction. The structure and function of oil were not changed in this process, suggesting maintenance of its antibacterial and antioxidant properties (Esmaeili & Asgari, 2015).

Thyme oil has also been mixed with chitosan in order to make biofilms for wound healing applications, due to their antimicrobial and antioxidant activities. The results confirmed the good potential of thyme oil to be incorporated into these antibacterial and permeable films, and vibrational

spectroscopy data showed that there was no interaction between the functional groups of chitosan and the active groups of thyme oil (Altiok et al., 2010).

In the same way, cinnamon essential oil was mixed with chitosan in order to make biofilms with lower moisture content than pure chitosan films, which also showed improved antimicrobial activity, solubility in water, water vapor permeability and elongation at break properties (Ojagh et al., 2010).

Another interesting natural extract is 4-hydroxy-3-methoxybenzaldehyde (vanillin), the primary component of the extract of the vanilla bean. Films containing chitosan and vanillin were obtained and could be used as antimicrobial and as flavor-release materials. The release kinetics of vanillin from these films were evaluated and the emulsifier influence on the flavor release was also assessed (Stroescu et al., 2015). Moreover, chitosan derivatives such as N-vanillyl chitosan and 4-hydroxybenzyl chitosan have also been prepared by reacting the amino groups of chitosan with these aldehydes to form a Schiff base intermediate, which was subsequently converted into N-alkyl chitosan by reduction with sodium cyanoborohydride (Figure 3.4). The antimicrobial activity of the modified chitosan films was studied against *Aspergillus flavus*, finding a clear reduction of the aflatoxins produced by the fungus (to 98.9% and non-detectable levels, for N-vanillyl chitosan and 4-hydroxybenzyl chitosan film discs, respectively) (Jagadish et al., 2012).

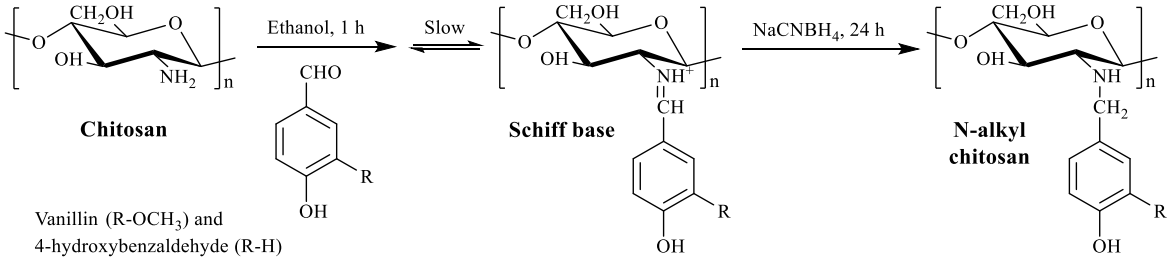

Figure 3.4. Synthesis of N-vanillyl chitosan, as an intermediate step towards the obtaining of N-alkyl chitosan films.

The effectiveness of chitosan coated polypropylene films against bacteria, either in its pure form or incorporating an ethanolic extract of propolis (EPP), has also been evaluated against six foodborne pathogens (*Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Cronobacter sakazakii* and *Bacillus cereus*), demonstrating that the chitosan-coated films had a broad-spectrum antibacterial activity. However, the mechanical properties of chitosan–propolis coated films are still subject of further research (Torlak & Sert, 2013). Likewise, a good antimicrobial activity was also exhibited by three formulations of propolis-based chitosan varnish, obtained by dissolution of propolis with chitosan in an hydro-alcoholic vehicle at different concentrations (Franca et al., 2014).

Matei et al. (2015) conducted the synthesis of chitosan oligomers/propolis/silver nanoparticles composite systems and studied their activity against a xylophagous fungus (*Diplodia seriata*). In this case, the vibrational spectra suggested the existence of hydrogen bonding between chitosan and propolis.

3.3.3. *Chitosan co-polymerized with synthetic species*

The nature of chitosan allows the incorporation of synthetic materials in its polymeric structure. In this way, a chitosan–chloroquinoline derivative was prepared by a Green-Chemistry technique, treating a chitosan solution in aqueous acetic acid with a 2-chloroquinoline-3-carbaldehyde solution to form a hydrogel, which was then subjected to solvent exchange. The antimicrobial activity of the derivative against different bacteria, viz. *Staphylococcus aureus, Escherichia coli* and *Candida albicans* was evaluated, concluding that the chitosan–chloroquinoline derivative holds promise for novel antimicrobial agents (Kumar et al., 2011).

Tahtat et al. (2011) investigated the antibacterial activity of polyvinyl alcohol hydrogels containing different concentrations of chitosan, cross-linked by γ-irradiation, against *Escherichia coli* and *Bacillus subtilis*, revealing a higher effectiveness in the inhibition of the growth of gram positive bacteria than for gram negative ones. They also found that both the chitosan content in the hydrogel and its MW had a direct influence on growth inhibition.

A bioactive coating consisting of a silica xerogel/chitosan hybrid -more hydrophilic with increasing silica xerogel content- was applied by Jun et al. (2010) to titanium at room temperature as a novel surface treatment for metallic implants. This hybrid coating layer induced the rapid precipitation of apatite on its surface when immersed in a simulated body fluid, thus affording excellent bone bioactivity. In comparison to a pure chitosan coating, the hybrid coating enhanced the viability of the cultured osteoblastic cells and promoted the alkaline phosphate activity of the cells, with optimum results for the composite containing 30% chitosan. Consequently, these silica xerogel/chitosan hybrids can potentially be utilized as room temperature bioactive coating materials on titanium-based implants.

Chitosan and bilayered – Rhodamine 6G impregnated silica–chitosan – coatings have also been prepared and investigated as a model for controlled drug release. Spectroscopic ellipsometry measurements showed that covalent cross-linking led to an increased swelling degree of chitosan layers. Despite the swelling behavior, both cross-linked chitosan layers showed significant retard effect on dye release from the bilayered coatings (Dabóczi et al., 2016).

Novel organic–inorganic hybrid materials consisting of chitosan and nanosilicas (plain silica, silica-titania and silica-alumina) were prepared by Podust et al. (2014) and assessed as adsorbents of biomedical relevance. The chitosan modified nano-oxides were obtained by equilibrium adsorption method. It was found that chitosan adsorption capacities of TiO_2/SiO_2 and Al_2O_3/SiO_2 were higher than that of the plain SiO_2 because the mixed oxides presented additional active sites on their surfaces.

Other research groups have conducted studies by combining chitosan with oils and/or plasticizers, to yield different types of composites or nanocomposites, such as edible films consisting of gelatin-chitosan incorporated with clove essential oil, whose antimicrobial activity was tested against six selected microorganisms (namely *Escherichia coli, Listeria innocua, Pseudomonas fluorescens, Shewanella putrefaciens, Lactobacillus acidophilus* and *Photobacterium phosphoreum*). The gelatin-chitosan film incorporating clove essential oil was then applied to fish during chilled storage, observing a significant reduction in the growth of gram-negative bacteria, in particular Enterobacteriaceae, while the impact on lactic acid bacteria was negligible. The addition of clove essential oil to the film also led to a substantial increase in water solubility, which can be ascribed to protein–polyphenol interactions that weaken the interactions that stabilize the protein net (Gómez-Estaca et al., 2010). The mechanical properties of the chitosan–gelatin edible films were assayed and the interaction between gelatin, chitosan and several plasticizers, pure or in binary combinations, was also investigated (Palma et al., 2016).

Furthermore, chitosan films with lauric arginate, cinnamon oil, and ethylenediaminetetraacetate (EDTA) have been prepared, and their combination results in a synergistic antimicrobial effect on gram-positive bacteria but with an antagonistic effect on gram-negative bacteria (Ma et al., 2016).

Stimuli-responsive nanocomposite-derived hydrogels have recently gained prominence in tissue engineering because they can be applied as injectable scaffolds in bone and cartilage repair. Due to the great potential of these systems, Moreira et al. (2016) synthesized and characterized novel thermosensitive chitosan-based composites, chemically modified with collagen and reinforced by bioactive glass nanoparticles, aimed at the development of injectable nanohybrids for regenerative medicine applications. The results demonstrated that the addition of collagen and bioactive glass increased the mechanical properties after the gelation process, with promising potential to be used as thermoresponsive biomaterials for biomedical applications including bone tissue regeneration.

Bionanocomposite film with enhanced properties were prepared by Sanuja et al. (2015) by incorporating different concentrations (0.1, 0.3 and 0.5%) of nano-ZnO and neem essential oil into chitosan by a solution cast method. The results showed that the 0.5% nano-ZnO incorporated composite film had improved tensile strength, film thickness, film transparency elongation, and decreased water solubility, swelling and barrier properties.

As mentioned above, the need to reduce the negative impact of conventional treatments on human health and on the environment has led to an increase in use of eco-friendly polymers as antimicrobial materials. The antifungal properties of films based on chitosan have been assessed in various studies, such as the work by Ziani et al. (2009), who tested its antifungal activity against *Aspergillus niger*, *Alternaria alternata* and *Rhizopus oryzae*, showeing that the antifungal activity largely depended on the particular type of fungus treated.

A one-pot kinetic uptake study of urea in aqueous solution with various chitosan sorbent materials such as pristine chitosan, cross-linked chitosan with glutaraldehyde from low to higher glutaraldehyde content, and a Cu(II) complex of a glutaraldehyde cross-linked chitosan material was recently studied by Xue and Wilson (2016). Cross-linked chitosan displayed relatively rapid urea uptake and greater adsorption capacity when compared with pristine chitosan. These results further illustrate the rational design of chitosan-based materials for the controlled uptake of urea in aquatic environments.

3.4. Synthesis methods for chitosan-based nanocomposites

Different chitosan products have different structures and physicochemical properties, that may result in novel bioactivities or new findings in known bioactive compounds (Zou et al., 2016).

As noted above, compared to the other natural polysaccharide polymers, chitosan also suffers from some disadvantages, one being its low water solubility at physiological pH. The transfection efficiency of native chitosan is also relatively low, and it lacks some functionalities that are highly desired for some applications. Thus, a number of chemical modification techniques have been used to overcome these drawbacks (Taşkın et al., 2014). Chemical modification of chitosan can be attained by N-substitution, by O-substitution, or by N,O-substitution, and also *via* chitosan association with small molecules or macromolecules.

The antimicrobial activity can be improved by chemical modifications of the amino group at the C-2 position of glucosamine with positively charged groups Figure 3.5(*a*) (Jeon & Kim, 2001). Chitosan acylation is a significant functionalization method used to prepare chitosan derivatives with good water solubility, biocompatibility, increased bioactivities and even improved antioxidant activity. A remarkable member of N-acyl chitosan group that exhibits good compatibility is N-succinyl-chitosan. This compound (see Figure 3.5(*b*)), obtained through a simple reaction between chitosan and succinic anhydride, preserves a series of biological properties such as biocompatibility and nontoxicity. Mercapto chitosan can be obtained by thioglycolic acid ($SHCH_2COOH$) thiolation reaction (Figure 3.5(*c*)). Chemical modification of chitosan by acylation and copolymerization are shown in Figure 3.5(*d*) and Figure 3.5(*e*), respectively.

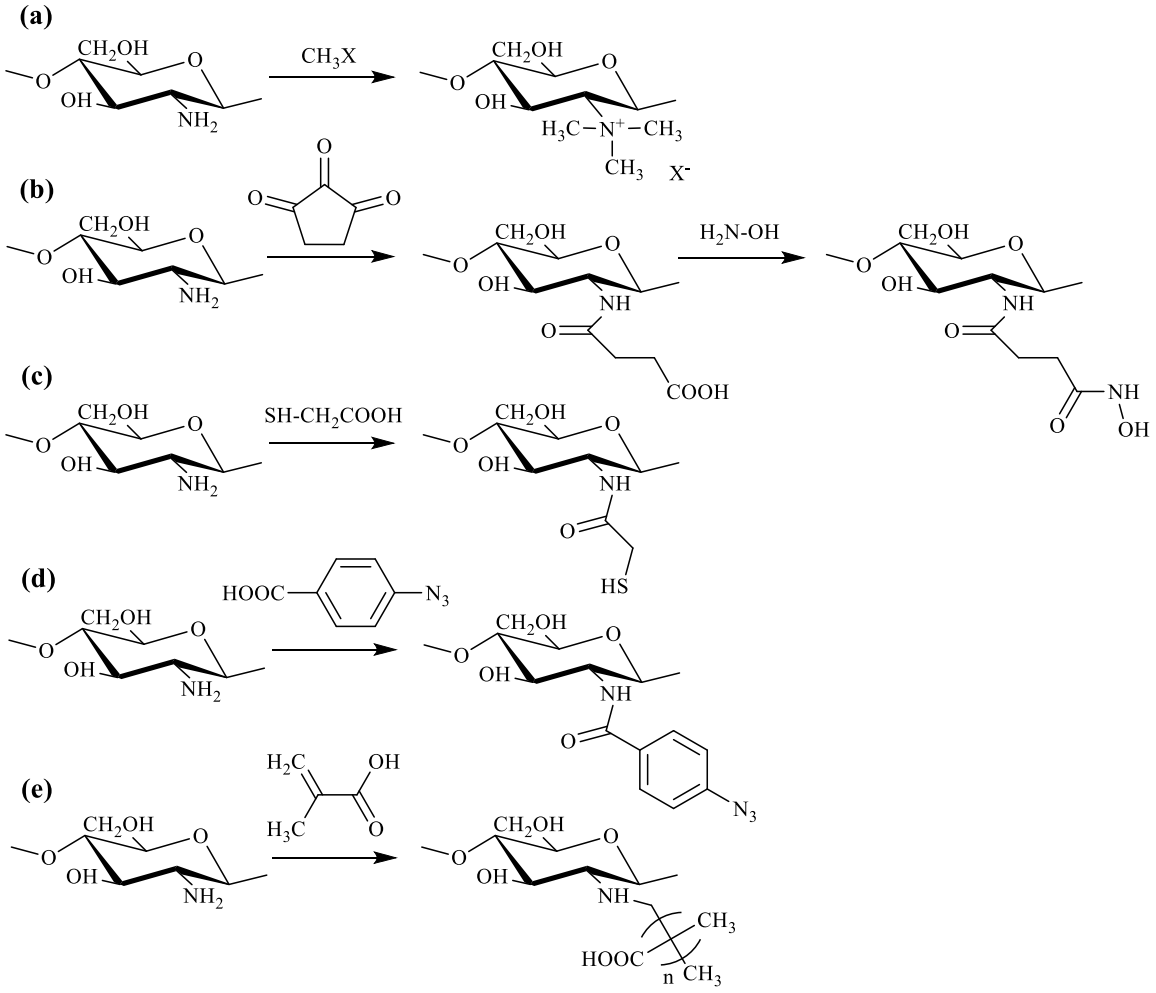

Figure 3.5. Synthetic methods for the chemical modification of chitosan.

Table 3.2. Synthesis methods for the obtaining of composites based on chitosan.

Type of synthesis	Synthetic methods	Materials	Applications	References
Biological	Biocatalysis with microbial transglutaminase	Chitosan /nisin	Antimicrobial	(Zhu et al., 2015)
	Enzymatic hydrolysis	Chitosan	Multiple applications	(Pan et al., 2016)
Physical	Ultrasonication	Chitosan	Food packaging	(Ho et al., 2016)
	Thermal treatment	Chitosan / nanosilver	Antimicrobial	(Wei et al., 2009)
	Microwave	Chitosan / TETA	Environmental	(Ge & Ma, 2015)
	Ultrasonication	Chitosan NPs	Multiple applications	(Gokce et al., 2014)
Chemical	Graft polymerization (ATRP)	Chitosan / lactic acid	Multiple applications	(Bhattarai et al., 2006)
	Catalysis	Chitosan / nanosilver	Antimicrobial	(Venkatesham et al., 2012)
	Acid hydrolysis	Chitosan acrylic NPs	Antimicrobial	(Torabi et al., 2016)
	Chemical coupling	Chitosan / nanosilver	Antimicrobial	(Gu et al., 2014)
	Acid hydrolysis / catalysis	Chitosan	Multiple applications	(Xia et al., 2013)
	Sol–gel method by freeze casting	Chitosan / bioactive glass	Biomedical	(Pourhaghgouy et al., 2016)
	Nucleophilic substitution	Chitosan / pyridine	Fungicide	(Jia et al., 2016)

3.4.1. *Biological methods*

The modification of chitosan with enzymes, in comparison to chemical modification, can be regarded as particularly attractive because of its specificity and also in terms of its environmental impact (Shukla et al., 2013). Among the methods using the enzymatic pathway for obtaining chitosan composites we have selected, based on their advocated effectiveness, one that uses microbial transglutaminase and nisin natural antimicrobial and another that employs papain (Table 3.2).

By using microbial transglutaminase as a biocatalyst, nisin grafted chitosan was prepared with high selectivity and efficiency, under mild reaction conditions and in an environmental friendly way. By adjusting the reaction temperature, the reaction time and the molar ratio of nisin to chitosan, it was possible to control the degree of substitution of nisin–chitosan, and the resulting product showed excellent solubility at different pH. This composite can find application in, for example, the pharmaceutical and food industry fields (Zhu et al., 2015).

Pan et al. (2016) obtained low molecular weight chitosan by the enzymolysis of chitosan by papain. Enzymolysis conditions, the initial chitosan concentration, temperature, pH and ratio of papain to chitosan were optimized by conducting experiments at three different levels using the response surface methodology to obtain high soluble reducing sugars concentrations. Meanwhile, the influence of chitosan substrate concentration on the activity of papain was assessed and chitosan exhibited substrate inhibition.

3.4.2. *Physical methods*

The physical modification of chitosan can be attained by blending, or by physically mixing, at least two polymers to create a new material with different physical properties. Four examples of synthesis methods that make use of ultrasonic pretreatment, heat treatment, microwave irradiation or ionic gelation with ultrasonication techniques are discussed below.

Self-aggregated chitosan particles are a promising candidate in stabilizing food-based emulsions because they are naturally-derived, edible, and inexpensive. In a recent study, the self-aggregated chitosan particles were synthesized from chitosan solution with and without ultrasonication pretreatment. Ultrasonication pretreatment caused depolymerization of chitosan, resulting in the formation of smaller and monodisperse chitosan particles, in comparison to the non-pretreated chitosan. These findings suggest that ultrasonication pretreatment on chitosan could reduce the hydrophobicity of the chitosan particles formed via self-aggregation, as confirmed by contact angle measurements (Ho et al., 2016).

Wei et al. (2009) synthesized chitosan-based Ag-NPs by reducing $AgNO_3$ with chitosan. The resulting Ag-NPs exhibited a high antibacterial activity toward both gram-positive and gram-negative bacteria, comparable with that of the highly active silver salts precursor. The Ag-NPs impregnated chitosan films, formed via thermal treatment, showed both faster and more long-lasting antibacterial effectiveness against *Escherichia coli* than pure chitosan films.

A novel triethylenetetramine/graphene oxide/chitosan composite was successfully synthesized by microwave irradiation method and compared with one prepared by conventional heating. The experimental results indicated that the product obtained using microwaves had higher yield and uptake than the one obtained by the conventional approach (Ge & Ma, 2015).

An ionic gelation method have also been used for the preparation of chitosan nanoparticles. The impact of freeze-drying, ultrasonication time and cryoprotectant (d-trehalose) utilization on the particle size, size distribution and stability of the nanoparticles as evaluated, concluding that freeze-drying caused the particles to agglomerate, that the addition of the cryoprotectant led to a decrease in the particle size and increasing ultrasonication time increased the nanoparticles size. Accordingly, Gokce et al. (2014) determined that controlled ultrasonic treatment and the use of cryoprotectant were effective for imparting nanoparticle stability.

3.4.3. *Chemical methods*

Physicochemical properties, such as electrostatic charging and permeation of polymeric surfaces, can be altered by chemical modification, which can take place in different ways. Amongst the many chemical modifications available, alkylation, acylation, hydroxylation, nitration, sulphonation, phosphorylation, xanthation, Schiff's base formation and graft copolymerization are the most popular approaches (Shukla et al., 2013).

Graft co-polymerization can be defined a method in which one polymer is covalently bonded to the other polymer chain (Benamer et al., 2011). Among the various graft copolymerization techniques used to change the surface characteristics of polymers such as chitosan and other materials for different applications, the technique of atom transfer radical polymerization (ATRP) is rapidly emerging as the first preference due to the advantages it offers over other techniques (Ifuku et al., 2013; Thakur & Thakur, 2014). Bhattarai et al. (2006) attained lactic acid modification of chitosan without using a catalyst by grafting D,L-lactic acid onto amino groups in chitosan.

The following are some representative examples of chemical synthesis methods that incorporate catalysis, acid hydrolysis, chemical coupling, sol-gel method and nucleophilic substitution procedures for the obtaining of composites based on chitosan.

Stable silver nanoparticles were synthesized by Venkatesham et al. (2012), without using any toxic chemicals, by using chitosan as both a reducing and a stabilizing agent. The antimicrobial activity of the resulting Ag-NPs was tested against *Micrococcus luteus* and *Escherichia coli*, confirming inhibiting properties.

Torabi et al. (2016) also reported an environmentally-friendly process for the preparation of acrylic/chitosan films with antibacterial activity and nontoxic properties by using a water-base acrylic

resin. It was found that the obtained films had enhanced antibacterial activity against *Escherichia coli* and *Staphylococcus areus*, and the cytotoxicity analysis showed a reasonably non-toxic behavior of the composite films.

Amphiphilic chitosan-graft-poly(ε-caprolactone) copolymers were synthesized by a homogeneous coupling method and characterized by ninhydrin assay and NMR and FTIR spectroscopies. The graft copolymers were subsequently self-assembled into micelles, which were measured by dynamic light scattering and transmission electronic microscopy. The particle size of the micelles decreased as the segment grafting fraction was increased. Thereafter, silver nanoparticles were prepared in the presence of chitosan-based micelles under ultraviolet irradiation. The films impregnated with the Ag-NPs showed a strong antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (Gu et al., 2014).

In the study by Xia et al. (2013), water soluble chitosan was prepared by hydrolyzation using H_2O_2 and phosphotungstic acid (PTA, $H_3PW_{12}O_{40}$) as a catalysis in homogeneous phase, and the various factors affecting hydrolysis and the optimal hydrolysis conditions were investigated. The resulting products were composed of chitooligosaccharides.

A recent study by Pourhaghgouy et al. (2016) focused on chitosan-based nanocomposite scaffolds preparation by freeze-casting method through blending of a constant chitosan concentration with different portions of synthesized bioactive glass nanoparticles. The biodegradation study showed that increase in bioactive glass nanoparticles content led to growth of weight loss amount, while the *in vitro* biomineralization studies confirmed the bioactive nature of all nanocomposites.

Jia et al. (2016) introduced pyridine moieties into chitosan by nucleophilic substitution to afford N-(1-carboxybutyl-4-pyridinium) chitosan chloride (pyridine chitosan). The antifungal activity of the resulting chitosan derivative was then examined by studying the inhibition of mycelia growth and spore germination. The results indicated that pyridine chitosan exhibited enhanced antifungal activity by comparison with pristine chitosan. Non-toxicity of pyridine chitosan was demonstrated by an acute toxicity study. These results are beneficial for assessing the potential utilization of this chitosan derivative and for exploring new functional antifungal agents with chitosan in the food industry.

3.5. Analytical techniques for the identification of the composite materials

The identification of characteristics such as the degree of deacetylation, MW, polydispersity and crystallinity are of utmost importance for the synthesis of chitosan derivatives. For instance, amongst the different analytical techniques available, atomic force microscopy (AFM), cryogenic transmission electron microscopy (cryo-TEM) and small-angle neutron scattering (SANS) analysis have been used to characterize the molecular shape and water soluble grafted chitosan (see Table 3.3) (Shukla et al., 2013).

Table 3.3. Methods for the determination of the physicochemical characteristics of chitosan and chitosan derivatives. Updated from that available in (Trutnau et al., 2011).

Physico-chemical characteristics	Determinations methods	Reference
Degree of deacetylation	Infrared spectroscopy (FT-IR)	(Martínez-Camacho et al., 2010)
	First derivative UV-spectroscopy	(Kasaai, 2009)
	Nuclear magnetic resonance spectroscopy (NMR)	(Aranaz et al., 2009)
	Titration (alkalimetric, conductometric, potentiometric)	(Trutnau et al., 2009)
	Differential scanning calorimetry (DSC)	(Jiang et al., 2003)
Molecular weight /Mw distribution	Viscosimetry	(Rhazi et al., 2000)
	Gel permeation chromatography	(Brugnerotto et al., 2001)
	Light scanning	(Chatterjee et al., 2005)
	Electrophoresis	(Muzzarelli et al., 1994)
Crystallinity	X-ray diffraction (XRD)	(Matei et al., 2015)
Moisture content	Gravimetric analysis	(Ogawa et al., 1993)
Ash content	Gravimetric analysis	(Ogawa et al., 1993)
Protein content	Bradford method	(Dornish et al., 2001)
Texture	Transmission electronic microscope (TEM)	(Pillai et al., 2009)
Morphological structures	Scanning electronic microscope (SEM)	(Sanuja et al., 2015)
Thermal stability	Thermo gravimetric analysis (TGA)	(Martínez-Camacho et al., 2010)
Mechanical properties	Near-infrared	(Palma et al., 2016)

3.6. Advanced applications of bionanomaterials based on chitosan

In this section some of the applications of the nanocomposites are discussed: as antimicrobial agents for the control of fungi and bacteria; in biomedical applications for wound dressings, drug delivery or tissue engineering; in food packaging; in environmental applications for the adsorption of metals and toxics or in wastewater treatment; in crop protection; and in the conservation cultural of heritage such as historic and artistic works in stone, wood, textile, etc. (Figure 3.6).

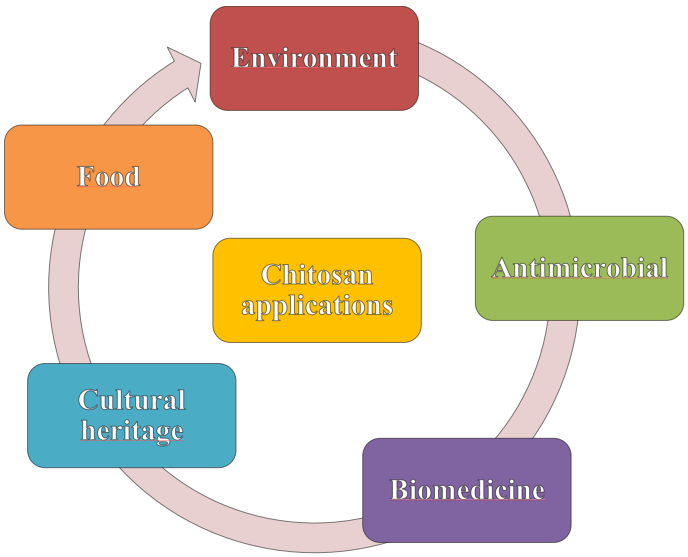

Figure 3.6. Chitosan applications in different fields.

3.6.1. *Antimicrobial applications*

Compounds of chitosan are being widely researched for their application as antimicrobial agents. Ziani et al., 2009 examined the effect of chitosan with different MWs at different concentrations as an antifungal in the control of phytopathogenic fungi *Aspergillus niger*, *Alternaria alternata* and *Rhizopus oryzae*. The highest inhibition was observed against *A. alternata* (97%).

As noted above, a chitosan-nisin composite also showed a marked inhibitory effect against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*, which is very promising for both pharmaceutical and food-related uses (Zhu et al., 2015). On the other hand, Torabi et al. (2016) illustrated that a blend of polyacrylic nanoparticles with chitosan is an easy and efficient method for obtaining anti-bacterial coatings, non-toxic and with a good control of the desired properties.

Several recent studies also demonstrate the antibacterial activity of other composites with chitosan (Esmaeili & Asgari, 2015; Gómez-Estaca et al., 2010; Gu et al., 2014; Ma et al., 2016; Tahtat et al., 2011; Torlak & Sert, 2013; Venkatesham et al., 2012). These compounds were tested for use as bactericides in food packaging, in the pharmaceutical, nutraceutical and cosmetic industries, confirming their inhibitory effect against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Cronobacter sakazakii*, *Listeria monocytogenes* and *Salmonella typhimurium*.

A compound of chitosan and antifungal pyridine was tested against *Fulvia fulva* and *Botrytis cinerea*, resulting in a superior antifungal activity compared with pure chitosan. This nontoxic compound can be deemed as very promising as a new antifungal agent in the food industry (Jia et al., 2016).

Matei et al. (2015) reported a ternary composite of chitosan, propolis and silver nanoparticles which was evaluated against *Diplodia seriata*, a very aggressive phytopathogenic fungus that attacks grapevines, evincing an effective antifungal activity.

Likewise, Saharan et al. (2013) investigated the antifungal effect of a compound of chitosan, saponin and copper nanoparticles in the control of phytopathogenic fungi, i.e., *Alternaria alternata*, *Macrophomina phaseolin* and *Rhizoctonia solani*. The results demonstrated that chitosan nanoparticles showed a very high inhibitory effect on the mycelial growth of *Macrophomina*

phaseolin in a concentration as low as 0.1%. Thus, it can have a great potential for development as a fungicide for crop protection.

3.6.2. *Biomedical applications*

3.6.2.1. *Antimicrobial activity of wound dressings*

Wound dressings made of chitosan are biomaterials with promising features in terms of biocompatibility, biodegradability, antibacterial properties and hemostatic characteristics. Nonetheless, the applicability of pure chitosan is limited due to its poor mechanical properties. This limitation can be overcome, for example, by resorting to poly(ethylene glycol) reinforced chitosan hydrogels, as reported by Chen et al. (2013).

Nanofibers of a chitosan/sericin composite, prepared by electrospinning technique, have been studied by Zhao et al. (2014) as potential wound dressings and showed good antibacterial properties. Similarly, Sweeney et al. (2014) prepared alginate–chitosan fibers via a one-step, direct wet-spinning extrusion process for wound care dressings applications, finding that they featured a high surface area for absorption, ease of fabrication and softness, biocompatibility, non-toxicity and bioactive properties.

Santos et al. (2013) developed membranes based on chitosan/soy and showed that they possessed valuable properties for wound dressings materials in terms of ease of handling, healing/repair stimulation and esthetic appearance.

3.6.2.2. *Drug delivery*

Chitosan is also a promising biopolymer for drug delivery systems because of its cationic character and primary amino groups, which are responsible for its many properties such as mucoadhesion, controlled drug release, transfection, *in situ* gelation, efflux pump inhibitory properties and permeation enhancement (Elgadir et al., 2014).

Advances in self-assembled chitosan nanomaterials for drug delivery hold great potential as carriers for the delivery of proteins and peptides, genes, small molecules and combinational drugs (Yang et al., 2014).

A study carried out by Franca et al. (2014) showed that a propolis-based chitosan varnish could also be used for drug delivery and controlled release, in addition to its antimicrobial activity against oral pathogen bacteria. The reported formulations could be suitable products for clinical application on the dental caries prevention field, although further clinical studies to confirm its *in vivo* activity are still required.

3.6.2.3. *Tissue engineering*

As mentioned above, Moreira et al. (2016) have recently reported some thermogelling chitosan–collagen–bioactive glass nanoparticle hybrids which could be used as injectable systems for tissue engineering. Their application as injectable scaffolds for regenerative medicine applications in bone and cartilage repair holds great promise.

3.6.3. *Food-related applications*

Chitosan and its derivatives –which exhibit high antimicrobial activity against pathogenic and spoilage micro-organisms, including fungi, and both gram-positive and gram-negative bacteria– have a significant potential in the food industry as biologically active bio-molecules because of the increasing concerns about the negative environmental impact of conventional packaging materials such as plastics and in view of contaminations associated with food products (Aider, 2010).

Chitosan is also being studied in applications to stabilize emulsions in the food industry (Ho et al., 2016). López-Mata et al. (2013) described the use of chitosan film with carvacrol (cymophenol), examining the mechanical and optical properties and the antibacterial capacity of the antioxidant film against *E. coli* and *S. typhimurium*. The compound showed high antioxidant capacity and a superior protective effect against the oxidation of red blood cells. These results suggest potential applications of chitosan films with carvacrol as containers for preserving food products.

In a study by Leceta et al. (2013), chitosan-based films with glycerol were obtained for packaging applications, incorporating different glycerol levels into the chitosan solutions to improve mechanical properties: the resulting films were transparent and resistant against ultraviolet light, which would have a preventive effect and retard product oxidation induced by UV light. Furthermore, the films were an effective barrier against water vapor and oxygen, which helped to maintain the quality of the product.

Aguirre-Loredo et al. (2016) evaluated the effect of moisture content on the thermal, mechanical and barrier chitosan films under various conditions of relative humidity. They concluded that increasing the moisture content of the material promoted the transport of water vapor molecules through the film, thus reducing the barrier capability.

Other studies have also evaluated compounds with chitosan for the manufacture of films for use in packaging in the food industry (Cruz-Romero et al., 2013; Martínez-Camacho et al., 2010; Pavinatto et al., 2010; Tan et al., 2016).

3.6.4. *Environmental applications*

3.6.4.1. *Metal absorption*

Chitosan can also be used to the fight the pollution of rivers and to control effluents discharged by the industry, since it is capable of extracting metals from water, such as copper, zinc, lead, cobalt and cadmium. In the study by Benamer et al. (2011), acrylic acid was grafted to chitosan by irradiation and tested for the adsorption of lead ions and cadmium -considered highly toxic-, confirming an improvement in the adsorption capacity.

In a similar study, a compound of chitosan with triethylenetetramine was synthesized by microwave irradiation and by conventional heating, and both were assessed in terms of their chromium adsorption capacity. The experimental results indicated that the product obtained by microwave irradiation showed higher efficiency and absorption than the compound obtained by conventional heating (Ge & Ma, 2015).

3.6.4.2. *Wastewater treatment*

Chitosan can be utilized for wastewater treatment because of its ability to chelate metal ion (Ravi Kumar, 2000). The size of the chitosan particles, the pH of the solution and the concentration of ions were be the main factors that influenced the degree of chelation. As regards the pH, the efficiency increased with increasing pH, although it even took place at pH levels as low as 2 (Burke et al., 2002).

In a study by Suarez Meraz et al. (2016), chitosan was tested in order to improve the efficiency of wastewater coagulation-flocculation. The performance of chitosan with two different molecular weights against wastewater containing high levels of organic carbon was compared, concluding that the low molecular weight chitosan was more efficient than the high molecular weight one.

3.6.4.3. *Agricultural crops*

Chitosan can also be used as a stimulant in plants, since it induces the formation of antioxidant enzymes such as superoxide dismutase, catalase and peroxidase (which attack the reactive oxygen species), it improves the tolerance to abiotic stress and it induces pathogen resistance.

Studies have demonstrated the use of chitosan as a coating for fruits in order to extend the postharvest life and maintain their quality. The chitosan coating induced the antioxidant capacity and also maintained the total phenolic content of the fruit (Ghasemnezhad et al., 2011; Ghasemnezhad et al., 2010).

Mondal et al. (2012) investigated the effect of foliar application of chitosan as a growth promoter in plant morphology, growth, biochemical and fruit yield of okra, reporting that the morphological attributes (height), growth (absolute growth rate), biochemical parameters (nitrate reductase and photosynthesis) and yield increased with increasing concentration of chitosan up to 25 ppm. In similar work conducted by Yin et al. (2012), the effects of chitosan oligosaccharides on growth and content of secondary metabolites in oregano were evaluated.

Sathiyabama et al. (2014) focused on the antifungal properties of chitosan and its role in protection against *Alternaria solani*, a fungus that causes damage in tomato leaf. They discovered that chitosan induced activity of the chitinase enzyme, which disrupted glycosidic linkages in the chitin molecules constituting the fungal cell wall, thus reducing the severity of the disease and playing a role in the activation of defense responses.

Warner and Andreescu (2016) described the use of chitosan as an immobilization matrix for acetycholinesterase enzyme in a biosensor aimed at detecting pesticides in the presence of a high content of organic solvents. This biosensor proved to be sensitive to paraoxon (a parasympathomimetic which acts as an acetylcholinesterase inhibitor), selected as a model pesticide belonging to the group of organophosphates.

3.6.5. *Applications in Heritage Preservation*

As noted above, chitosan can chelate metal ions, which makes it a viable option for immobilizing metal ions in archaeological wood (reducing the catalytic effect that these have on many degradation processes), provided that treatment times are usually quite long. The beneficial effect of chitosan would be two-fold, since it would not only stop metal ions in the wood, but would also reinforce it (Christensen et al., 2012).

Abdel-Kareem et al. (2015), evaluated the use of a cross-linked chitosan coated Ag-loading nano-SeO_2 composite in the consolidation of ancient Egyptian linen textiles and it showed good bacterial resistance.

On the other hand, chitosan can also be useful for the preservation of the aesthetic appearance and historical value of stone in industrial countries and damp temperate climates. Micro- and macroorganisms can deteriorate stone chemically and mechanically, causing irreversible damage (such as biocorrosion, pitting, cracking, detachment) at the surface and inside the stone. Eyssautier-Chuine et al. (2015) developed a preventive treatment using a sol-gel process applied onto a clean surface. Tetraethoxysilane (TEOS), commonly used as a consolidant, was used here as a matrix to which active components such as chitosan and silver nitrate were then added for their biocide effect, together with hydrophobic silica as a water-repellent. They were tested in laboratory conditions with a green alga of the widespread genus *Chlorella* on limestone, with a view to preventing the weathering and biofouling of stone monuments. Results revealed different patterns of algal development according to treatment efficacy. The combination of silver nitrate and hydrophobic silica, both at high dosages, provided the best biocide effect, but when chitosan was added, a similar biocide effect was obtained using a lower concentration of chemicals. This synergy was not observed when hydrophobic silica was either absent or present at a higher dose.

3.7. Conclusions

Amongst natural polymers, chitosan is deemed as the most promising for the preparation of novel nanocomposite materials, since it is significantly more versatile than chitin or cellulose due to the presence of amino groups. These groups afford an alkaline character that determines an enhanced tendency to form collateral bonds and to react with aldehydes and ketones so as to produce biofilms. Furthermore, since chitosan oligomers can be synthesized from chitosan under mild reaction conditions, it is possible to take advantage of their improved properties to prepare better grafted composites. In fact, these chitosan oligomers show a marked affinity for biological products such as liposomes or acetylcholinesterase, natural extracts such as essential oils (propolis, thyme, cinnamon, vanillin), and natural antimicrobial agents (as nisin), thus enabling the synthesis of entirely eco-friendly or biocompatible composites.

An additional advantage is the proclivity of chitosan/chitosan oligomers to be processed into gels, membranes, nanofibers, beads, microparticles, nanoparticles and scaffolds through several synthesis methods that involve biological, chemical and physical processes, e.g., enzymatic hydrolysis, graft polymerization, electrospinning, γ-irradiation, microwave or ultrasonic techniques. In this chapter, special emphasis has been placed on the novel synthesis methods for chitosan functionalized co-polymers, in particular those which incorporate extracts from natural products and which improve the solubility of chitosan and its antimicrobial activity.

On the other hand, the incorporation of metallic nanoparticles or metal oxides to the graft copolymers also holds great promise for applications in the biomedical, drug-delivery, biosensors, pharmaceutical, food, agricultural, conservation and environmental fields.

The state-of-the-art presented herein could drive substantial research interest in the development of new sustainable nanomaterials for advanced applications based on the assembling of chitosan oligomers with natural extracts, antimicrobial agents and nanometals.

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3.9. References

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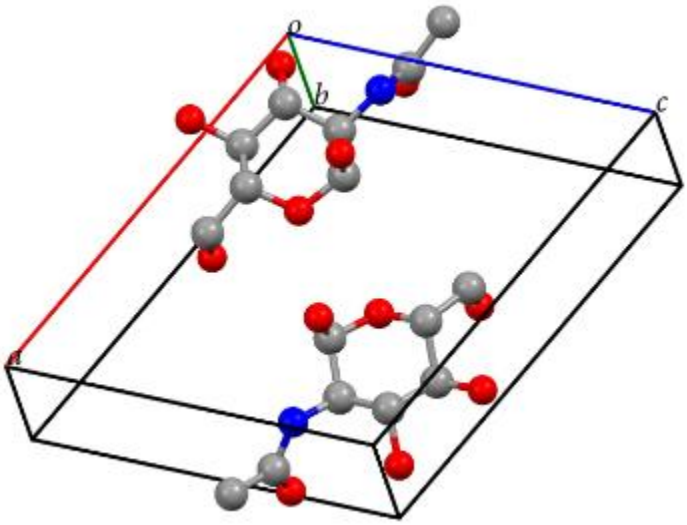
Artículo 1

Un estudio de espestroccopía de la N-Acetil-D-Glucosamina usando THz-TDS, Far-FTIR y métodos semiempíricos de química cuántica



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A study of the far infrared spectrum of *N*-acetyl-D-glucosamine using THz-TDS, FTIR and semi-empirical quantum chemistry methods

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Abstract

The far-infrared spectrum of *N*-acetylglucosamine has been studied by combining THz–TDS and FT-IR characterization techniques with theoretical studies based on semi-empirical quantum chemistry methods. A strong spectral peak at 1.8 THz has been identified, which constitutes the main signature of the material in the terahertz band. Calculated molecular vibrations are in good qualitative and semi-quantitative agreement with both the THz-TDS and FT-IR experiments. In comparison to previous DFT-based studies, the semiempirical approach chosen herein, suitable for parallel multi-core and GPU acceleration, allows for a full study using periodic boundary conditions and no further approximations within a constrained computing time.

Keywords: FIR spectroscopy; FTIR spectroscopy; *N*-acetylglucosamine; THz-TDS spectroscopy; semiempirical methods.

1. Introduction

N-acetylglucosamine (or D-GlcNAc, or NAG) is a derivitized glucose monomer found in polymers of bacterial cell walls, chitin, hyaluronic acids and various glycans. As the monomeric unit of chitin polymer, it forms the outer coverings of insects and marine crustaceans and it is the major component of the cell walls of most fungi. Chitosan (deacetylchitin) is a form of *N*-acetylglucosamine that has been chemically altered.

N-acetylglucosamine derivatives -chitin and chitosan- have become of great interest not only as underutilized resources, but also as new functional materials of high potential in various fields [1-3]: in biomedical applications (antimicrobial agents, drug and gene delivery, wound dressings and tissue engineering), in waste water treatment (purification and toxic ion removal), agriculture (seed coatings and controlled agrochemical release), in food industry (packaging and preservative materials) and in cosmetics, to name a few.

The structure of *N*-acetylglucosamine [4] is shown in Figure 1. Crystals are monoclinic, with space group $P2_1$ and unit cell parameters a=11.25 Å, b=4.82 Å, c=9.72Å, and β=113.7º. Mercury software package [5] has been used for the representation.

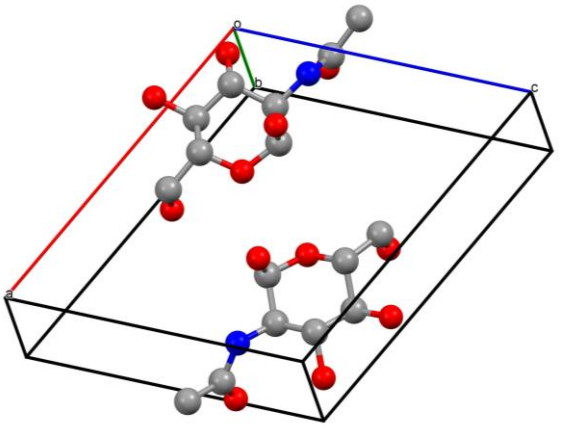

Figure 1. Crystal cell of *N*-acetyl-D-glucosamine.

The far infrared (FIR) spectra of polycrystalline mono- and poly-saccharides is dominated by lattice modes involving molecular hydrogen bonds or/and van der Waals forces. Therefore, FIR spectroscopy techniques in general and, particularly, terahertz time-domain spectroscopy (THz-TDS) are notably useful in the structural characterization of carbohydrates [6-8].

Computational quantum chemistry is a highly valuable tool for the interpretation of FIR spectra due to the complexity of the associated vibration modes. *Ab-initio* and density functional theory (DFT) methods are very accurate in the prediction of molecular vibrations [9]. Nevertheless, solid-state calculations over large supra-molecular systems are typically required due to the relevance of long-range interactions, thus limiting the use of these computationally costly methods. On the other hand, semi-empirical methods [10,11], especially with the recently developed parallel implementations for shared-memory multiprocessor and massively-parallel graphics processing units architectures [12], permit to study the vibrations in large molecular crystals at reduced computation times. Semi-empirical quantum chemistry methods have been previously used in the interpretation of the terahertz spectra of various carbon nitride [13] and carbon [14] materials.

A study of the IR spectrum of NAG was previously presented in [15]. The experimental characterization was performed using a FT-IR spectrometer and the theoretical modeling using a DFT method. Due to the high computer cost of such calculations, a single molecule was studied and the intermolecular hydrogen bond interactions were simulated with intramolecular interactions. In this work, we focus on the far-infrared spectrum of NAG. We combine THz–TDS and FT-IR techniques for the characterization of the spectral regions between 10 cm^{-1} and 80 cm^{-1} and 80 cm^{-1} and 420 cm^{-1}, respectively. THz-TDS measurements have permitted to identify one strong spectral peak at 1.8 THz, which is the main signature of the material in the terahertz band. Theoretical studies have been performed using semi-empirical quantum chemistry methods. The use of parallel multi-core and GPU acceleration allows for a full study using periodic boundary conditions and no further approximations within a constrained computing time.

2. Materials, experimental and theoretical methods

2.1. *Materials*

N-acetyl-D-glucosamine (CAS No. 7512-17-6, ≥99%) was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). The sample material was mixed with ultra-high molecular weight surface-modified, 53-75 µm particle size polyethylene (PE, CAS No. 9002-88-4), also from Sigma-Aldrich, at 18 wt%. The mixture was pressed at 5 tm for 3 minutes to make a 13 mm-diameter pellet.

2.2. *Spectroscopic characterization*

A Menlo (Martinsried, Germany) Tera K15 spectrometer was used for the THz-TDS analysis. The system is based on a 1560 nm fiber laser that generates 90 fs pulses at a repetition rate of 100 MHz. This provides a compact fiber-coupled setup. The system was operated in a nitrogen rich atmosphere in order to avoid the signature of water absorption in the recorded samples. Ten sample and ten reference measurements were performed in each case in order to reduce the noise in the measurements.

The material parameters in the spectral range of interest were calculated from the time domain photocurrent traces measured with the spectrometer. These time domain waveforms depend not only on the material data but also on the width of the pellets, due to the contributions from multiple reflections at the pellet-air interfaces. Signal processing techniques similar to those described by Duvillaret *et al*. [16], with a flat-top window [17], were employed in order to obtain the THz spectra of the materials.

The vibrational spectrum of the material in the 80-420 cm^{-1} spectral range was measured using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 FT-IR Spectrometer. At shorter wavelengths the measurement was saturated because of the attenuation associated to the PE matrix.

2.3. *Quantum chemistry computations*

The semiempirical quantum chemistry computations were performed with the PM6 method [18] using the parallel implementation for multi-threaded shared-memory CPUs and massively parallel GPU acceleration [12] of the MOPAC2012 [19] software package. A Fedora Linux server with a 12-core Intel Xeon processor and a NVIDIA Tesla K20 GPU were used for the computations.

3. Results and discussion

3.1. *FIR spectrum of N-acetyl-D-glucosamine*

The results of the THz-TDS measurements are shown in Figure 2. The terahertz spectrum in the spectral range from 0.3 THz to 2.5 THz (80 cm^{-1}) was characterized by a dominant peak at 1.8 THz. Two very weak peaks could also be observed at 1.36 THz and 2.15 THz, respectively.

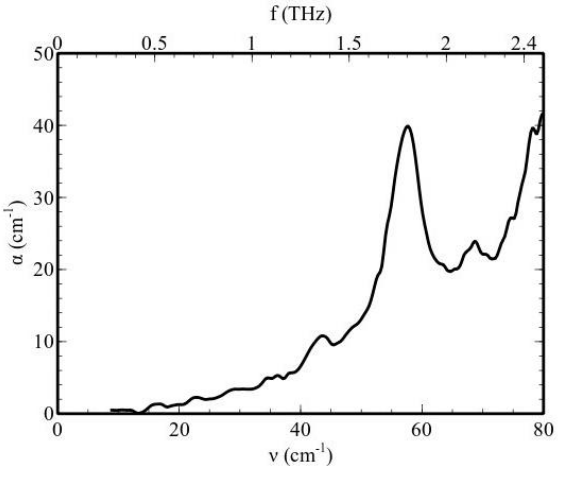

Figure 2. *N*-acetyl-D-glucosamine THz-TDS measurement results.

The FT-IR measurement results in the spectral range from 80 cm^{-1} to 420 cm^{-1} are shown in Figure 3. The solid line corresponds to the sample material dispersed in PE powder and the dashed line to a pure polyethylene reference pellet. The polyethylene absorption displayed the expected Rayleigh scattering monotonic increase of attenuation with frequency associated with the finite particle size, and the PE band at 110 cm^{-1} [20]. A second faint PE absorption band was also found close to 230 cm^{-1}. The spectrum in the whole range is shown in Figure 4.

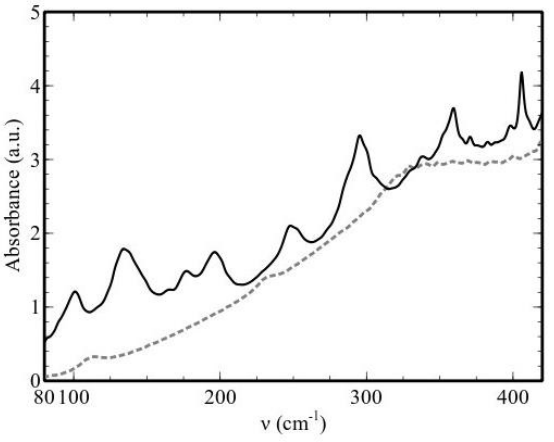

Figure 3. FT-IR spectrum in the spectral range from 80 cm^{-1} to 420 cm^{-1}. The solid line corresponds to *N*-acetyl-D-glucosamine dispersed in polyethylene powder at 18 wt% and the dashed line to a reference pellet of pure polyethylene.

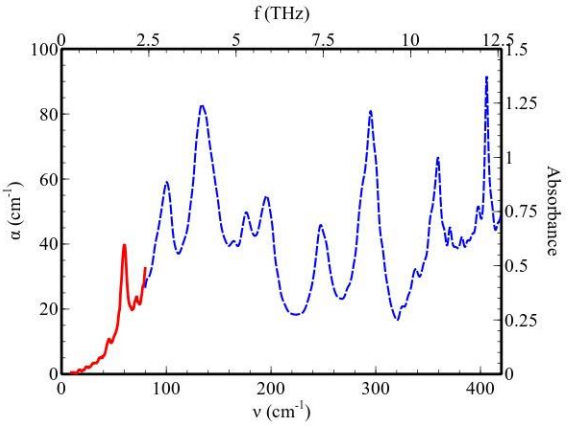

Figure 4. Measured FT-IR spectrum (*dashed blue line*), after subtraction fo the contribution to the absorbance of the PE matrix, and THz-TDS measurement (*red solid line*).

3.2. *Semi-empirical quantum chemistry results*

The solid-state geometry of NAG was optimized using the PM6 Hamiltonian [18] using the actual crystal geometry [4] as the initial condition. Accurate calculations using MOPAC with periodic boundary conditions require a computational domain at least capable of fitting a sphere with a diameter of 8 Å. A minimal domain spanning 1×2×1 crystal unit cells was used by setting the keyword MERS=(1,2,1). The unit cell dimensions of the resulting optimized geometry a=11.400 Å, b= 4.616 Å and c=9.040 Å were in relatively good agreement with the actual values a=11.25 Å, b=4.82 Å, and c=9.72 Å. Nevertheless, the cell angles α=94.56º, β=114.09º and γ=85.51º showed a noticeable deviation from the monoclinic unit cell, even though β was very close to the experimental value of 113.7º. The calculated vibrations for the crystal geometry optmized with the PM6 Hamiltonian did not include any imaginary frequency. PM7 [21] calculations provided a better fit with the actual crystal unit cell angles, but the method failed to converge to a true ground state geometry.

The PM6 geometry of one of the two molecules of the crystal unit cell is compared with the experimental structure in Figure 5. The two molecules have been plotted using VMD [22]. A relative shift has been introduced to facilitate the visual comparison due to the highly overlapping geometries. Bond lengths are compared in Table 1, and angles in Table 2. We have used the atom labels shown in Figure 6.

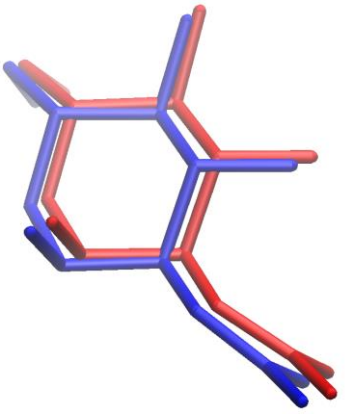

Figure 5. Comparison of the PM6 optimized geometry with the experimental X-ray geometry of *N*-acetyl-D-glucosamine.

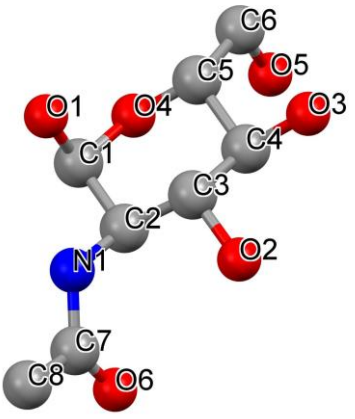

Figure 6. Atom labels used in the comparison of the molecular geometries.

Table 1. Comparison of the experimental bond lengths (in Å) with those of the geometry optimized with the PM6 method. The unsigned mean error (UME) was 1.30%.

Bond	Experimental distance	PM6-calculated distance	Error
C1-C2	1.5281	1.5499	1.43%
C2-C3	1.5367	1.5477	0.72%
C3-C4	1.5344	1.5464	0.78%
C4-C5	1.5504	1.5392	0.72%
C5-C6	1.5201	1.5298	0.64%
C7-C8	1.5126	1.4903	1.47%
C2-N1	1.4763	1.4745	0.12%
C7-N1	1.3800	1.3819	0.14%
C1-O1	1.3935	1.4167	1.66%
C3-O2	1.4511	1.4319	1.32%
C4-O3	1.4685	1.4382	2.06%
C1-O4	1.4593	1.4248	2.36%
C5-O4	1.4598	1.4581	0.12%
C6-O5	1.3908	1.4402	3.55%
C7-O6	1.2177	1.2465	2.37%

Table 2. Comparison of the experimental bond angles (in degrees) with those of the geometry optimized with the PM6 method. UME=1.54%.

Angle	Experimental	PM6	Error
C2-C1-O1	108.33	107.52	0.75%
C2-C1-O4	108.01	111.06	2.82%
O1-C1-O4	107.95	108.70	0.69%
C1-C2-C3	110.61	109.53	0.98%
C1-C2-N1	108.92	109.16	0.22%
C3-C2-N1	108.17	111.48	3.06%
C2-C3-C4	108.60	110.39	1.65%
C2-C3-O2	108.19	108.51	0.30%
C4-C3-O2	105.90	105.73	0.16%
C3-C4-C5	106.85	110.18	3.12%
C3-C4-O3	107.75	109.13	1.28%
C5-C4-O3	105.94	106.38	0.42%
C4-C5-C6	113.88	115.29	1.24%
C4-C5-O4	105.53	108.03	2.37%
C6-C5-O4	104.94	104.08	0.82%
C5-C6-O5	111.44	110.08	1.22%
C8-C7-N1	113.15	118.45	4.68%
C8-C7-O6	123.10	120.88	1.80%
N1-C7-O6	123.62	120.59	2.45%
C2-N1-C7	119.01	121.15	1.80%
C1-O4-C5	116.51	117.14	0.54%

The use of two crystal cells for the calculation of the vibrations resulted in the inclusion of modes with wavectors sampling different points of the first Brillouin zone of the crystal. Nevertheless, only $k \cong 0$ solutions are relevant for the optical spectrum. The in-phase oscillating modes were selected from the global set of vibrations by projecting the mass-scaled eigenvectors components of the two cells in the computation domain, and selecting only those vibrations for which the scalar product was larger than 0.9.

3.3. *Frequency assignment*

The frequency assignment from our experimental and theoretical analysis is shown in Table 3, where the results are compared with those of the former study by Kovács *et al.* [15]. Due to the complexity of many of the vibration modes, a graphical description has been included in Figure 7, where the mass-scaled eigenvectors are plotted for one of the molecules in the crystal cell.

In our assignment, the calculated results are in good qualitative and semi-quantitative agreement with the experiments. We find larger errors for modes with a strong lattice-type vibration contribution, where the whole molecules experience large displacements. This is the case, for example, in the lowest (terahertz) band of the spectrum. On the other hand, very good agreement is found when the strongest contributions are due to localized intra-molecular vibrations. This is the case, for instance, in the upper band between 350 cm^{-1} and 420 cm^{-1}. These results can be attributed to the highly accurate molecular geometry predicted with the PM6 Hamiltonian and, on the other hand, to the slightly distorted predicted crystal unit cell.

There is also a good agreement between the description of ref. [15] and our results. One difference is the signature peak at 1.8 THz, which we have relabeled as of medium (instead of weak) intensity. In our measurements, we have not observed the shoulder at 242 cm^{-1} detected in ref. [15] and the resonance at 251 cm^{-1} in [15] is resolved in our measurements into two components: one peat at 247 cm^{-1} and a shoulder at 252 cm^{-1}. Moreover, there are two new weak peaks between 360 cm^{-1} and 410 cm^{-1} that had not been previously measured and assigned. The lowest lying weak resonance at 45 cm^{-1} had not been previously reported and we have not been able to assign it to any of the calculated modes.

In order to improve the theoretical description in the terahertz band, a geometry optimization with the PM6 method keeping the crystal cell parameters constant was performed. In general, optimization with this type of constraints does not guarantee that the resulting geometry is a true ground state. Nevertheless, no imaginary frequencies were found for this geometry. Besides keeping the actual crystal parameters, the quality of the molecular geometry was comparable in terms of their respective UME for bonds and angles to that of the former opmitization. The results, which are shown in brackets in Table 3, permitted to assign the resonance at 45 cm^{-1} after some relaxation of the selection criterion for in-phase resonances, and to improve the accuracy in the assignment in the terahertz band, but a worsening was observed in the description for frequencies above 80 cm^{-1}.

Table 3. Frequency assignment and comparison with the theoretical and experimental results of ref. [15]. The following abbreviations have been used in the description of the vibrations: *s*, strong; *m*, medium; *w*, weak; *sh*, shoulder; *υ*, stretching; *β*, in-plane bending; *δ*, deformation; *ω*, wagging; *τ*, twisting.

Experimental [this work] FT-IR		Experimental [this work] THz-TDS		PM6	Experimental [15] FT-IR		DFT [15]	Characterization [15]
406	m			410	404	m	397	42% δ_{ring}, 21% τCO, 10% νCO
398	w			394				
382	w			386				
359	m			360	359	m	373	30% wCO, 13% wCC_{CH2}, 13% νCC, 10% βCO_{CH2}
338 325	w			315	336	w	320	20% βCN, 14% νCC, 12% $\delta_{ring,}$ 12% wCO, 11% βCO_{CH2}, 11% βCO
295	s			301	295	s	300	33% βCO, 21% νCC, 15% βCN
274	sh			269	274	sh	277	30% wCN, 22% βCO, 10% νCC, 10% βCN
252	sh			258				
247	m			235	251	m	251	48% βCO, 26% βCN
				230	242	sh	236	26% βCN, 23% βCO, 24% δ_{ring}
196	m			225	195	m	218	41% τ_{ring}, 10% βCN, 10% τCN, 10% wCO
176	m			216	175	m	181	28% βCC_{CH2}, 17% βCN, 13% δ_{ring}, 10% βCO_{CH2}
164	w			165				
134	s			144	135	s	140	30% τ_{ring}, 18% wNH, 10% wCO, 10% τCN
100	m			100	100	m	120	42% τCH_2, 21% βCN, 18% τ_{ring}
90	sh			95				
82	sh			86	84	sh	86	24% τCH_3, 24% τ_{ring}, 21% τCN, 13% τCH_2
		72	w	80 (76)	70	w	65	33% τCN, 23% wNH, 18% τ_{ring}
		60	m	70 (63)	58	w	51	43% τ_{ring}, 42% βCN
		45	w	(45)				

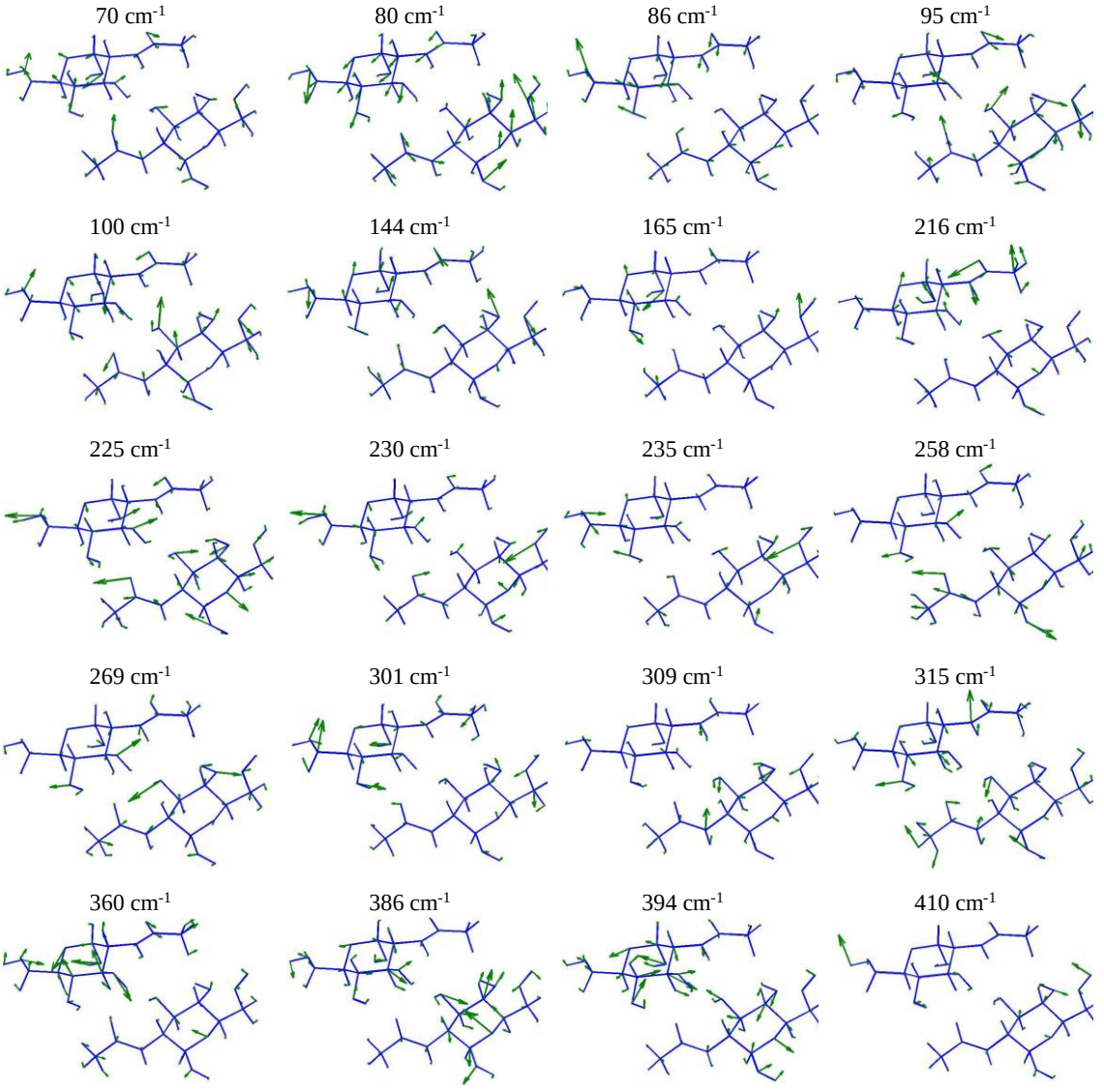

Figure 7. Mass-scaled normal modes of the frequencies in the far infrared spectrum of N-acetyl-D-glucosamine.

4. Conclusions

A combination of THz–TDS and FT-IR spectroscopies with theoretical studies based on semi-empirical quantum chemistry methods has been used to study the far-infrared spectrum of *N*-acetylglucosamine (NAG). The terahertz spectrum in the spectral range from 0.3 THz to 2.5 THz (80 cm^{-1}) was characterized by a dominant peak at 1.8 THz. On the other hand, the high-resolution ATR-FTIR spectrum from 80 cm^{-1} to 420 cm^{-1} permited the identification of some additional bands in comparison to those reported in the literature. PM6 Hamiltonian semi-empirical calculations, by making use of the recently developed parallel implementations for shared-memory multiprocessor and massively-parallel GPU architectures, allowed to study the molecular vibrations at a reduced computation time (*vs.* DFT or *ab-initio* approaches), obtaining a good qualitative and semi-quantitative agreement between the calculated results and the experiments. The largest errors were found for modes with a strong lattice-type vibration contribution, while very good agreement was found when the strongest contributions were due to localized intra-molecular vibrations.

5. Acknowledgements

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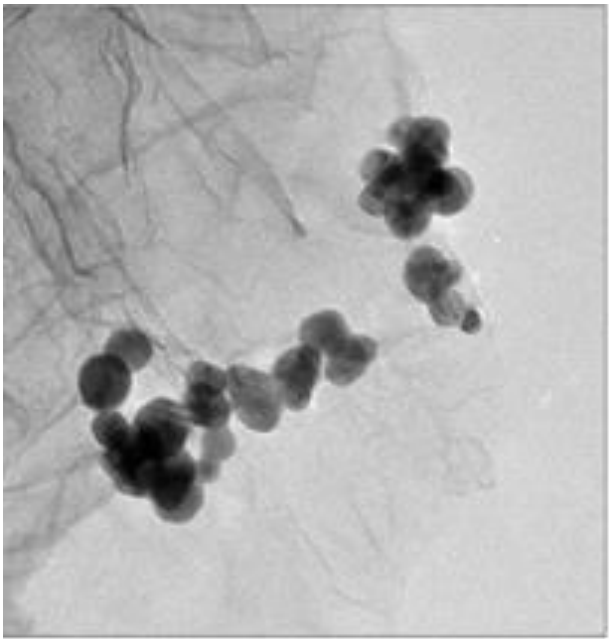
Artículo 2

Nanoagregados de plata en óxido de grafeno reducido funcionalizado en quitosano, utilizando radiación de microondas



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Silver Nanoaggregates on Chitosan Functionalized Reduced Graphene Oxide using Microwaves Radiation

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Abstract

A reduced graphene oxide (rGO)/chitosan oligomers (CSO)/silver nanoparticle (AgNPs) composite was designed and prepared via a self-assembly process with rGO through a microwave-assisted method. The material, isolated as a nano-crystalline graphenic material, was characterized by ATR-FTIR and UV-Vis spectroscopies, X-ray powder diffraction, TEM microscopy and energy-dispersive X-ray spectroscopy. The nano-rGO/CSO/AgNPs composite can be described as a biopolymer in which AgNPs have been anchored on the surface of CSO-functionalized graphitic rGO sheets.

Keywords: chitosan; nanocomposite; reduced graphene oxide; self-assembly; silver nanoparticles

1. Introduction

Mesoporous networks in graphene oxide (GO) composite structures are particularly appropriate for the formation of nanoparticles in comparison with traditional routes [1-7]. On the other hand, chitosan (CS), a well-known compound resulting from chitin N-deacetylation, shows functional amine segments (carboxyl, hydroxyl, epoxy, and $–NH_2$ groups) in the molecular skeleton that can form nanostructures with GO or reduced graphene oxide (rGO) via interactions such as hydrogen bonding. Morphological characterization of rGO/GO-CS composites have demonstrated the formation of porous 3D nanostructures which feature a high adsorption capacity.

By contrast, negatively charged silver nanoparticles (AgNPs) are prone to form aggregates on GO via electrostatic interaction [8] and are susceptible to be enveloped by chitosan, CS [9]. Thus, the integration of aforementioned materials in a one nanomaterial can be deemed as an achievable goal. In fact, GO/CS/AgNPs and rGO/CS/AgNPs ternary nanocomposite systems have been designed so that anchoring of AgNPs to CS functionalized graphitic GO/rGO sheets can occur [8-10].

The present study is focused on investigating the preparation of rGO/CSO/AgNPs nanocomposites (were CSO stands for chitosan oligomers) through a microwave-assisted method, which has not been reported before to the best of the authors' knowledge. This emerging synthesis technique has already proved to be a valuable alternative to selectively prepare materials and nanomaterials, in almost quantitative yields and with greater precision than using conventional heating [11-13].

2. Experimental section

2.1. *Materials and methods*

High quality reduced graphene oxide (rGO), produced from helical-ribbon carbon nanofibres by chemical methods, was supplied by Grupo Antolín Ingeniería (GRAnPH Nanotech, Burgos, Spain). The sample consists of few layer (up to five) graphene oxide nanoplatelets, highly crystalline and with a large cross section (above 10 square microns) [14].

Medium molar mass chitosan (CAS No. 9012-76-4) was purchased from Hangzhou Simit Chemical Technology Co. Ltd (Hangzhou, China). Silver nitrate (CAS No. 7761-88-8) was supplied by Merck Millipore (Darmstadt, Germany).

The formation and assembly of rGO/CSO/AgNPs was carried out in a Milestone Ethos-One microwave. An ultrasonic machine, model CSA 20-S500, 20 kHz was used for the sonication of the solutions.

Infrared spectra were recorded with a Thermo Nicolet 380 FT-IR apparatus, equipped with Smart Orbit Diamond ATR system, in order to identify the chemical functional groups. Optical absorption spectra in the UV-Vis region were recorded with a Shimazdu UV-2450 UV-Vis spectrophotometer. X-ray powder diffractograms of the samples were obtained using a Bruker D8 Advance Bragg-Brentano diffractometer, in reflection geometry. Transmission electron microscope (TEM) micrographs were collected with a JEOL JEM-FS2200 HRP equipped with an Oxford instruments INCA Energy TEM 250 EDS probe.

2.2. *Preparation*

2.2.1. *Chitosan oligomers preparation*

An aqueous solution of chitosan oligomers (CSO) was prepared from chitosan with average molecular weight (140000-300000 g/mol) in 2% AcOH at pH 4-6 with the addition of 0.3 M H_2O_2, under constant agitation for 12 hours. Subsequently, it was subjected to 6 intermittent periods of sonication, of 5 minutes each, keeping the temperature below 60 °C. The molecular weight of the resulting oligomer was about 2000 g/mol, in accordance with the tests reported by Sun *et al.* [15].

2.2.2. *Silver NPs preparation*

Silver nanoparticles were prepared by a sonication method, without resourcing to UV stabilization (used, for example, in [16]), as follows: an aqueous solution of $AgNO_3$ (50 mM) was treated with sodium citrate (30 mM) and the resulting solution was cooled and stirred at a temperature between 5 and 10 °C. Subsequently, it was deoxygenated with an inert gas (N_2) for over 30 minutes and the pH was adjusted between 7 and 8. Polyvinylpyrrolidone was added to prevent the silver nanoparticles aggregation. A 10 mM solution of $NaBH_4$ (reducing agent) was then added dropwise: the first droplet made the solution turn from colorless to yellowish and successive droplets led to an intensification of the yellow color (care had to be taken so as to avoid an excess of reducing agent, which would lead to a brownish color). After vigorous stirring for one hour, the yellowish solution was sonicated for 3-5 minutes and then allowed to rest and stabilize for at least 24 hours in a refrigerator at 5 °C.

2.2.3. *Microwave synthesis of the composites*

The rGO/CSO/AgNPs composites preparation procedure was as follows: 17 mg of reduced graphene oxide were mixed with 2 mL of the silver nanoparticles solution (170 µg/mL), 100 µL of the chitosan oligomers solution (22.7 mg/mL) and ethylenglicol (1 mL). Water was then added till a volume of 10 mL was obtained. A microwave treatment was subsequently conducted at 60 ºC or at 120 °C for 10 minutes with stirring (heating ramps of 5 and 10 min, respectively). The obtained products were centrifuged, decanted and washed with ethanol for characterization by analytical techniques.

3. Results

3.1. *Vibrational characterization*

The vibrational spectra of the rGO/CSO/AgNPs composites prepared by microwave treatment at 60 ºC and 120 ºC are depicted in Figure 1. The two spectra were very similar to each other (only showed small differences in the absorbance intensity), indicating that they corresponded to the same material. Typical peaks such as -NH stretching of CSO, carboxyl C=O and C-O, and alkoxy C-O groups could be readily identified in the spectra, while the peak assigned to epoxy C-O at 1226 cm^{-1} had disappeared. In addition, amide peaks could be clearly observed at 1636 cm^{-1} and less clearly at 1412 cm^{-1}. Thus, according to Jiao *et al.* [10], the FTIR results demonstrate the presence of rGO and the successful synthesis of Ag nanoparticle-containing rGO-based composite materials.

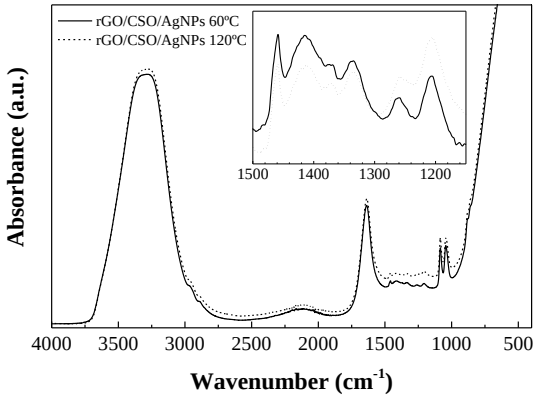

Figure 1. ATR-FTIR spectra of the rGO/CSO/AgNPs composite material

3.2. *Optical absorption*

To get an insight into the dynamics of the formation and assembly of rGO/CSO/AgNPs, intermediate UV–vis absorption spectra at different reaction stages were collected (see Figure 2). Whereas the spectrum of rGO revealed an absorption peak centered at 230 nm and a shoulder peak at about 300 nm [17,18], a new peak appeared at about 430 nm, associated to AgNPs decorated onto the rGO/CSO, which implies the formation of rGO/CSO/AgNPs. It is also worth noting that the material obtained at 120 °C showed higher absorbance than that obtained at 60 °C.

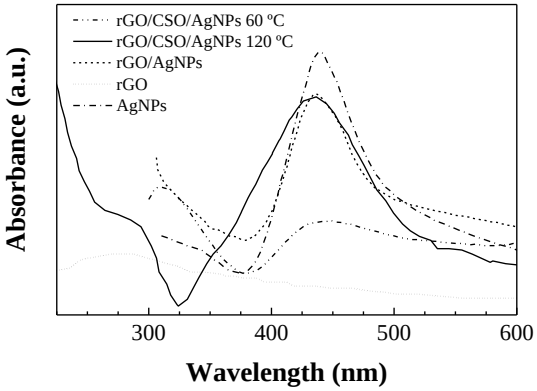

Figure 2. Visible absorption spectra for rGO/CSO/AgNPs composite and its intermediate products

3.3. *X-ray powder diffraction*

The XRD pattern of the composite (Figure 3) exhibited a main peak at $2\theta \approx 22^{o}$ (which, after deconvolution, corresponded to two peaks at d=4.44 Å and d=3.70 Å) followed by a minor peak at 38º (d=2.36 Å). These peaks indicates the presence of the three components of the nano-composite: the peaks at 21º and 24º are to be assigned to CSO and rGO, respectively, in very good agreement with those reported by Matei *et al.* [19] and Park *et al.* [20], while the peak at 38º is assigned to AgNPs according to JCPDS patterns [21].

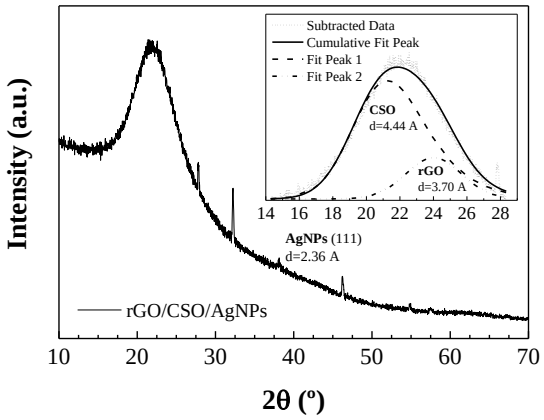

Figure 3. XRD pattern of nano-rGO/CSO/AgNPs composite showing CSO, rGO and AgNPs peaks

3.4. *Imaging of Debye-Scherrer rings*

On the basis of d-spacing from Debye-Scherrer rings (Figure 4), three assignations could be made: 1.94 Å to (200) reflection of AgNPs; 1.677 to graphite (004) reflections; and 3.237 Å to planar stacking of graphene sheets.

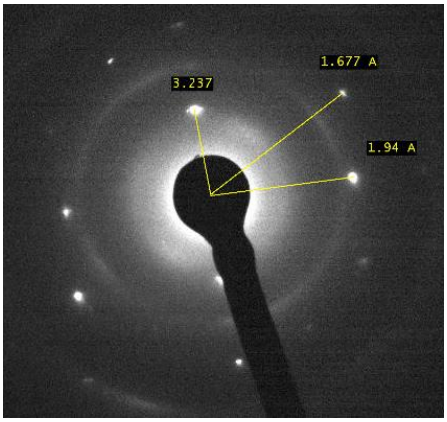

Figure 4. Debye-Scherrer rings of nano-rGO/CSO/AgNPs composite

3.5. *Textural properties and EDS analysis*

TEM images of a well-dispersed and stabilized sample of the synthesized composite are shown in Figure 5. It could be observed that spherical silver nanoparticles with a diameter ranging from 10 to 30 nm were embedded in a matrix of CSO functionalized graphitic rGO sheets and also that no particles were observed outside this matrix.

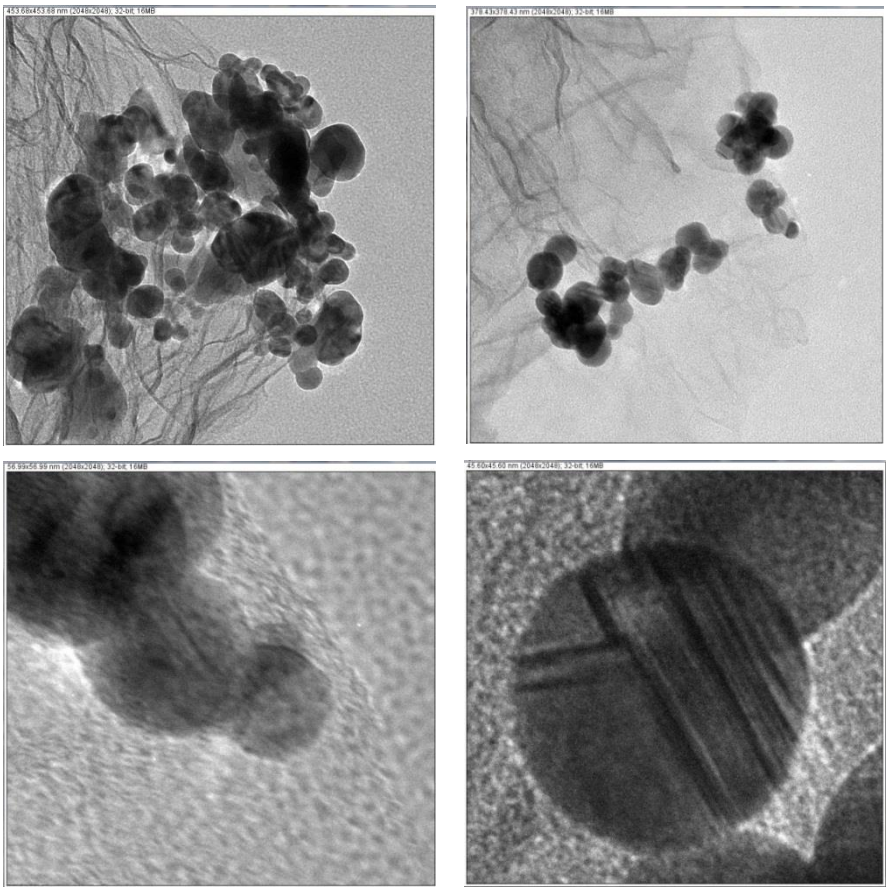

Figure 5. TEM photographs showing the spherical Ag nanoparticles on the CSO functionalized graphitic rGO sheets

EDS analysis (Figure 6) further confirmed the claim of the presence of AgNPs in the composite.

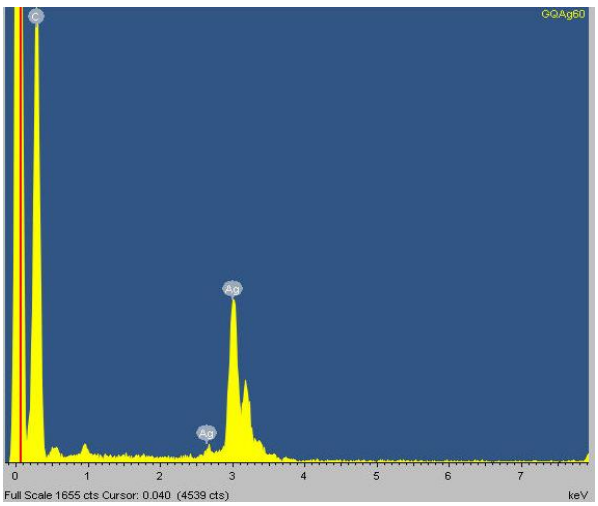

Figure 6. EDS scan showing the presence of C and Ag elements

4. Discussion

The nano-composite reported in this paper has different characteristics from those of similar composites prepared from GO and rGO with chitosan and silver nanoparticles [8,10]. The main feature of the composite discussed herein is to be a crystalline solid of graphenic texture and not a hydrogel [10]. This difference must be referred to the low proportion of chitosan in our nanocomposite (11.6%) and to the fact that the chitosan is in the form of oligomers, instead of in the form of high or medium molecular weight chitosan. Another important difference is determined by the very low presence of nanosilver in our composite (2%), in contrast to the very high percentages used by other authors, reaching proportions of 88% in weight [8].

Regarding the operating conditions followed in the microwave reactor for synthesizing nanocomposite variants, the results indicate that it is sufficient to apply a program with a temperature as low as 60 °C (for 10 minutes, with stirring) to obtain the desired material. The differences in morphology, properties and yield versus the material obtained at 120 ºC cannot be deemed as significant.

5. Conclusion

The facile design and synthesis of rGO/CSO/AgNPs composite material assisted by microwave radiation has been reported. The CS molecule was chosen due to its functional amine segments in the molecular skeleton that can form porous nanostructures via electrostatic interactions and by hydrogen bonding. Morphological characterizations of the obtained composites show the formation of silver nanoaggregates on CSO functionalized rGO sheets by a self-assembly process. The *in situ* formed silver nanoparticles appeared uniformly anchored on rGO sheets. The use of microwave in the preparation of the reported nanohybrids provides a novel method for the development of new multifunctional nanocomposites on the basis of the existing nanomaterials.

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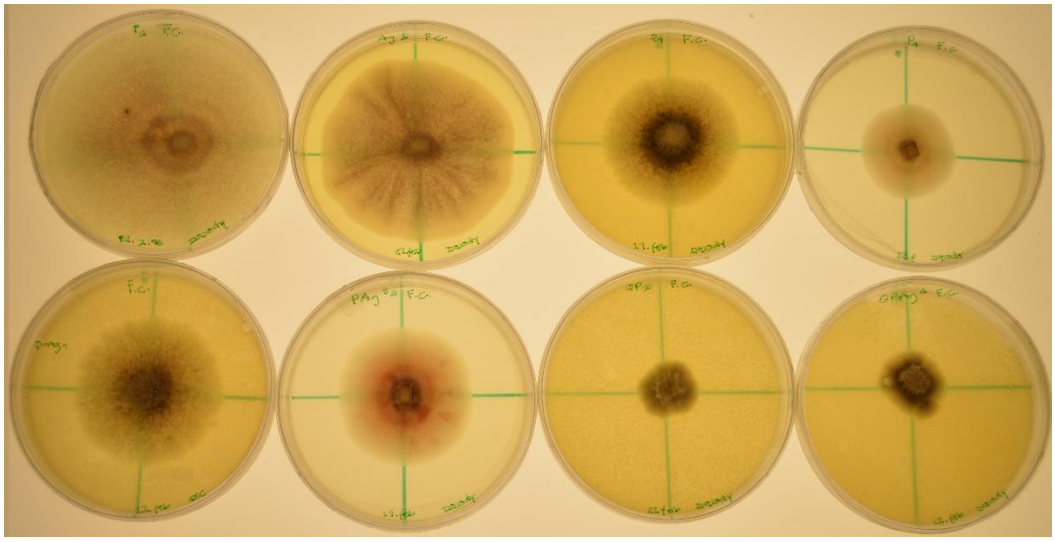
Artículo 3

Control potencial de enfermedades de especies forestales con soluciones de oligómeros de quitosano, propóleo y nanoplata



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Potential control of forest diseases by solutions of chitosan oligomers, propolis and nanosilver

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Abstract

There is a growing necessity to replace chemical agents with ecofriendly materials, arising from their impact on the environment and/or human health, which calls for the design of new broad-spectrum fungicides. In this work, chitosan oligomers (COs), propolis (Ps) and silver nanoparticles (AgNPs) mixtures in solution were assessed to control the growth of different phytopathogenic fungi and oomycetes *in vitro*. Binary solutions of COs-Ps and COs-AgNPs evinced the highest antifungal effect against *Fusarium circinatum* and *Diplodia pinea* fungi, respectively, with a *ca*. 80% reduction in their mycelial growth. The COs solution by itself also proved to be greatly effective against *Gremmeniella abietina*, *Cryphonectria parasitica* and *Heterobasidion annosum* fungi, causing a reduction of 78%, 86% and 93% in their growth rate, respectively. Likewise, COs also attained a 100% growth inhibition on the oomycete *Phytophthora cambivora*. On the other hand, Ps inhibited totally the growth of *Phytophthora ×alni* and *Phytophthora plurivora*. The application of AgNPs reduced the mycelial growth of *F. circinatum* and *D. pinea*. However, the AgNPs in some binary and ternary mixtures had a counter-productive effect on the anti-fungal/oomycete activity. In spite of the fact that the anti-fungal/oomycete activity of the different treatments showed a dependence on the particular type of microorganism, these solutions based on natural compounds can be deemed as a promising tool for control of tree diseases.

Keywords: anti-fungal; anti-oomycetes; forest pathogens; natural compounds.

1. Introduction

Phytopathogenic microorganisms are responsible for major economic losses and ecological impacts, affecting from seedling nurseries to mature trees in plantations, seed orchards, landscape plantings, or native forests (Hirooka and Ishii 2013; Gordon et al. 2015). All over the world, several species of conifers are affected by common ascomycete fungi, such as *Fusarium circinatum* Nirenberg & O'Donnell, responsible for pitch canker disease (Wingfield et al. 2008); *Diplodia pinea* (Desmaz.) J. Kickx fil. (= *Sphaeropsis sapinea* (Fr.) Dyko & Sutton), which causes *Diplodia* tip blight and stem canker disease (Gibson 1979; Adamson et al. 2015); and *Gremmeniella abietina* (Lagerberg) Morelet (anamorph: *Brunchorstia pinea* (P. Karsten) Höhnel) that produces shoots dieback and cankers on stems and trunks (Kaitera and Jalkanen 1992; Romeralo et al. 2015), causing the death of conifers including spruce, fir, larch, pine and juniper. In the same way, another of the most important pathogens in coniferous forests is *Heterobasidion annosum* (Fr.) Bref. (= *Fomes annosus* (Fr.) Cooke) basidiomycete, which causes root and butt rot (Asiegbu et al. 2005; Garbelotto and Gonthier 2013).

Other main forest pathogens include *Cryphonectria parasitica*, one of the most undesirable introduced plant pathogens, which causes chestnut blight on species in the genus *Castanea* (Heiniger and Rigling 1994; González-Varela et al. 2011); and oomycetes species such as *Phytophthora*. These latter comprise *P. cambivora* (Petri) Buisman, also a common pathogen of *Castanea*, *Fagus* and other hardwoods (Jung et al. 2005); *P.* ×*alni* (Brasier & S.A. Kirk) Husson, Ioos & Marçais, nothosp. nov., which cause alder decline by dieback, small sparse and yellowish leaves, excessive fructification, and tarry and rusty exudates (Husson et al. 2015); and *P. plurivora* T. Jung and T.I. Burgess, which causes aerial canker and collar rot in several species, including beech, oaks and alders (Jung and Burgess 2009; Haque et al. 2014; Haque et al. 2015).

To date, control of plant diseases has typically been performed by application of high toxic chemicals, whose excessive use has occasioned undesired impacts on the environment and on human health (Hirooka and Ishii 2013). Moreover, regulations are increasingly limiting the utilization of high toxic chemicals and promoting the use of integrated pest management and non-chemical alternatives to pesticides (Directive 2009/128/EC).

Chitosan is a natural polymer composed of randomly distributed β-(1-4) D-glucosamine and N-acetyl-D-glucosamine units. It can be found in the form of chitin in the shells of crustaceans and in

the cell walls of fungi (Jayakumar et al. 2011). This cationic biopolymer is characterized by being biocompatible, biodegradable, non-toxic and features antimicrobial, antiviral and antifungal properties (Ngo et al. 2015). Indeed, all fungi are expected to be vulnerable to chitosan, except those containing chitosan as a major wall compound (i.e. zygomycetes) (Leuba & Stössel, 1986, cited in Laflamme et al. (2000)).

Another natural compound that has been widely used for its antiseptic properties, mainly in traditional medicine, but also in plant protection (Özcan et al. 2004), is propolis. It is a resinous material collected by bees from different parts of plants, buds and exudates, which –once mixed with their own enzymes– is used as a void sealant or as a sanitization agent in the hive (Marcucci 1995). Propolis is rich in flavonoids, polyphenols, steroids, aldehydes, amino acids and quinones, which account for its strong antimicrobial power (Farooqui 2012; Mărghitaş et al. 2013).

Regarding silver nanoparticles, they have gained attention in the past decade as a very promising bactericidal and antifungal agent, with a much higher activity than silver ions (Kashyap et al. 2012). Silver nanoparticles have the ability to destroy the cellular walls and interfere with bacterial DNA replication and protein production processes (Wei et al. 2009).

Natural alternatives based on chitosan products have been widely studied against plant diseases, meanly in the crop protection, such as rice (Boonlertnirun et al. 2008), soybean (Zeng et al. 2012) and potatoes (Kurzawińska and Mazur 2006), to name a few. So that, the development of application strategies such as seeds coating, foliar treatment and soil amendment is very broad (El Hadrami et al. 2010). Nonetheless, against forest diseases there are very few reports regard of chitosan uses (e.g. Reglinski et al. 2004; Fitza et al. 2013).

In this work, the anti-fungal/oomycete activity of chitosan oligomers (COs), propolis (Ps) and silver nanoparticles (AgNPs) and their binary and ternary combinations in solution has been assessed against eight forest pathogens through an *in vitro* study. The information on their effectiveness against each of the pathogens could pave the way for the development of novel natural compound-based antifungals, useful in an integrated management approach.

2. Materials and methods

2.1. *Fungal material and reagents*

To assay *in vitro* the effects of the different mixtures, eight species –five fungi and three oomycetes– were chosen. All these pathogens were isolated in natural areas in the North-West of

Spain (see Table 1). The pathogens were kept in dark at 25 ºC in Potato Dextrose Agar (PDA) culture medium in order to preserve the standard mycelial growth before the treatments.

Table 1. Assayed fungi and oomycetes, isolated in previous studies.

Species	Isolate	Host tree	Origin	Isolation year	References
Fungi					
Fusarium circinatum	FcCa1	*Pinus radiata*	Cantabria	2009	(Martínez-Álvarez et al. 2012)
Diplodia pinea	HP154	*Pinus radiata*	Cantabria	2009	(Martínez-Álvarez et al. 2016)
Gremmeniella abietina	VAI-13	*Pinus halepensis*	Valladolid	2003	(Botella et al. 2010)
Cryphonectria parasitica	EU1	*Castanea sativa*	Zamora	2005	(Zamora et al. 2012)
Heterobasidion annosum	A14009-AFZAPR001	*Pinus pinaster*	Zamora	2014	
Oomycetes					
Phytophthora cambivora	PH14012-LR2-2	*Quercus ilex*	Segovia	2014	
Phytophthora ×alni	PA02	*Alnus glutinosa*	Zamora	2012	(Zamora-Ballesteros et al. 2016)
Phytophthora plurivora	SORLDD4	*Alnus glutinosa*	Soria	2012	(Haque et al. 2014; Zamora-Ballesteros et al. 2016)

Unless otherwise stated, all chemicals and reagents were supplied by Sigma-Aldrich Química S.A. (Tres Cantos, Madrid) and were used without further purification. Chitosan with medium molar mass was purchased from Hangzhou Simit Chemical Technology Co. (Hangzhou, China). Propolis with a content of polyphenols and flavonoids of *ca*. 10% (w/v) came from Burgos (Spain).

2.2. *Synthesis of chitosan-based mixtures in solution*

The synthesis of the solutions based on COs with Ps and AgNPs was conducted according to the procedure described by Matei et al. (2015), with some modifications. COs aqueous solutions were prepared from medium molecular weight commercial chitosan (140000–300000 g/mol) in AcOH 2% at pH 4–6, after neutralization with KOH. However, when the final pH of the substrate was close to 6, there was no influence on the growth of the pathogen (Jönsson-Belyazio and Rosengren 2006). Then, 0.3 M H_2O_2 was added to obtain 2000 g/mol oligomers. Ps extraction was carried out by grinding raw propolis to fine powder and by maceration in a hydroalcoholic solution 7:3 (v/v), which was subsequently percolated (1 L/min) and filtrated with a stainless steel 220 mesh to remove any residues. AgNPs were prepared with the procedure described by Venkatesham et al. (2012), where the nanoparticles from an aqueous solution of $AgNO_3$ (50 mM) were obtained with chitosan acting as both reducing and stabilizing agent without using any toxic chemicals. The reaction was carried

out in an autoclave at 120 °C for 15 min to obtain a clear yellow color indicating the formation of silver nanoparticles.

COs-Ps, COs-AgNPs, Ps-AgNPs binary and COs-Ps-AgNPs ternary solutions were prepared by mixing –under vigorous stirring– the necessary volumes of each solution in order to obtain a concentration of 10 mg/mL of COs, 1 mg/mL of Ps and 10 µg/mL of AgNPs in every solution. The AgNPs content was kept to a minimum to preserve the stability of the nanoparticles.

In order to characterize the mixtures and identify the interaction of the chemical functional groups, the samples in solution were freeze-dried (lyophilized) for 24 hours and their infrared spectra in the 400-4000 cm^{-1} spectral range was measured using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 FT-IR Spectrometer, equipped with an in-built diamond attenuated total reflection (ATR) system.

2.3. *In vitro experiments*

To assay the anti-fungal/oomycete activity, a typical *in vitro* mycelial growth inhibition test was performed. Indeed, the experimental design consisted of a factorial scheme with three factors: (1) COs (presence/absence), (2) Ps (presence/absence) and (3) AgNPs (presence/absence). So, the anti-fungal/oomycete activity of the three compounds separately and their binary and ternary combinations was analyzed for each pathogen (Figure 1). Each solution was uniformly incorporated at a ratio of 1:10 (v/v) into PDA after its sterilization for 20 min at 121 °C, as described by Wang et al. (2014), obtaining a final concentration of 1 mg/mL of COs, 0.1 mg/mL of P and 1 µg/mL of AgNPs in every treatment. These concentrations correspond to the minimum inhibitory concentrations used in other similar studies (Yoksan and Chirachanchai 2010; Torlak and Sert 2013; Olicón-Hernández et al. 2015). 20 mL of the mixtures were spread in Petri dishes (9 cm in diameter) setting four replicates for each treatment.

Once the culture medium had solidified, an inoculum of every pathogen (a 5×5 mm^2 plug cut from the margins) was placed at the center of the Petri dish. Then, Petri dishes were sealed and incubated at 25 °C in the dark. The mycelial growth (g) was measured on a daily basis until the day in which the dishes of the control treatment were fully covered with mycelium (n).

The radial growth rate was calculated according to the following equation:

$$\text{Radial growth rate} = \frac{\sum_{i=1}^{n} g_i}{n} \qquad \text{Eq. (1)}$$

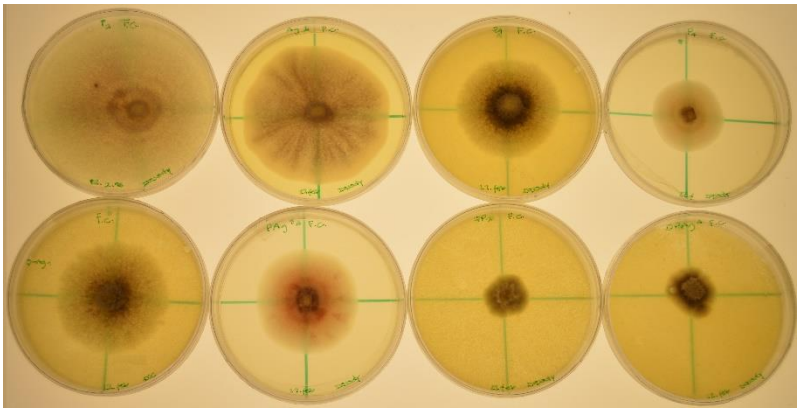

Figure 1. *In vitro* growth inhibition test for *Fusarium circinatum* (day 7). Control and treatments with AgNPs, COs, Ps, COs-AgNPs, Ps-AgPNs, COs-Ps, COs-Ps-AgNPs (*left-to-right, top-to-bottom*).

2.4. *Statistical analyses*

Analyses of variance (ANOVAs) and multiple comparison procedures were performed to test the effect of three different anti-fungal/oomycete agents (chitosan, propolis and nanosilver) and their combinations on the mycelia growth of the eight forest pathogens. As the raw data violated two ANOVA assumptions (normality and homogeneity of variances), robust methods were applied (García Pérez 2011). In particular, three-way fixed factor ANOVAs were performed under non-normality and inequality of variances, using the generalized Welch procedure, a 0.2-trimmed mean transformation and alpha value of 0.05. ANOVAs were carried out using the "Wilcox' Robust Statistics (WRS2)" package, in particular the functions "t3way" and "lincon" (see Wilcox (2016)), implemented in the R software environment (R Development Core Team 2016).

3. Results

3.1. *Aqueous solutions characterization*

Insight into the interaction of COs with the functional groups (phenolic and acids) from Ps and into the chelation of AgNPs in the binary and ternary aqueous solutions was gained by attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy. The vibrational spectra of the COs, COs-Ps binary and COs-Ps-AgNPs ternary mixtures were depicted in Figure 2.

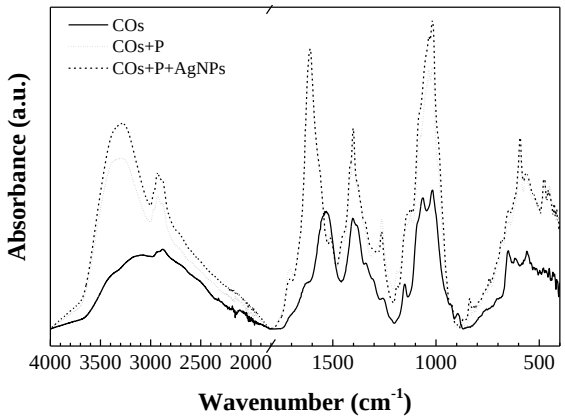

Figure 2. ATR-FTIR spectra of chitosan oligomers (COs), binary solution of chitosan oligomers and propolis (COs-Ps) and ternary solution of chitosan oligomers, propolis and silver nanoparticles (COs-Ps-AgNPs). A break has been inserted in the *x*-axis at 1800 cm^{-1} to allow a clearer representation of the fingerprint region.

The COs spectrum (solid line in Figure 2) showed the characteristic absorption peaks of chitosan at 3256 cm^{-1} (stretching vibration of the O-H and N-H bonds); at 1633 and 1550 cm^{-1} (amide I (C=O stretching) and to N-H (amine) vibration overlapped to amide II (N-H vibration), respectively); at 1152 cm^{-1} (C–O in oxygen bridges resulting from deacetylation of chitosan); and at 1065 and 1018 cm^{-1} (C-O-C and C-O vibrations).

The spectrum of the binary mixture of COs-Ps (dotted line in Figure 2) sensitized the interaction between the two components by significant changes *vs*. the COs spectrum, caused by the bonded Ps components (mainly flavonoids and lipids). An increase in the intensity of the bands at 1165 cm^{-1} (C–O and C–OH vibration), 1434 cm^{-1} (C–H vibration), 1508 and 1610 cm^{-1} (aromatic ring deformations), and 1681 cm^{-1} (C = O stretching) took place. Another important difference between the COs-Ps and COs spectra was a shift of the band associated to $\nu(C_{\Phi}\text{-}O)$ from 1257 cm^{-1} to 1263 cm^{-1}, which occurs when hydrogen bonding between COs and phenolic groups from Ps components takes place.

The lyophilizate of the ternary mixture COs-Ps-AgNPs (dashed line in Figure 2) showed a very similar pattern to the infrared spectrum of the COs-Ps binary mixture, albeit with a decrease in intensity for the bands at 1721, 1271 and 1130 cm^{-1}. This change, unaccompanied by a shift in the bands, suggests weak bonding of NH_2-AgNPs.

3.2. *Anti-fungal activity*

All individual agents and some mixtures demonstrated the ability to reduce the mycelial growth of fungi (Figure 3), in particular those with COs, however, their antifungal activity was dependent on the particular type of pathogen assayed. With regard to *F. circinatum* fungi (Figure 3*a*), an interaction

amongst the three agents was observed in the Post-hoc analysis (F=354.6, p<0.001). Nevertheless, the binary mixture of COs-Ps showed the best antifungal effect, where the radial growth rate (1.4 mm·day^{-1}) was 5.6 times lower than of the control treatment (8.0 mm·day^{-1}), corresponding to 82% of inhibition. In this case, the addition of AgNPs did not increase the effectiveness of the COs-Ps binary solution, whereas it reduced the effect of Ps and COs as individual compounds, which presented a high capacity to inhibit the mycelial growth, with a 68% and 53% of inhibition, respectively. However, AgNPs by itself, although in a lesser extent, also inhibited the mycelial growth regarding the control (27% of inhibitions).

The treatments against *D. pinea* (Figure 3*b*) also showed an interaction amongst the three agents in the Post-hoc analysis (F=7.4, p=0.02). The binary mixture COs-AgNPs showed the best antifungal effect, with a radial growth rate of 0.9 mm·day^{-1}, over 4.3 times lower than the control treatment (3.9 mm·day^{-1}), that is equivalent to 77% of inhibition. No significant differences were found considering the addition of Ps in the ternary mixture (COs-Ps-AgNPs). Nonetheless, the binary mixture of COs-Ps showed a 69% of inhibition. The separate application of COs, Ps and AgNPs also evinced some antifungal activity, with lower radial growth rates of 1.7, 2.4 and 3.2 mm·day^{-1}, i.e., 55, 37 and 18% of inhibition, respectively.

With regard to *G. abietina* (Figure 3*c*), there was an interaction between COs and AgNPs (F=6.6, p=0.03). The best antifungal effect was associated to COs, which caused a 78% reduction of the growth rate. On the contrary, the addition of AgNPs to COs not only did not improve the antifungal activity, but had a counter-productive effect. No significant differences were found with AgNPs solution in comparison to the control.

In relation to *C. parasitica* ascomycete (Figure 3*d*), the results showed an interaction between COs and Ps (F=371, p=0.001), and the effectiveness of COs both with and without Ps was around 93%. This study seems to point out that there is no advantage in adding Ps to the COs, in spite of that the inhibition percentage of the individual Ps solution was also high (2.35 mm day^{-1}, 64% inhibition).

The treatments on *H. annosum* basidiomycete (Figure 3*e*) showed a high antifungal activity of individual COs solution (86% of inhibition), without any interactions amongst the three elements, so it may be inferred that the use of Ps and AgNPs, by themselves, or in addition to COs did not significantly increase the inhibitory effect.

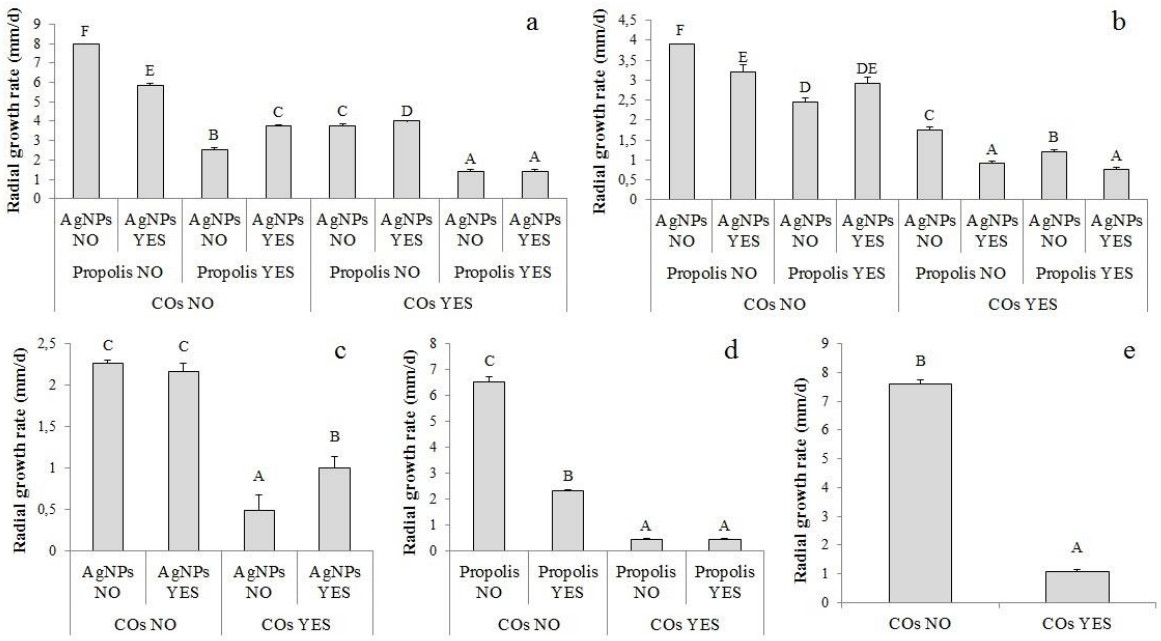

Figure 3. Radial growth rate and interaction among treatments based on chitosan oligomers (COs), Propolis and silver nanoparticles (AgNPs) against (**a**) *F. circinatum*; (**b**) *D. pinea*; (**c**) *G. abietina;* (**d**) *C. parasitica*; and (**e**) *H. annosum* fungi. Different letters above bars indicate significantly different means (generalized Welch procedure 0.2 trimmed means, a = 0.05). Error bars show the standard deviation. Note: Only significant interactions from the Post hoc analyses are shown.

3.3. *Anti-oomycete activity*

As regards the assays conducted with oomycetes (Figure 4), a remarkable inhibitory activity was attained for COs and Ps. Treatments against *P. cambivora* (Figure 4*a*) evidenced an interaction among the three agents (F=64.1, p=0.001), but all treatments with COs (individual, binary and ternary mixtures) presented 100% of growth inhibition. The treatment with the individual Ps solution also showed growth inhibition (43%), but AgNPs and Ps-AgNPs treatments did not exhibit any significant differences *vs*. the control.

On the other hand, an interaction between COs and Ps was found in treatments against *P.* ×*alni* and *P. plurivora* (F=23, p=0.002 and F=722.8, p=0.001, respectively). While the application of COs and Ps (individual or mixed) resulted in a similar growth inhibition for *P.* ×*alni*, the addition of Ps played a leading role in the growth inhibition for *P. plurivora* (Figure 4*b* and Figure 4*c*).

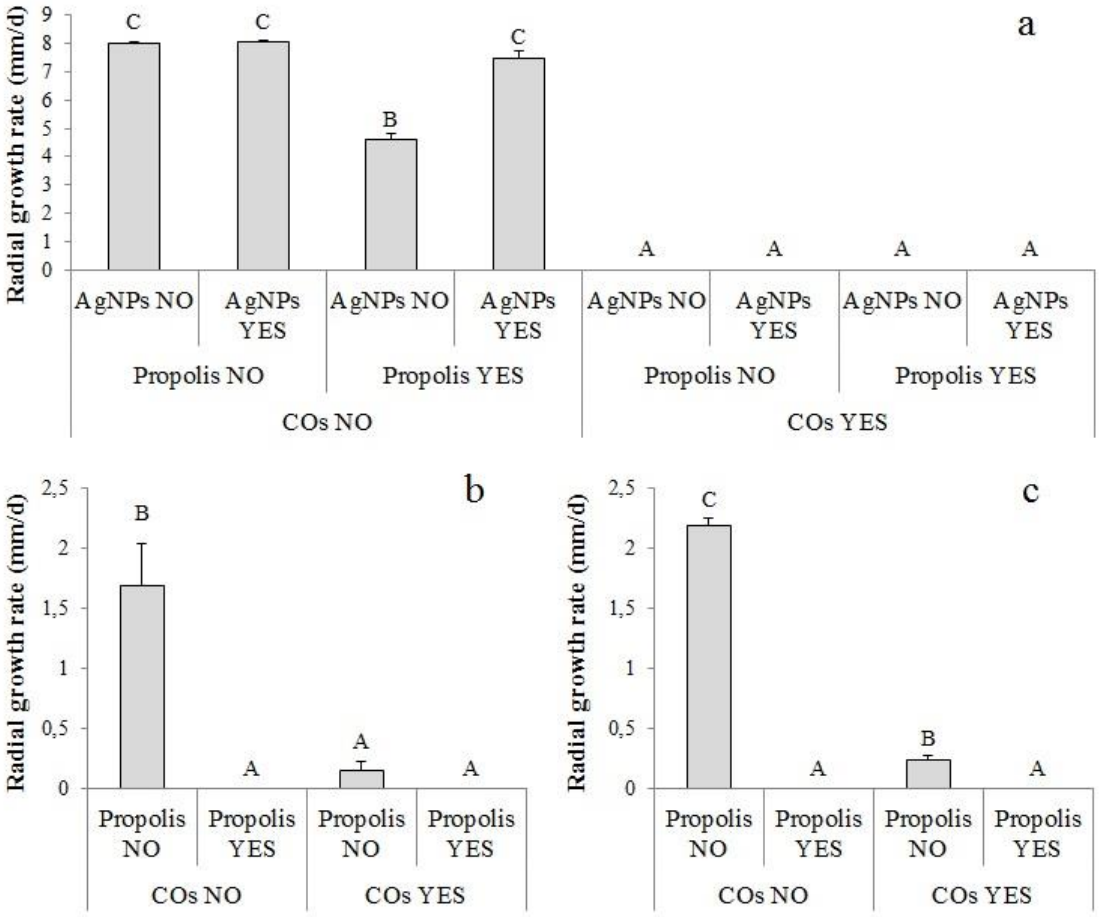

Figure 4. Radial growth rate and interaction among treatments based on chitosan oligomers (COs), Propolis and silver nanoparticles (AgNPs) against (**a**) *P. cambivora*; (**b**) *P. ×alni*; and (**c**) *P. plurivora* oomycetes**.** Different letters above bars indicate significantly different means (generalized Welch procedure 0.2 trimmed means, a = 0.05). Error bars show the standard deviation. Note: Only significant interactions from the Post hoc analyses are shown.

4. Discussion

The present study has demonstrated that the three compounds (COs, Ps and AgNPs) have an antifungal effect on different forest pathogens. COs by itself showed an inhibitory effect on the mycelial growth of all pathogens tested. Although the antifungal activity of chitosan polymer has been already reported by other authors both in *in vitro* and *in vivo* experiments, for example, chitosan applications to increase the resistance of pine seedlings to *F. circinatum* and *D. pinea* (Reglinski et al. 2004; Fitza et al. 2013), this study confirms the importance of the use of low molecular weight chitosan such as COs. It is worth noting that when chitosan with higher molecular weight than that used in this study (e.g., 50,000 Da instead 2,000 Da) are applied, a lower antifungal activity is attained, with 35% of reduction of mycelial growth of *D. pinea* in the first day as reported by Singh

et al. (2008). This is consistent with the results reported by Avelelas et al. (2014), Qiu et al. (2014) and Cobos et al. (2015), who demonstrated that chitosan antifungal activity increased in inverse proportion to its molecular weight. Consequently, COs of molecular weight under 2,000 Da, might be a preferable option as compared to commercial 'low molecular weight' chitosan (i.e., 50,000 to 190,000 Da, CAS Number 9012-76-4) in terms of its activity against *D. pinea*. On the other hand, in an *in vitro* study by Ziani et al. (2009), the use of chitosan solutions proved to be more effective against *Aspergillus niger*, *Alternaria alternata* and *Rhizopus oryzae* than the use of films, where presumably, the chitosan solution had positive charges on the quaternary amino groups that interacted with the fungal cell walls, while for the films a protonation loss occurred.

The inhibitory effect of Ps was demonstrated on most of the pathogens tested (*F. circinatum*, *D. pinea*, *C. pararisitica*, *P. cambivora*, *P. ×alni* and *P. plurivora*). The use of propolis has not been as well studied as chitosan, although its inhibition capacity against *F. circinatum* was already reported by Iturritxa et al. (2013). However, they reported a fungicidal effect, whereas in this study a growth inhibition effect was found.

The application of AgNPs by itself also reduced the mycelial growth of *F. circinatum* and *D. pinea*, which is consistent with the results reported by Narayanan and Park (2014), who observed slight to moderate inhibition against wood-degrading fungi when a low dose of AgNPs was used. Nevertheless, AgNPs had not a significant anti-oomycete activity on the species tested in this study, contrasting with Mahdizadeh et al. (2015), who found that another oomycete (*Pythium aphanidermatum* (Edson) Fitzp.) was the most sensitive pathogen to nanosilver among the six tested species.

The effect of the binary solutions of the compound tested varies according to the species. While the COs-Ps binary solution showed the highest antifungal effect against *F. circinatum*, the result of the application of AgNPs in the binary solutions varies according to the pathogen. Indeed, the binary solution COs-AgNPs recommended by Wang et al. (2015) was the most promising mixture in order to control *D. pinea*. Nevertheless, the use of COs-AgNPs and Ps-AgNPs solutions had a counter-productive effect on the anti-fungal/oomycete activity against *G. abietina* and *P. cambivora*, respectively. This is in contrast to other studies in which nanosilver was also incorporated into chitosan, although in higher doses. For example against ascomycete *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., the mixture showed excellent results: the inhibitory action increased from 44% to 100% as the AgNPs concentration was increased from 0.1 up to 100.0 μg/mL (Chowdappa et al. 2014). It is also noteworthy that the solution consisting only of AgNPs did not show statistically significant difference *vs*. the control treatment, in contrast to the study by Narayanan and Park (2014),

who observed slight to moderate inhibition against wood-degrading fungi when a low dose of AgNPs was used. In the same vein, Saharan et al. (2013) and Saharan et al. (2015) found that the nanocopper-chitosan complex showed growth inhibition against other ascomycota such as *Fusarium oxysporum* and *A. alternata*. They suggested that addition of nanometals increased the surface charge density and provided more electrostatic interaction with fungal membrane.

Differences in the inhibitory behavior of the similar COs-Ps-AgNPs mixture have been reported for other fungal species and different application procedures: the COs-Ps-AgNPs ternary complex did not improve the antifungal/anti-oomycete activity compared to the binary solutions in this study, which contrasts with the complete inhibition obtained using similar COs-Ps-AgNPs mixtures applied to *D. seriata* and *Bipolaris oryzae* (Breda de Haan) Shoemaker (Matei et al. 2015; Araujo-Rufino et al. 2016). This discrepancy may be associated to that the gel phase used was ascribed to the higher concentrations of chitosan oligomers in the gel (20-25 mg/mL) *vs.* the aqueous solution of this study (1 mg/mL).

The bands in the ATR-FTIR spectrum of the COs-Ps-AgNPs composite evidenced a weak interaction among COs and AgNPs, even weaker than that reported for chitosan-AgNPs thin films and nanocomposites manufactured by spin-coating (Wei et al. 2009; Wang et al. 2015), whose infrared spectra showed shifts between 5 and 10 cm^{-1}. On the other hand, the spectrums of COs and COs-Ps showed very similar bands to those reported in other works for chitosan (Matei et al. 2015; Stroescu et al. 2015; Branca et al. 2016) and propolis extracts (Franca et al. (2014); Siripatrawan and Vitchayakitti (2016).

A differential feature of this investigation in comparison to the literature was that in the preparations described above Green Chemistry procedures were used, without need for the addition of chemical bond reinforcing agents, widely used in other works (Gu et al. 2014; Jemec et al. 2016). Accordingly, these eco-friendly compounds could be useful in management strategies based on integrated approach, for example in the use of appropriate nursery hygiene practices. Likewise, the application of chitosan had been suggested using the chitosan-based Biochikol 020 PC, a biological agent with fungicidal properties and resistance stimulator, in order to control *P. xalni* complex in forest nurseries (Oskazo 2007).

In conclusion, from the results of the *in vitro* growth inhibition experiments respect the anti-fungal/oomycete effect of individual, binary and ternary mixtures of COs, Ps and AgNPs, assayed against eight plant pathogens, it could be inferred that: (*i*) the inhibitory activity against fungi and oomycetes of the individual low molecular weight COs solutions was significantly high (reaching growth rate reductions of up to 78, 86, 93% and 100% against *G. abietina, C. parasitica, H. annosum*

and *P. cambivora*, respectively); (*ii*) the growth inhibition is enhanced by association of COs with Ps (e.g., *F. circinatum*) and COs with AgNPs (e.g. *D. pinea*); and (*iii*) the COs-P-AgNPs ternary complex did not improve the antifungal/anti-oomycete activity compared to the binary solutions. Thus, the weak interactions that appear in solution amongst the three components (evidenced by FTIR) suggested that strong interactions are necessary to achieve the desired anti-fungal/oomycete effect. Additionally, further studies are essential to determine the effect of the COs-Ps-AgNPs combinations on seeds, tree seedlings and mature trees infested by different pathogens, as an innovative application system useful in an integrated management approach.

5. Acknowledgments

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6. Compliance with Ethical Standards

The authors declare that they have no conflict of interest.

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Artículo 4

Control de la roya de las hojas del café con oligómeros de quitosano y propóleo

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CONTROL OF COFFEE LEAF RUST BY CHITOSAN OLIGOMERS AND PROPOLIS

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Abstract

Broad scale use of chemical pesticides is known to have produced broad scale environmental impact worldwide and, at the same time, discovery and registration of new molecules to be used as insecticides, herbicides and fungicides have slowed significantly along the last decades, reducing the number of options for pest control by the farmers. Searches for novel environmentally friendly products are a recognized priority. In this work, aqueous solutions of chitosan oligomers (COs) and propolis (P), individually or as binary mixtures, were tested against Hemileia vastatrix, the fungus which causes coffee leaf rust, the worst disease of coffee. Germination of H. vastatrix was inhibited up toll 99 % by COs, followed by the mixture of COs-P and P individually (germination inhibition of 96 % and 54 % respectively). Testing those products on detached coffee leaf discs also produced promising results when COs were applied before H. vastatrix inoculation. These results may lead to the discovery of new antifungal products for the control of coffee leaf rust.

***Key words**:* chitosan oligomers, *Hemileia vastatrix*, natural fungicide, propolis.

INTRODUCTION

Coffee, a tropical crop, one of the most important agricultural products in economic terms, which consumption reached more than 9 thousand of millions of tons last year (ICO, 2017), is especially affected by the differences in their environmental conditions (Cristancho et al., 2012). Coffee leaf rust (CLR), caused by *Hemileia vastatrix* Berk. & Br., a biotrophic basidiomycete fungus, is the most devastating disease affecting coffee crop (Morris, 1880). It was reported from the first time in cultivated coffee in Sri Lanka in 1869, and occurs in almost all producing countries from 1984 (Talhinhas et al, 2017). An unusual increase in disease incidence and high leaf severities was observed from 2008 to 2013 in Central and South America (Avelino et al., 2015) over *Coffea arabica*, the main cultivated variety (ICO, 2017).

Currently, control methods are based on improving varieties to increase plant resistance (Gatica-Arias et al., 2017), escaping the disease through planting coffee in higher altitudes and, fungicide applications. Chemical pesticides are known to have produced broad scale environmental impact worldwide and, at the same time, discovery and registration of new molecules to be used as insecticides, herbicides and fungicides have slowed significantly along the last decades, reducing the number of options for pest control by the farmers (Ștefan et al., 2015; Cosoveanu et al., 2016). However, the integration of natural products with antifungal activity against *H. vastatrix*, that reduces or eliminates the impact of the application of conventional fungicides on the environment, represents one more alternative for the management of CLR.

In that sense chitosan, a polysaccharide obtained from deacetylation of chitin, the second natural polymer most abundant (Azuma et al., 2015; Miteluț et al., 2015), is promising. Nonetheless, the size of the polymer chain plays a key role (Harish et al., 2007) chitosan oligomers have showed a great antifungal activity against many phytopathogens fungi (Silva-Castro et al., 2018, Matei et al., 2010). Another natural product with prospects of

being used to control agricultural diseases is propolis, its ethanolic extracts contains flavonoids and polyphenols, compounds attributed to it antifungal activity (Matei et al., 2015). In this work, solutions of chitosan oligomers (COs), propolis (P) and their mixture were assessed against *H. vastatrix* such as potential alternative to control CLR.

MATERIALS AND METHODS

Aqueous solutions

Chitosan oligomers (COs) were obtained from powered medium molecular weight chitosan (Hangzhou Simit Chemical Technology Co., Ltd., Hangzhou, China)) dissolving 10 g in 500 mL of acetic acid (1%) under constant stirring at 60°C. Once dissolved, hydrogen peroxide (0.3 mol/L) was added for the degradation of the polymer chains, obtaining oligomers of less than 2000 Da (Sun et al., 2007). Propolis (P), from the Duero basin (Burgos, Spain) was introduced in a hydroalcoholic solution 7:3 (v:v) and kept in vigorous agitation until its complete dissolution. In order to obtain the binary mixtures, both solutions were mixed at desired concentration.

Fungal pathogen

Urenidiospores of *H. vastatrix* were collected from infected leaves of *C. arabica* (var: Caturra) from a greenhouse plantation of the Departament of phytopathology-UFV, MG, Brazil. The spores were suspended in sterile water with Tween 80 (10%) at 1×10^{-6} spore mL^{-1}. This concentration of spore solutions was used for all experiments.

Bioassay on spore germination

The inhibitory effect of chitosan oligomers and propolis on germination of urenidiospores of *H. vastatrix* was tested *in vitro* on glass slides placed 5×10^{-5} spore mL^{-1}, 25 µL of spore suspension and 25 µL of each solution: COs P, COs-P and water (control). Four replicates of each treatment were placed in a humid chamber at 22°C and 90 % relative humidity (RH) in dark for 6 hours. The germination inhibition percentage was calculated by the following equation:

$$\text{Germination inhibition \%} = (c - x / c) \times 100$$

Where, c=germinated spores in the control and x=germinated spores in the treatments.

Note: When the germ tube size of the urediniospore was larger than the diameter of itself, it was considered germinated.

Bioassay on disease severity

The inhibitory potential of the COs, P and their mixture on spore germination of *H. vastatrix* was tested measuring the severity of disease on *Coffea arabica* leaves using the leaf disc method (Eskes, 1989). An aliquot of 25 µL of inoculum (urenidiospores suspension) was placed on leaf discs (15 mm diameter) in three different times: 24 hours before (T1), 24 hours after (T2), and at the same time (T3) that application of 25 µL of COs (2 mg mL^{-1}), P (0.2 mg mL^{-1}), COs-P (2 and 0.2 mg mL^{-1} respectively) and water (control). The leaf discs were placed inside plastic box filled with 100 mL of sterile water in order to obtain a high humidity. The boxes were kept for 30 days under controlled conditions (photoperiod: 12 h light/12 h dark and 22 °C). Twelve repetitions were used for each treatment and the experiment was repeated twice. The severity of the disease was evaluated 30 days after inoculation following the scale proposed by Silva et al., 2012 (Table 1).

Table 1. Coffee leaf rust severity scale on leaves

Class	Scale	Severity (%)
0	0	0
1	> 0 a 2.5	1,25
2	> 2.5 a 5	3,75
3	> 5 a 15	10
4	> 15 a 25	20
5	≥ 25	25

Data analysis

Analyses of variance (ANOVAs) and multiple comparison procedures were performed to test the effect of COs, P and COs-P against *H. vastratrix* in both experiments. Under non-normality and homoscedastic data, for the disease severity assay, were used Kruskal-

Wallis tests implemented in the R software environment (R Development Core Team 2016).

RESULTS AND DISCUSSIONS

Effect on spores' germination

The efficacy of aqueous and hydroalcoholic solution based on chitosan oligomers and propolis against the urenidiospore germination of *H. vastatrix in vitro* assay are depicted in Figure 1 and 2.

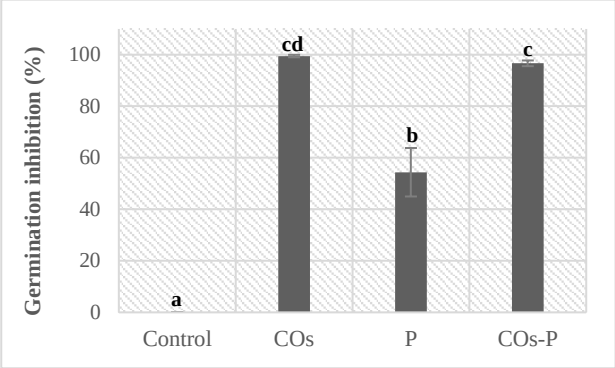

Figure 1. Percentage of germination inhibition of *H. vastatrix* urenidiospore *in vitro* assay. Letters above bars indicate significantly different means (*p* value = 0.05). Error bars show the standard deviation.

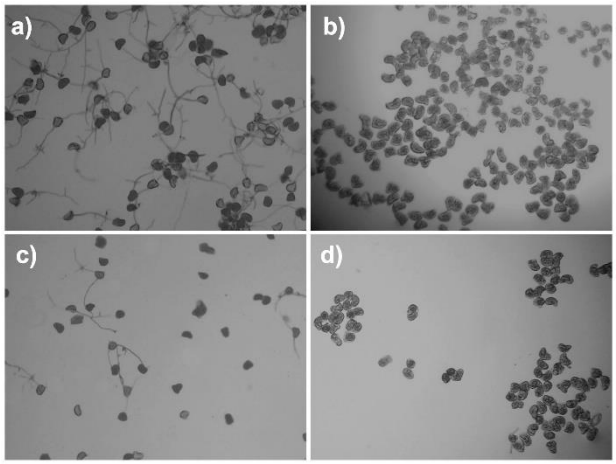

Figure 2. Inhibitory effect on germination of *H. vastatrix* by aqueous solutions: **a** control (H_2O), **b** chitosan oligomers (COs), **c** propolis (P) and **d** their mixture (COs-P). Optical microscope 20x (a,c and d) and 40x (b).

All three treatments exhibited a reduction of percentage of germination compared to control (F=118.7, p<3.48e-09). In fact, chitosan oligomers (COs) at 2 mg mL^{-1} inhibited 99,5 % of the germination of *H. vastatrix* spores (Figure 2-b). The high efficiency of low molecular weight chitosan in inhibition of spore germination have been reported for *Botrytis cinerea* and *Mucor piriformis*. Also, abnormal swelling of the conidia of *M. piriformis* and granular inclusions in the cytoplasm of both fungal species was found after chitosan application (Rahman et al., 2015).

The mixture chitosan oligomers and propolis (COs-P), showed a slight percentage of germination inhibition (96.6 %), less effective than COs individually (F=6.391, p=0.0448), that could mean that the addition of propolis extract produces an interaction with chitosan polymer chains, reducing the antifungal activity of COs (Silva-Castro et al., 2018).

Otherwise, propolis solutions (P) 0.2 mg mL^{-1} inhibited 54.4 % the germination of the fungus. The effect caused by propolis is very relevant considering the solution concentration ten times less than COs, actually, propolis have been a great antimicrobial and also antifungal applying low doses (Silva-Carvalho et al., 2015).

Effect on disease severity

The effectiveness of COs, P and their mixture on CLR severity was tested in different times. The T1 assay (pathogen was firstly placed and after 24 hour the treatments were applied), does not shown significant differences compared to control and the other treatments (Figure 3).

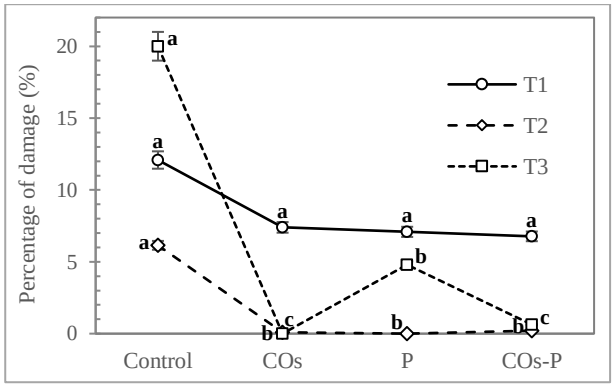

Figure 3. CLR severity on coffee leaves under different treatments and timing of application. Letters above bars indicate significantly different means (*p* value = 0.05). Error bars show the standard deviation.

Due to the fact that fungus infection on the leaf occurs during the first 24 hours of contact (Avelino et al., 2015), the subsequent presence of COs, P and COs-P did not cause a significant impact against it. Finally was

registered a percentage of damage between 7 and 12 % over leaves.

The T2 assay, designed to prevent fungal contamination (treatment applied 24 hou before fungal inoculation), presented the highest antifungal capacity of COs, P and COs-P, which allowed just 0.2 % of damage on leaves after 30 days, no significant differences among this three treatments being recorded Hence, this application suggests the use chitosan as preventive treatment, which could be very useful in the disease management program. That represents an easy-to-reach tool, meanwhile, the studies to learn more and bette about the evolutionary genetics of the fungus and the factors that intervene in the disease continue to develop (Toniutti et al., 2017 and Silva et al., 2018).

In the T3 assay (treatments in the same time with fungal inoculation) , there was differences between COs and P applications, leaves with COs shown zero percentage of damage, versus leaves with P, that revealed 5 %. However, the effect of this treatment was very important in comparison with control, where *H. vastatrix* caused until 20% of damage on coffee leaves (Figure 4).

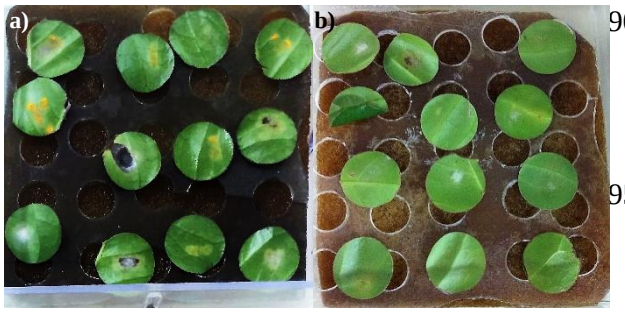

Figure 4. Effect of chitosan oligomers over *H. vastatrix* (caused CLR) on coffee leaves discs: **a** control (H_2O) and **b** chitosan oligomers (COs), 30 days after of treatments application.

Applications of low molecular weight chitosan, have been evaluated in another bioassays with strawberry flowers, in which chitosan oligomers reduced until 60 % of flower infection caused by *B. cinerea* (Rahman et al., 2015) similar results than the present study, where the damage was reduced from 75 % until 100 % in the T2 case.

CONCLUSIONS

The chitosan oligomers, as have been proved in this study, have a great capacity to inhibit urenidiospore germination of *H. vastatrix*. Also propolis, another product of natural origin, exhibit antifungal activity in both *in vitro* and *in vivo* assays, however, its effect could be improved increasing the doses to apply.

Combinations of both products show more or less the same result than chitosan individually. Nonetheless, products based on chitosan oligomers are a promise tool, especially in the prevention of diseases, to add to the health management program of coffee production in order to combat the coffee leaf rust caused by *H. vastatrix*.

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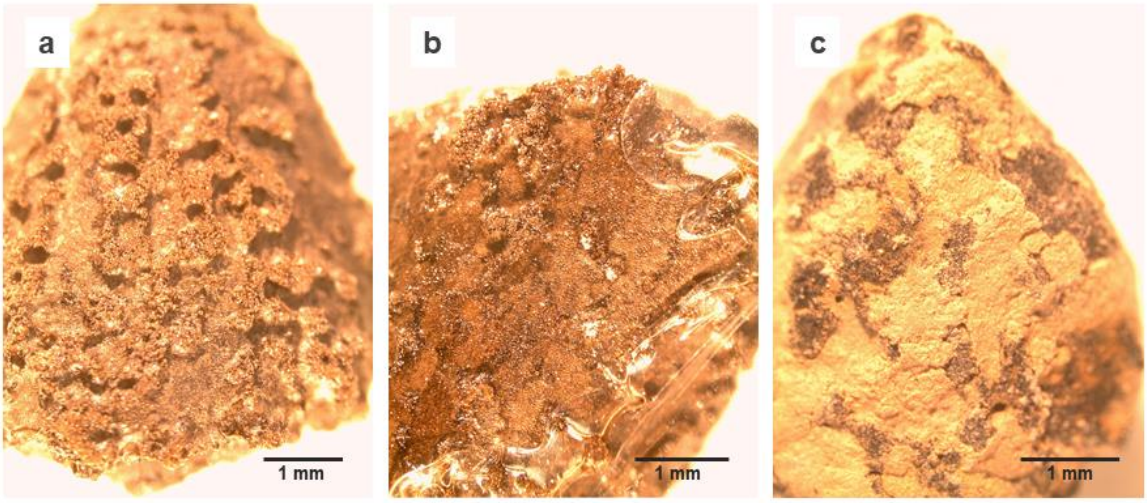
Artículo 5

Aplicación de recubrimientos bioactivos de quitosano y propóleo para la protección de especies de pinos contra *Fusarium circinatum*



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Article

Application of Bioactive Coatings Based on Chitosan and Propolis for *Pinus* spp. Protection against *Fusarium circinatum*

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Abstract: Pine pitch canker (PPC) is a major threat to pine forests worldwide because of the extensive tree deaths, reduced growth, and degradation of timber quality caused by it. Furthermore, the aggressive fungus responsible for this disease (*Fusarium circinatum*) can also infect pine seeds, causing damping-off in young seedlings. This study proposes an approach based on coating treatments consisting of natural products to ensure seed protection. Seeds from two pine species (the most sensitive to this disease, *Pinus radiata* D. Don, and a more resistant one, *Pinus sylvestris* L.) were coated with single and binary mixtures of low and medium molecular weight chitosan and/or ethanolic-propolis extract. The germination rate, pre- and post-emergence mortality, total phenolic content, and radical scavenging activity were assessed. All treatments, and especially the one based on chitosan oligomers, had a beneficial impact on *P. sylvestris* seedlings, significantly enhancing survival rates and displaying a positive influence on the total phenolic content and on the seedlings' radical scavenging activity. Conversely, non-significant negative effects on germination percentages were observed in the case of *P. radiata* seeds. The proposed treatments show promise for the protection of *P. sylvestris* seedlings against PPC.

Keywords: antifungal; antioxidant; natural coating; seed protection; total phenolic content

1. Introduction

Fusarium circinatum Nirenberg & O'Donnell is a quarantine fungus according to the European and Mediterranean Plant Protection Organization (EPPO) [1] that causes pine pitch canker (PPC) and which has been deemed as one of the most damaging pathogens for *Pinus* spp. throughout the world [2]. In forest nurseries, *F. circinatum* causes pre- and post-emergence damping-off, wilting of seedlings, shoot and tip dieback, and it finally leads to the death of the infected seedlings [3]. *F. circinatum* can be found in nurseries of North and South America, South Africa, Asia, and Southern Europe [4]. The use of seeds from orchards poses a serious threat of spread of this fungus to

nurseries worldwide [5]. At present, there are no effective means of controlling PPC in nurseries and forest plantations. An integrated management plan should therefore include both adequate quarantine measures and appropriate nursery and silvicultural management strategies. Thus, the implementation of seed protection in nursery health practices would be of paramount importance.

Seed-coating technology may act not only as a phytosanitary against pests and diseases but may also enhance germination rates and crop yield [6]. Chemicals such as imidacloprid and tebuconazole [7,8] have been extensively used as coatings for seeds, but nowadays their use in forests is highly restricted (Directive 2009/128/EC). Consequently, natural substances and biological agents—such as starch [9] and *Trichoderma* spp. [10,11], respectively—are receiving increasing attention as environmentally friendly alternatives [12,13].

Amongst these natural products, chitosan and propolis have shown great promise for plant protection purposes, and, in particular, against *F. circinatum* [14–16]. Chitosan, obtained from chitin's deacetylation, is an organic polymer with a cationic character, which confers numerous physicochemical and biological properties, such as copolymerization, filmogenicity, biocompatibility, biodegradability, and also antibiotic properties [17–20]. In turn, propolis is a chemically very complex resinous bee product with many biological properties [21]. Due to its composition, mainly flavonoids and phenolic acids, it is able to alter membrane permeability and inhibit protein synthesis in microorganisms [22].

For agricultural applications, chitosan is applied as an elicitor (inducer of plant resistance) and antifungal product because of its ability to induce the synthesis of phenolic compounds [23], which are involved in tolerance mechanisms against biotic or abiotic stressors [24]. Among the large number of phenolic antioxidants, flavonoids, for instance, can directly inhibit microbial enzymes production [25]. Other specific flavonoids, such as anthocyanins, are known to increase the antioxidant activity, reducing the susceptibility to fungi [26]. The total phenolic content (TPC) and/or the radical scavenging activity (RSA) are properties commonly analyzed in order to identify responses caused by elicitors in plants [27,28].

The molecular weight of chitosan plays a key role in its fungicide properties [29], in such a way that low molecular weight chitosan (i.e., oligomers) is more effective at inducing a set of plant defense responses than its higher molecular weight counterpart (i.e., polymers) [30]. In spite of the fact that chitosan oligomers feature better antimicrobial activity than high molecular weight chitosan [31,32], the later has a higher viscosity [33], which explains why it is more frequently used as a coating [34,35].

In view of the chemical affinity and well-established synergies between chitosan and propolis [36–38], the main aim of the work reported here was to evaluate the protection conferred by bioactive seed coatings based on chitosan with two different molecular weights—medium (CM_{MW}) and low (CL_{MW})—and propolis ethanolic extract (PEE) composites against *F. circinatum* in the relatively resistant *Pinus sylvestris* L. and in the highly susceptible *Pinus radiata* D. Don.

2. Materials and Methods

2.1. Fungal and Plant Materials

The *Fusarium circinatum* isolate FcCa6 used in this study was obtained from the collection of the Forest Entomology and Pathology Laboratory at the University of Valladolid, Spain [39–43]. Plant material consisted of seeds of *P. radiata* and *P. sylvestris* (see provenance in Table 1).

Table 1. Provenance of plan material.

Seed Species	Provenance	Provided by
Pinus radiata (Monterey pine)	"Galicia montañas meseta Interior" (Spain)	Consellería do Medio Rural (Xunta de Galicia, Spain)
Pinus sylvestris (Scots pine)	"Sierra de Guadarrama" (Spain)	El Serranillo Nursery (Ministry of Agriculture and Environment, Spain)

2.2. Seed-Coating Preparation

2.2.1. Reagents

In order to obtain the coating material, medium molecular weight chitosan powder, purchased from Hangzhou Simit Chemical Technology Co. (Hangzhou, China), and propolis with a content of poly-phenols and flavonoids of ca. 10% (*w*/*v*) from Burgos (Spain) were used. High specific surface (70–85 m^2/g) halloysite in powder form, from Dunino mine, with reduced iron content (ca. 5%), was purchased from Intermark (Gliwice, Poland). All other reagents (viz., acetic acid, hydrochloric acid, hydrogen peroxide, ethanol, Tween 80, Folin–Ciocalteu reagent, 2,2-diphenyl-picrylhydrazyl, etc.) were of analytical grade and were purchased from Sigma-Aldrich Química S.L. (Madrid, Spain).

2.2.2. Preparation of the Seed Coating Solutions

Due to differences in viscosity, and therefore in the adhesion to the seed surface, chitosan with two different molecular weights was assessed. The medium molecular weight chitosan (CM_{MW} 60–130 kDa) was prepared by dissolving 2 g of commercial chitosan in 100 mL of acetic acid solution (1% *v*/*v*) under constant stirring at 60 °C for 2 h until its complete dissolution. To obtain the low molecular weight chitosan (CL_{MW} 20 kDa), it was necessary to add hydrogen peroxide (0.3 M) to the chitosan solution obtained in the previous step, keeping the same conditions until a brown and less viscose solution was obtained after 1 h [44]. Propolis ethanolic extract (PEE) composites were prepared by introducing the finely grinded resin into a hydroalcoholic solution (7:3 *v*/*v*). After stirring for 72 h at room temperature, the insoluble particles were filtered [45].

To obtain the first composite (CM_{MW}-PEE), Tween 80 was added dropwise to a 10 mg·mL^{-1} chitosan solution, followed by the addition of 1 mg·mL^{-1} of propolis solution. The mixture was sonicated with a probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) for 3 min in cycles of 1 min with sonication and 1 min without sonication to keep the temperature below 40 °C [37]. To obtain the second composite (CL_{MW}-PEE), a similar process was followed, albeit replacing Tween 80 with halloysite, a natural clay innocuous to seeds and fungus. Halloysite was added to the less viscous solutions (CL_{MW}, PEE and CL_{MW}-PEE) in order to improve their adherence to the surface of the seeds.

2.2.3. Seed Coating Application

Prior to coating application, seeds underwent the following pre-germination procedure according to Martín-García et al. [40]: they were initially soaked in water for 24 h (renewing the water after 12 h), followed by soaking in hydrogen peroxide (3%) for 15 min, triple-washing with sterile distilled water, and an immersion in sterile distilled water for another 30 min (in order to clear away any remaining hydrogen peroxide). Subsequently, the seeds were placed in a laminar flow hood in order to dry them.

Six treatments were applied: (1) Control (sterile water), (2) CM_{MW}, (3) CL_{MW}, (4) PEE, (5) CM_{MW}-PEE, and (6) CL_{MW}-PEE. Halloysite (1 g to 100 mL of solution) was applied in treatments 3, 4, and 6. Then, the seeds were dried again to form a film on their surface (Figure 1) and were kept in sterile flasks until sowing. Seventy seeds (replicates) of each pine species were prepared per treatment (i.e., a total of 840 seeds).

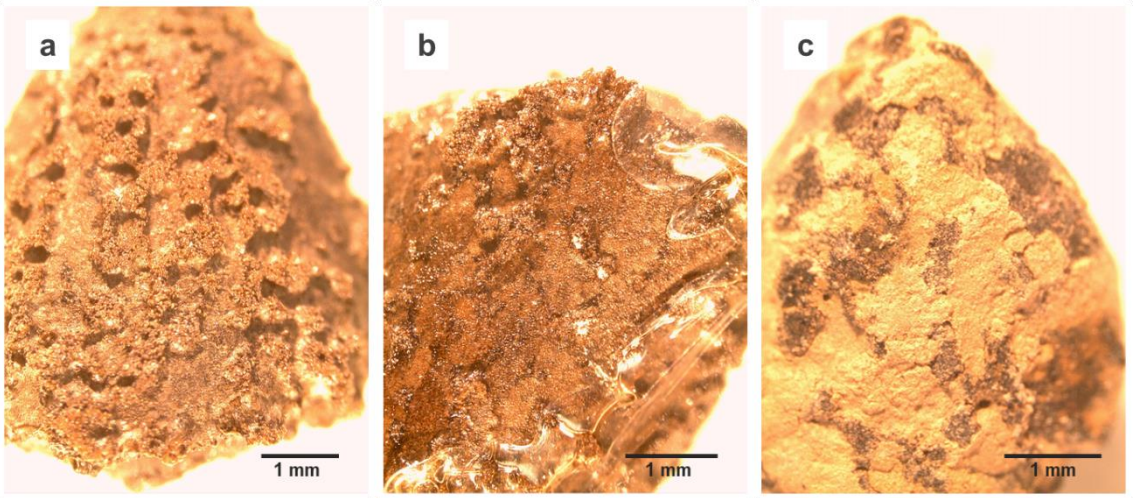

Figure 1. Film formation on seed surface of *P. radiata* seeds: (**a**) control (sterile water), (**b**) coating based on medium molecular weight chitosan (CM_{MW}) and (**c**) coating based on low molecular weight chitosan (CL_{MW}) with halloysite.

2.3. Pathogenicity Tests

Following the procedure presented in Martín-García et al. [41], a spore suspension of *F. circinatum* (Fc) was cultured on potato dextrose broth (PDB). Five mycelial agar plugs (5 mm in diameter) were added to 1 L of PDB and were placed on an orbital shaker at 180 cycles for 24 h at 25 °C. After that, the liquid medium was filtered twice through sterile cheesecloth to remove hyphae and the spore concentration was adjusted to 1×10^3 spores·mL^{-1} with a Neubauer hemocytometer.

Seventy seeds per each type of coating (plus 70 without coating) and per pine species were individually sown in germination trays (96 mL) containing a twice-autoclaved (105 kPa, 120 °C, 30 min) mixture of peat and vermiculite (1:1, *v*/*v*). For half of the seeds with each type of coating, the spore suspension of *F. circinatum* was added to the substrate when the seeds were sown. The other half of the seeds were mock-inoculated with sterile distilled water. Thus, the experimental design consisted of twelve treatments: (*i*) control, (*ii*) CM_{MW}, (*iii*) CL_{MW}, (*iv*) PEE, (*v*) CM_{MW}-PEE, (*vi*) CL_{MW}-PEE, (*vii*) Fc, (*viii*) CM_{MW}-Fc, (*ix*) CL_{MW}-Fc, (*x*) PEE-Fc, (*xi*) CM_{MW}-PEE-Fc, and (*xii*) CL_{MW}-PEE-Fc.

Incubation of the germination trays was conducted in a growth chamber under controlled conditions (temperature: 21.5 °C; photoperiod: 16/8 h light/dark). They were watered every two days, with equal water doses, all over the period of study. Seed germination and subsequent seedling mortality were monitored on a daily basis.

2.4. Determination of Total Phenolic Content (TPC) and Radical Scavenging Activity (RSA)

The extracts were obtained according to the following process: four asymptomatic seedlings were collected from each of the trays 30 days after the sowing date, including control (*i*) and inoculated treatments (*vii*) to (*xii*). These seedlings were dried (40 °C, 7 days) and grinded. Then, 20 µg of each sample in powder form was added to 2 mL of methanol (70%, vol.) acidified with a few drops of HCl (1 M). The mixture (methanol and sample) was kept in shaking condition for 2 h and filtered to get the extracts [27] that were used in both analyses (TPC and RSA).

The TPC was evaluated with a modified Folin–Ciocalteu procedure [46]: firstly, 100 µL of the sample methanolic extract was mixed with 450 µL of distilled water and 50 µL of Folin–Ciocalteu reagent. After 10 min, 400 µL of Na_2CO_3 was added and the samples were kept in dark for 90 min. The absorbance was measured at 765 nm using a Thermo Scientific Multiscan Go Microplate Spectrophotometer (Waltham, MA, USA). A calibration curve was prepared with standard gallic acid ($y = 0.0407x - 0.0595$; $r^2 = 0.99$) and used to express the results as gallic acid equivalents (GAE, in mg of gallic acid per mL of extract).

The RSA was determined by the 2,2-diphenyl-picrylhydrazyl (DPPH) method, according to the procedure described by Chiang et al. [47], with some modifications. Briefly, 50 μL DPPH radical solution (1×10^{-4} M) was added to 450 μL of the sample methanolic extract, and the reaction mixtures were kept at room temperature for 30 min. Absorbance was recorded at 517 nm. The radical scavenging activity was expressed as a percentage (RSA%) relative to the control, using the following equation:

$$RSA\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100 \quad (1)$$

where A_{blank} is the absorbance of the blank (distilled water) and A_{sample} is the absorbance of the sample extracts.

2.5. Statistical Analyses

All analyses were performed using R software environment (R Foundation for Statistical Computing, Vienna, Austria). Chi-square tests (χ^2) were carried out using the mock-inoculated treatments—(*i*) to (*vi*)—to test the effect of the coatings on germinative capacity. Likewise, chi-square tests (χ^2) were carried out using the control (*i*) and inoculated treatments—(*vii*) to (*xii*)—to test the protective effect of seed coatings on the pre-emergence mortality caused by *F. circinatum*. To prevent overestimation of statistical significance for small data, Yates' correction for continuity was applied for counts smaller than 5. To test the post-emergence mortality up to the end of the experiment (when no seedling from Fc treatment (*vii*) was alive, 40 and 30 days after sowing for *P. sylvestris* and *P. radiata*, respectively; in the case of *P. radiata*, the last four living seedlings were removed to carry out the TPC and RSA analyses), a survival analysis based on the Kaplan–Meier non-parametric estimator [48] was carried out using "Survival" package [49]. "Sirvfit" and "Survdiff" functions, also available in the same package, were used to create survival curves and to analyze the differences between the curves, respectively. Analyses of variance (ANOVAs) and Tukey's HSD (honestly significant difference) post-hoc test were carried out to assess the effect on TPC and RSA of seedling from control (*i*) and inoculated with *F. circinatum* treatments—(*vii*) to (*xii*)—as a function of the seed coatings at 30 days after sowing. These analyses were only performed in the pine species (*P. sylvestris*) in which changes on mortality rates as a result of seed coating were demonstrated in the previous step. All analyses were performed using R software environment (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Germination Test

Data on the germination percentage (GP) for each of the species and coatings is shown in Figure 2. The highest GP (85.7%) for *P. sylvestris* was attained for the control and PEE treatments. The use of chitosan, either individually or in combination with PEE, did not enhance the GP. In fact, CM_{MW} and CM_{MW}-PEE treatments led to lower GP values (60% and 62.9%, respectively) than that of the control treatment (Figure 2a). In the case of *P. radiata* seeds, the use of chitosan and propolis did not enhance the GP either, and the binary CL_{MW}-PEE composite decreased the GP vs. the control treatment (Figure 2b). No significant differences in terms of GP were found between Monterey pine and Scots pine ($\chi^2 = 1.43$, $p = 0.23$).

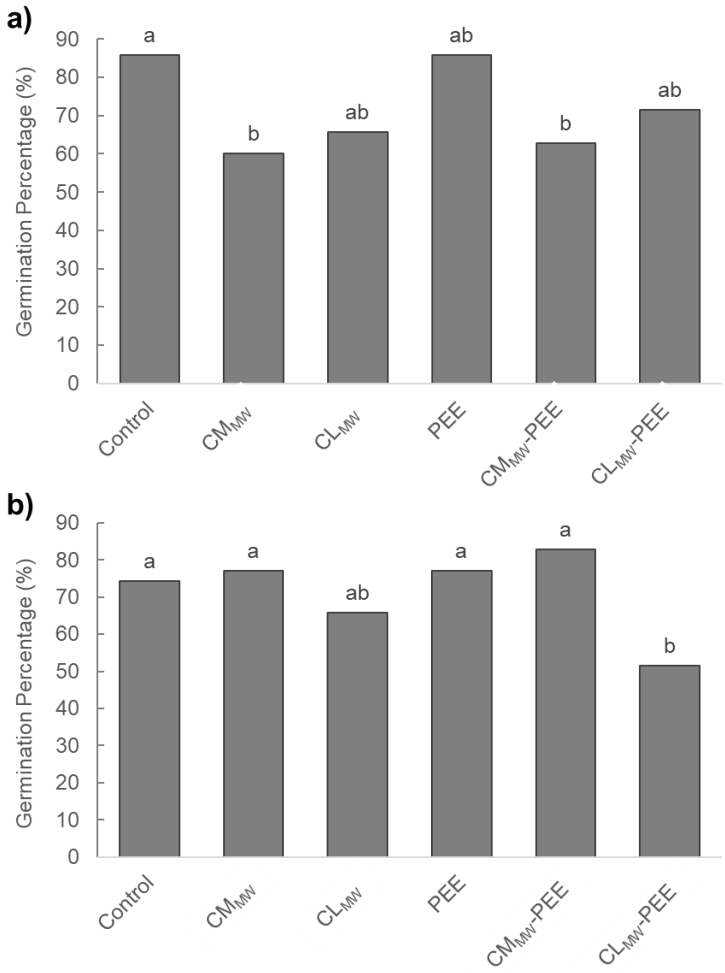

Figure 2. Germination percentage (GP) of (**a**) *P. sylvestris* and (**b**) *P. radiata* seeds coated with medium and low molecular weight chitosan (CMMW and CLMW, respectively) and/or with propolis ethanolic extract (PEE) at 40 and 30 days after sowing, respectively. Averages with the same letter were not significantly different according to the Chi-square test (χ^2) ($\alpha \leq 0.05$).

3.2. Pathogenicity Test

Inoculations with *F. circinatum* did not cause pre-emergence mortality for either *P. sylvestris* (χ^2 = 0.85, p = 0.36) or for *P. radiata* (χ^2 = 0.85, p = 0.36). Nonetheless, survival analyses demonstrated significant differences in post-emergence mortality among treatments in *P. sylvestris* (χ^2 = 49.7, p < 0.001). No seedlings of the Fc treatment survived beyond 40 days after inoculation (dai), whereas no mortality was recorded in the control seedlings. All coating treatments improved the survival of *P. sylvestris* inoculated seedlings, resulting in survival rates of over 50% for the CMMW-Fc, CLMW-Fc, and CMMW-PEE-Fc treatments. Chitosan-only treatments (CLMW-Fc and CMMW-Fc) showed similar protection efficacies as PEE-Fc (χ^2 = 1.4, p = 0.23; and χ^2 = 1.3, p = 0.26, respectively). However, in the CMMW-PEE-Fc and CLMW-PEE-Fc binary composites, the addition of PEE did not improve the survival rate in comparison to CMMW-Fc and CLMW-Fc (χ^2 < 0.001, p = 0.88; and χ^2 = 3.2, p = 0.07, respectively) (Figure 3a).

Mortality of inoculated *P. radiata* seedlings was faster than that of *P. sylvestris* seedlings. In fact, just four seedlings from the non-coated seeds inoculated with *F. circinatum* survived until 30 dai. Significant differences in post-emergence mortality were found between control treatment and inoculated seedlings, regardless of the coating treatment (χ^2 = 67.7, p < 0.001). However, none of the coating treatments was able to significantly improve the survival of the inoculated seedlings, neither the individual components CMMW-Fc, CLMW-Fc, and PEE-Fc (χ^2 = 1.7, p = 0.19; χ^2 = 0.2, p = 0.64; and χ^2

= 0.2, p = 0.66, respectively) nor the CMMW-PEE-Fc and CLMW-PEE-Fc binary composites (χ^2 = 2.5, p = 0.11; and χ^2 = 0.2, p = 0.69, respectively) (Figure 3b).

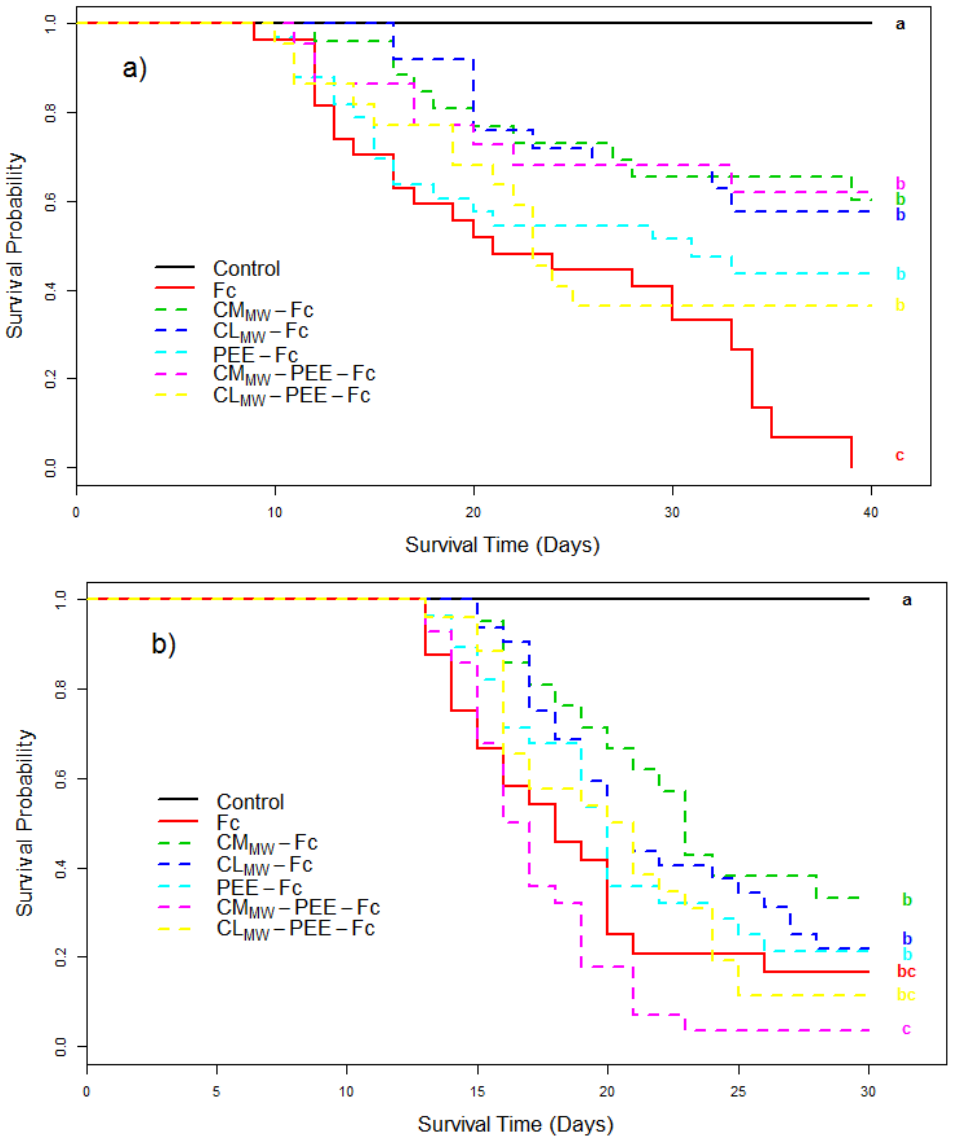

Figure 3. Plot of survival probability, determined using the Kaplan-Meier estimate of the survival function, for (**a**) *P. sylvestris* and (**b**) *P. radiata* seedlings inoculated with *Fusarium circinatum* (Fc) as a function of the seed coating treatments. CMMW and CLMW stand for medium and low molecular weight chitosan, respectively, PEE stands for propolis ethanolic extract. No mortality was registered for mock-inoculated and coating treatments (i.e., (*ii*) to (*vi*)). These curves were not shown to avoid multi-overlaps with the curve of the control treatment. Averages with the same letter were not significantly different according to the Kaplan–Meier estimator ($\alpha \leq 0.05$).

3.3. Total Phenolic Content and Radical Scavenging Activity

The TPC varied significantly in *P. sylvestris* seedlings as a function of the coating treatment (F = 4.99, p < 0.01). The mean TPC of the control treatment was 6.8 mg·mL^{-1} of GAE, which was significantly higher than the value obtained in the Fc treatment (2.7 mg·mL^{-1}), evidencing a significant TPC reduction caused by the pathogen (Figure 4a). TPC values did not change in inoculated-coated seeds in comparison to the control treatment, with the exception of the CMMW-Fc treatment. In particular, the CLMW-Fc treatment led to a TPC value comparable to that of the control treatment.

The negative effect caused by the pathogen was also evidenced in the low antioxidant capacity of inoculated seedlings in comparison to the high antioxidant activity shown by the control treatment (83.5%). This RSA value was just preserved by the CL_{MW}-Fc and PEE-Fc treatments, which did not vary significantly in comparison with the control treatment (Figure 4b). However, CM_{MW} and the binary composites did not succeed in reverting the aforementioned negative effect exerted by the pathogen.

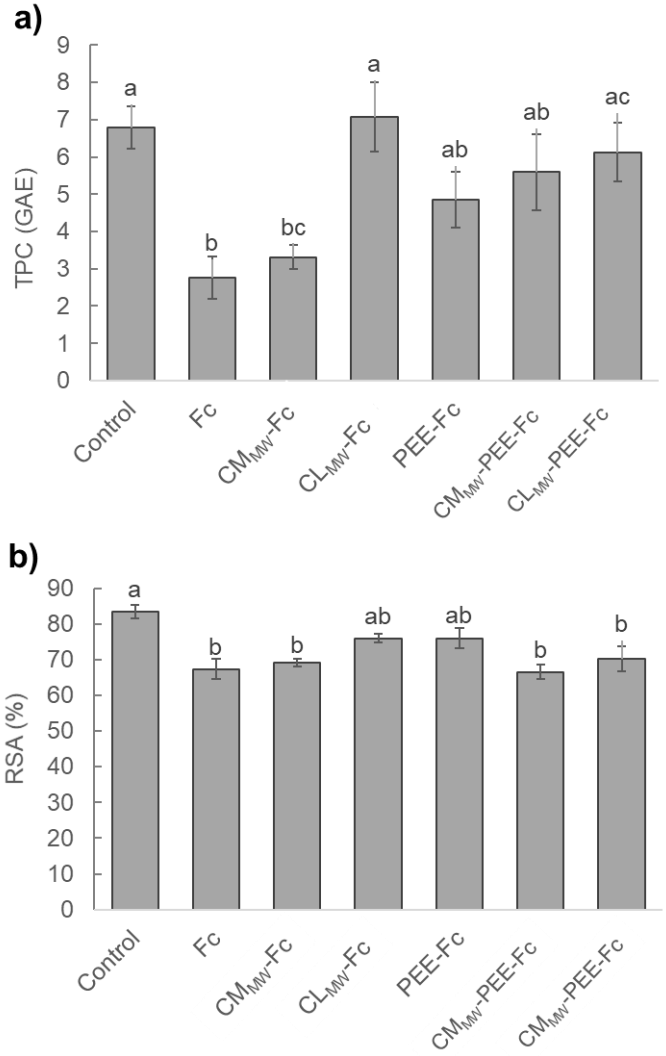

Figure 4. (**a**) Total phenolic contents (TPC) in gallic acid equivalents (GAE, mg·mL^{-1}) and (**b**) percentage of radical scavenging activity (RSA%) of *P. sylvestris* seedlings from control (*i*) and inoculated with *Fusarium circinatum* (Fc) treatments—(*vii*) to (*xii*)—as a function of the seed coatings at 30 days after sowing. CM_{MW} and CL_{MW} stand for medium and low molecular weight chitosan, respectively, PEE stands for propolis ethanolic extract. Averages with the same letter were not significantly different according to the Tukey's HSD test ($\alpha \leq 0.05$).

4. Discussion

At least 60 species of *Pinus* along with *Pseudotsuga menziesii* (Mirb.) Franco are known to be susceptible to PPC. Amongst them, *P. radiata* is recognized as the most susceptible [3], while *P. sylvestris* has also been reported to present a high susceptibility [41,43,50–53]. In this study, seed coatings based on chitosan—with two different molecular weights—and propolis were applied in order to test if these bioactive products were able to confer resistance against *F. circinatum* in the two species mentioned above.

It is well-known that chitosan application on seeds is beneficial in order to enhance the germination rate and/or yield in many agricultural crops, such as maize [54], wheat [6], soybean [55], canola [10], artichoke [35], or ajowan [56]. However, the application of chitosan and propolis did not enhance the germination rates in the present study. In fact, germination decreased with CM_{MW} and CM_{MW}-PEE in *P. sylvestris* and with CL_{MW}-PEE in *P. radiata* seeds. This discrepancy may actually be due to the type of species/provenance, since a previous study on *P. sylvestris* seeds treated with a commercial chitosan product did not result in an increase in seedling emergence percentage either [57]. Likewise, in an in vitro test on orchid *Dendrobium bigibbum* seeds with low viability (32.6%) that were treated with high and low MW chitosan, an improvement in seed germination with respect to the control did not occur either. There were no significant differences between polymers and oligomers of chitosan [23]. Differences among studies may also be ascribed to the coating process used, the type and concentration of chitosan [57], and the inoculum dose [16]. It should also be taken into consideration that, although chitosan's excellent filmogenic properties are well-established (it forms a semipermeable film on the seed surface which can maintain the seed humidity and absorb moisture from the soil [55]), the exact mechanism through which chitosan promotes germination is still unknown.

No pre-emergence mortality was observed in either *P. sylvestris* or in *P. radiata* seedlings as a result of *F. circinatum* inoculation. Conversely, Martínez-Álvarez et al. [43] reported lower emergence rates of both *P. sylvestris* and *P. radiata* when their substrate was inoculated with *F. circinatum* in comparison with controls. Differences on pre-emergence rates of seeds placed in infested substrates have been previously related to the genetic effect of pines [40]. However, in this case, the discrepancy may be due to the inoculum dose, since Martínez-Álvarez et al. [43] tested Scots pine seeds with the same provenance and *F. circinatum* isolate, but the inoculum dose was 1×10^6 spores·mL^{-1} instead of 1×10^3 spores·mL^{-1} applied in the present study. Thus, a higher inoculum dose may speed up the infection process, killing the germlings even before emergence.

Inoculations with *F. circinatum* caused post-emergence mortality in both *P. sylvestris* and *P. radiata*, which concurs with the results obtained by Martínez-Álvarez et al. [43]. Post-emergence mortality was reduced as a result of seed coating only in *P. sylvestris*, the species which featured a slightly lower susceptibility to *F. circinatum*, as noted above. Survival rates higher than 50% were found for seeds coated with chitosan (both CM_{MW} and CL_{MW}) at 40 dai, whereas all inoculated non-coated seedlings (Fc treatment) died. Although several studies have demonstrated that low molecular weight chitosan is more effective at inducing a set of defense responses and antifungal protection than higher molecular weight chitosan in crops [30,32], no significant differences as a function of the molecular weight were found in this study. This inconsistency could be due to the plant material, since conifers seem to show a different pattern: for instance, Fitza et al. [15], who evaluated chitin and chitosan as inducers of resistance to *F. circinatum* in *P. patula* seedlings, found that chitin—with higher molecular weight than chitosan—resulted in a higher percentage of healthy stems for lesion lengths caused by the artificial inoculation of the fungus. The survival rate also increased with propolis application. This antifungal effect of seed coatings based on propolis extracts has been already demonstrated for the postharvest treatment of papaya [45] and chilli [58] during storage. In vitro experiments demonstrated that the binary combination of chitosan and propolis was an efficient combination to deal with *F. circinatum* [14], *Diplodia seriata* [37], and *Hemileia vastatrix* [59]. Likewise, this binary combination has also been successfully used for the protection of food packaging materials [60,61] and against foodborne pathogens [36]. Nevertheless, in this study, the binary combinations did not improve the post-emergence mortality vs. the individual application of chitosan or propolis. It may be possible that the activity of propolis had been reduced due to the degradation of the extracts along the assay and that constant irrigation had washed the PEE coating. On the other hand, seed coatings based on chitosan and/or propolis failed to protect the *P. radiata* seedlings against *F. circinatum*. This seems to point out that the quick progression of the infection in this species (the most susceptible species to PPC [3]) may mean that the activation of induced resistances takes place too late.

In order to determine the influence of chitosan and propolis coatings on the host response mechanisms against the pathogen, TPC and RSA were measured on the seedlings at 30 dai. A decline in TPC and RSA was observed in the Scots pine seedlings infected by *F. circinatum*, which is consistent with the results obtained in *P. halepensis* seedlings inoculated with *Gremmeniella abietina* [27]. However, such an effect was not observed when the seeds were coated, except for the CM_{MW} treatment. This suggests that CL_{MW} and, to a lesser extent, PEE and the binary combinations would prevent phenolic compounds from decreasing when seedlings are infected by *F. circinatum*. Similarly, coatings based on chitosan showed an increase in TPC on tomato fruit infected by *Botrytis cinerea* and *Penicillium expansum* [62,63]. Although it is well known that phenolic content is an indicator of the activation of defense mechanisms in plants [28], there are other frequent responses that include increase of peroxidase, glucanase, and chitinase activity; higher lignin production; existence of toxic proteins and inhibitors of enzymes; among others [24]. Actually, chitosan has been reported as an elicitor of plants, with capacity to promote the production of phenolic compounds (e.g., the induction of phenylalanine ammonia-lyase) [23]. However, it has also been shown that chitosan affects many other plant responses, increasing the production of hydrogen peroxide, the activities of chitinase, the transcription of defense-related genes β-1,3-glucanase and chitinase, and the accumulation of pathogen-related protein (PR1) [30].

On the other hand, the antioxidant capacity of chitosan and its oligomers has been well-studied [44,64], as well as that of propolis [65], which is well-known in traditional medicine. This capacity to scavenge free radicals is related to the antimicrobial activity; for instance, anthocyanins are commonly induced under stress conditions and upon infection by pathogens in plants [66]. In this work, it was found that chitosan oligomers and propolis maintained the level of antioxidants in Scots pine seedlings under pathogen presence. Other works also found a high level of inhibition of free radicals in fruit exposed to fungus attack [26,34].

Future experiments should be carried out to elucidate the response mechanism that chitosan and propolis activate as seed protectors in the resistance to *F. circinatum* in pines, and to assess the effectiveness of these coatings in combination with other environmentally friendly methods used for seed protection, such as biological control agents (e.g., *Trichoderma* spp.) [40,67] and heat water treatments [68–70]. Other factors, such as the genetic resistance of plant material, including maternal effects and morphological traits of seeds, could also be studied [71,72].

5. Conclusions

The study presented herein demonstrated that the application of bioactive coatings based on chitosan and propolis could be helpful for protecting certain *Pinus* species' seeds against *F. circinatum*. In spite of the fact that their efficacy against pine pitch canker was limited in the case of *P. radiata,* which is recognized as the most susceptible species to this pathogen, the coatings significantly reduced the post-emergence mortality of *P. sylvestris* seedlings, resulting in survival rates higher than 50%, while all inoculated non-coated seedlings died at the end of the experiment. Seed coatings also had a positive influence on total phenolic content, leading to similar values to those found in non-inoculated seeds, and helped preserve the seedlings' radical scavenging activity. No significant differences in the germination percentages were observed. Among the various coating treatments proposed, the one based on low-molecular weight chitosan led to the best results, suggesting that more complex formulations including propolis would not be needed, and that medium molecular weight chitosan—despite its higher viscosity—would not be the preferred option. Notwithstanding these promising findings, further studies are needed to confirm the effectiveness of the seed coatings against *F. circinatum* in a wide range of environmental conditions (not only on sterilized substrate), their long-term persistence, and their potential use in combination with other environmentally friendly approaches.

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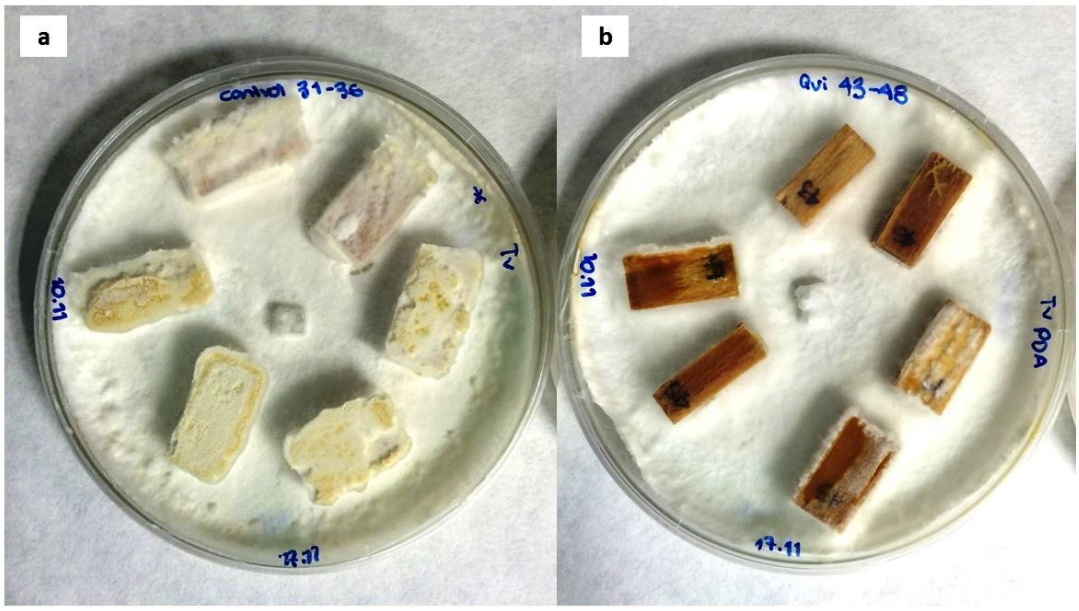
Artículo 6

Recubrimientos a base de quitosano para prevenir la descomposición de madera de *Populus* spp. causada por *Trametes Versicolor*



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Article

Chitosan-based coatings to prevent the decay of *Populus* spp. wood caused by *Trametes versicolor*

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Abstract: Chitosan and chitosan oligomers are receiving increasing attention due to their antimicrobial properties. In the present study, they were assayed as a preventive treatment against white-rot decay of *Populus* wood (very important in economic and environmental terms), caused by *Trametes versicolor* fungus. Their capacity to incorporate different chemical species into the polymer structure with a view to improving their anti-fungal activity was also assessed by mixing oligo-chitosan with propolis and silver nanoparticles. The minimum inhibitory concentration of medium-molecular weight chitosan (MMWC), chitosan oligomers (CO), propolis (P), nanosilver (nAg), and their binary and ternary composites against *T. versicolor* was determined *in vitro*. Although all products exhibited anti-fungal properties, composites showed an enhanced effect as compared to the individual products: 100% mycelial growth inhibition was attained for concentrations of 2.0 $mg \cdot mL^{-1}$ and 0.2 $mg \cdot mL^{-1}$ for the CO-P binary mixture, respectively; and 2 $\mu g \cdot mL^{-1}$ for nAg in the ternary mixture. Subsequently, MMWC, CO, CO-P and CO-P-nAg composites were tested on poplar wood blocks as surface protectors. Wood decay caused by the fungus was monitored by microscopy and vibrational spectroscopy, evidencing the limitations of the CO-based coatings in comparison with MMWC, which has a higher viscosity and better adhesion properties. The usage of MMWC holds promise for poplar wood protection, with potential industrial applications.

Keywords: chitosan composites; FTIR; natural protectors; poplar wood; white-rot fungus

1. Introduction

In the field of wood protection, significant efforts have been devoted over the last decades to the assessment of natural products that can pose an alternative to other traditionally used chemical compounds, which have toxic effects on human beings and the environment [1]. In this sense, renewable polymers have attracted intense industrial interest, and, amongst them, chitosan has particularly promising application perspectives [2]. This natural polysaccharide derived from chitin is the second most abundant polymer in nature. Chitosan consists of chains of N-acetyl-D-glucosamine and D-glucosamine, and features a cationic character, which confers unique properties on it [3]. Among its main characteristics, such as its bioactivity, non-toxicity or biodegradability, its antimicrobial effect is especially relevant [4–6]. Its activity against different fungi, gram positive and

gram negative bacteria, is ascribed to the positive charge of amino group (NH_3^+), which interacts electrostatically with the surface of the cellular membrane, destabilizing it. As a consequence, the presence of chitosan inside the cell can lead to intracellular responses such as inactivation or blocking of enzymes activities, and of DNA transcription and translation [7,8].

The antifungal activity of chitosan not only depends on the fungus species, but also on its molecular weight (MW), polymerization degree (PD) or deacetylation degree (DD). For instance, chito-oligosaccharides with low MW, PD and DD have been reported to be more effective on phytopathogenic fungi than chitosan with higher MW, PD or DD [9–11].

Another known advantage from chitosan and its oligomers is associated with their sorption and chelating properties. The cationic nature of the polymer allows it to bind to different chemicals species, ranging from organic macromolecules to metal nanoparticles [12–14]. Natural oils or extracts of natural products can be incorporated into the chitosan matrix, e.g., by cross-linking, producing solutions, films or beads [15–17]. In the particular case of propolis, its polyphenols have been reported to form hydrogen bonds and covalent bonds with the functional groups in chitosan [18]. This results in a stabilization of the propolis components in the polymer and in an enhancement of the antimicrobial activity due to a synergistic behavior [19,20]. In the case of nanometals, e.g., silver nanoparticles, chitosan and its derivatives can also be used in their synthesis, provided that chitosan can act as reducer as well as a stabilizer : the polymer matrix facilitates the nanoparticles preparation in one-step process, resulting in nanosilver with an ideal particle size [21]. Further, the resulting chitosan-nanosilver composites present a higher antimicrobial effect [22].

Chitosan-based composites have been successfully applied to the preservation of wood against mold fungi, as well as brown-rot and white-rot fungi [23–26], both *in vitro* and over beech and fir [27], Monterrey pine [28], Scot pine [29] and other hardwood and softwood species. Nonetheless, to the best of the authors' knowledge, to date no studies have focused on chitosan applications in order to protect poplar species. *Populus* spp., belonging to the Salicaceae family, are one of the most cultivated woody plants for industrial purposes. Its wood is one of the least expensive hardwoods and, although rarely used in the production of fine furniture, it is extensively used for pulp, panel productions and many other commercial applications [30,31]. These fast-growing trees therefore have a large economic impact worldwide, together with a significant importance from an environmental perspective [32]. According to EN 350:2016 rule, poplar wood is a non-durable species [33], and some studies have evidenced that it is highly susceptible to *Trametes versicolor* [34,35]. Therefore, chitosan-based composites can be applied as a potential tool to avoid the white-rot decay of *Populus* spp. wood, improving its natural durability and increasing its technological uses.

The first objective of the study was to investigate the *in vitro* antifungal activity against *T. versicolor* of two different molecular weight chitosan treatments: medium molecular weight chitosan (MMWC) and chitosan oligomers (CO); the latter –which resulted in a better minimum inhibitory concentration (MIC) value– was also tested in binary and ternary combinations with propolis (P) and nanosilver (nAg). In a second stage, once the MIC values had been determined, the best treatments were evaluated on poplar wood blocks as surface protectors to prevent wood decay, with a view to their practical application. Wood biodegradation was monitored by microscopy and by tracking chemical alterations. For this latter purpose, Fourier-transform infrared spectroscopy (FTIR) technique was chosen, provided that it is quickly consolidating as an easy-technique to characterize and evaluate the decay of wood [36–38].

2. Materials and methods

2.1. Reagents, fungal isolate and wood

Medium molecular weight chitosan (CAS No. 9012-76-4), with 60-130 kDa and 90% deacetylation, was acquired from Hangzhou Simit Chemical Technology Co. (Hangzhou, China). Propolis, with a content of ca. 10% w/v of polyphenols and flavonoids, came from the Duero river basin region (Burgos, Spain). Silver nitrate (CAS number 7761-88-8) was supplied by Merck Millipore

(Darmstadt, Germany). All the other reagents used to synthetize or prepare the solutions were of analytical grade.

The isolate of *T. versicolor* (L.) Lloyd 1920 (DSM 3086 strain, CECT 20804) was supplied by the Spanish Type Culture Collection (Valencia, Spain) and was cultivated on potato dextrose agar (PDA) (supplied by Scharlau, Barcelona, Spain) for all assays.

The wood blocks were supplied by the Wood Technology Laboratory at Universidad de Valladolid from *P. euroamericana* I-214 clone.

2.2. Chitosan-based solutions

The solutions of MMWC, CO, P, nAg and the CO-based binary and ternary mixtures -tested *in vitro* for the inhibition of *T. versicolor* mycelial growth and as wood coatings- were prepared starting from commercial medium MW chitosan, which was dissolved in acetic acid (1 %) under constant stirring at 60 °C for 2 h. The pH value was then adjusted from 4.5 to 6.5 with potassium methoxide. Subsequently, oligo-chitosan was prepared by oxidative degradation of the solubilized MMWC by addition of hydrogen peroxide (0.3 M), obtaining a MW of 2 kDa [39]. Propolis constituents soluble in ethanol were extracted by grinding the resin and adding it to a hydroalcoholic solution (ethanol 30%), followed by stirring for 72 h at room temperature, and by filtration with a stainless steel 220 mesh to remove insoluble particles [40]. Silver nanoparticles were synthetized by a sonication method: 50 mL of silver nitrate (50 mM) were first mixed with 50 mL of sodium citrate (30 mM) as a reducing agent, and the solution was heated up to 90 °C until it turned from colorless to pale yellow, which then became more intense. The yellowish solution was sonicated for 3-5 minutes and allowed to stabilize for at least 24 h in a refrigerator at 5 °C [41].

The binary and ternary solutions were prepared according to Martín-Gil et al. [42], by mixing of the solutions described above at the desired concentrations (Table 1), followed by sonication for 1 min with a probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz).

Table 1. Concentrations used in the *in vitro* experiments (MMWC, COs and P are given in mg·mL^{-1}, and that of nAg in μg·mL^{-1}).

Treatment	Concentration				
Control	0.0				
MMWC	1.0	2.0	4.0	7.0	10.0
CO	1.0	1.5	2.0	2.5	3.0
P	0.1	0.2	0.3	0.4	0.5
nAg	1.0	1.5	2.0	2.5	3.0
CO-P	0.5 – 0.05		1.0 – 0.1		2.0 – 0.2
CO-nAg	0.5 – 0.5		1.0 – 1.0		2.0 – 2.0
P-nAg	0.05 – 0.5		0.1 – 1.0		0.2 – 2.0
CO-P-nAg	0.5 – 0.05 – 0.5		1.0 – 0.1 – 1.0		2.0 – 0.2 – 2.0

2.3. In vitro assays

Each solution was incorporated into PDA at a ratio of 1:10 (v/v). PDA had been sterilized for 20 min at 121 °C, and had been cooled down to 50 °C before the addition of the treatments (or of distillated and sterilized water in the case of control). 20 mL of the medium were then spread in Petri dishes and, once they had solidified, 5 mm in diameter discs of young mycelia of *T. versicolor* were placed on the center of each Petri dish, followed by incubation in the dark at 25 °C and 65% HR. Three replicates were performed for each treatment. Finally, radial growth was measured on a daily basis until the mycelium reached the edges of the control Petri dish (8 days), and the inhibition growth percentage was calculated.

2.4. Wood coating assays

On the basis of the *in vitro* study results, the best treatments (either individual, binary or ternary mixtures) were assayed as a coating over poplar wood in order to prevent its degradation against *T. versicolor* fungus.

Small poplar wood blocks (5×5×20 mm), previously dried at 103±2 °C to determinate their initial dry weight, were soaked in the different solutions: water (control), MMWC, CO, CO-P and CO-P-nAg (36 blocks per treatment) for 12 h at room temperature. A concentration of 20 mg·mL^{-1} for MMWC, 20 mg·mL^{-1} for CO, 2 mg·mL^{-1} for P, and 20 μg·mL^{-1} for nAg were used. These doses, which were ten times higher than the obtained MIC values from binary and ternary mixtures, were chosen on the basis of adsorption, penetration and fixation tests, so as to compensate for the influence of the dipping time on the uptake of the preservative solution and to minimize wood swelling, as reported by Eikenes et al. [29] and Larnoy et al. [43]. Excess of liquid was then removed and the wood blocks were dried in a chamber for 24 h at 45 °C. The coated blocks were placed on Petri dishes -six per plate- newly covered until the edges by *T. versicolor* mycelia, which were again incubated at 27 °C and 70% of relative humidity (RH) for 30 days.

One Petri dish, with six blocks, of each treatment was removed every 5 days for analysis. The mycelia on the wood surface were carefully cleaned, the small blocks were then dried at 103±2 °C to quantify their dry weight after fungal attack, and the mass loss was determined by subtracting it from the initial weight according to EN 113:1996 [44].

2.5. Microscopy and FTIR studies

In order to monitor wood degradation, a Leica (Wetzlar, Germany) microtome was used to cut thin wood sections (30 μm), which were analyzed by optical microscopy (with a Leica DMLM Transmission Optical Microscope). Thin cut sections were also grinded and mixed with KBr (in 1:300 ratio) to prepare pellets to study the FTIR spectrum by direct transmittance technique, using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 FTIR spectrometer. The spectra were collected in the 400-4000 cm^{-1} region at room temperature with a 1 cm^{-1} spectral resolution; a total of 64 scans were co-added and the resulting interferogram was averaged. The obtained spectra were corrected using the advanced ATR correction algorithm available in OMNIC software suite (Thermo Scientific), and were normalize using the 1048 cm^{-1} band, assigned to CO stretching, in agreement with Colom et al. [36].

2.6. Statistical analyses

All the statistical analyses were performed using R software (v. 3.4.4) (R Development Core Team, 2018). A total of 216 data, corresponding to 15 different individual groups (6 samples × 3 preservative agents × 5 concentration levels), 1 control group (6 samples), 3 binary groups (6 samples × 3 groups × 3 concentration levels) and 1 ternary group (6 samples × 3 levels) were analyzed. Prior to the analyses, the assumptions of independence, normality and homoscedasticity were checked for all groups. Since the data structure did not meet the normality requirement, checked with Shapiro-Wilks test, or the homoscedasticity requirement, checked with Bartlett's test, the usual comparative analysis (ANOVA) could not be used. Bootstrapping, Welch's test and robust homogenous groups were used instead.

3. Results and discussion

3.1. Minimum inhibitory concentration

The antifungal activity of the chitosan-based composites against *T. versicolor* was evaluated in a typical *in vitro* inhibition growth experiment. The results, collected after seven days of incubation, are shown in Table 2. MMWC presented a moderate activity against mycelial growth, with significant differences in comparison with the control treatment. However, low doses resulted in a low inhibition and higher concentrations (up to 10 mg·mL^{-1}) were needed to approach 100% mycelial growth inhibition. In contrast, CO exhibited a high inhibitory activity even at the lowest doses; e.g., 1 mg·mL^{-1} of CO inhibited 79% of mycelial growth. As expected, the increase in CO concentration resulted in

an increase of *T. versicolor* mycelial growth inhibition, attaining 100% inhibition (MIC) at a concentration of 3 mg·mL^{-1}.

As regards the differences observed between the activities of MMWC and CO, the results obtained in this study would be in agreement with other *in vitro* assays reported in the literature, such as those mentioned in the introduction or those by Matei et al. [41] and Rahman et al. [9], who found a better antifungal activity for low MW (less than 20 kDa) chitosan than for medium or high MW (more than 140 kDa) chitosan. Nonetheless, it should be clarified that this behavior may not be extrapolated to all fungi: for instance, Younes et al. [10] found that fungal growth decreased with increasing MW for *Fusarium oxysporum*, thus pointing at the need to evaluate the optimum MW for each species.

In relation to the MIC, Stössel and Leuba [45] reported that –for native chitosan– MIC values could vary from 0.001 to 2.5 mg·mL^{-1}, depending on the phytopathogenic fungus. According to Rabea et al. [46], the MIC value depends on factors such as the type of chitosan, the fungus under study, the chemical or nutrient composition of the substrate and the environmental conditions. Another work on *Sphaeropsis sapinea* and *Trichoderma harzianum* wood-degrading fungi suggested that the application of 1 mg·mL^{-1} of low-molecular weight chitosan reduced growth rate by a factor of 3 with respect to the control [47], in line with the results reported by Silva-Castro et al. [40], who found that 1 mg·mL^{-1} of CO inhibited 86% of the mycelial growth of *Heterobasidium annosum* basidiomycete fungus. Thus, in view of aforementioned MIC values, it may be inferred that *T. versicolor* would show an intermediate sensitivity (i.e., would be moderately sensitive) to chitosan/CO alone, albeit such resistance would not be as high as that of, for example, *Macrophomina phaseolina* (for which MIC values of water-soluble chitosan as high as 12.5 mg·mL^{-1} were found [48]). Consequently, it seemed necessary to explore binary or ternary combinations with other compounds with antifungal activity in order to search for synergies and to attain lower MIC values.

Propolis exhibited a higher antifungal activity against *T. versicolor* than CO: in propolis-based treatments, 85% growth inhibition was attained with 0.1 mg·mL^{-1}, with a MIC value of 0.5 mg·mL^{-1} (six times lower than MIC of CO). The use of propolis as an antifungal agent is well-known, and its effectiveness has been proved against *Candida* spp. and other human pathogens [49–51]. This has promoted its use in the production of films to control foodborne fungi [52,53]. With regard to phytopathogenic oomycetes (*Phytophthora alni* and *Phytophthora plurivora*), 0.1 mg·mL^{-1} of propolis ethanolic extract resulted in 100% inhibition [40]. Although there is no data concerning propolis action against white-rot fungi (a thorough search of the relevant literature did not yield any reports on the antifungal activity of propolis ethanolic extracts to control *T. versicolor* growth), other natural products were assessed by Zhang et al. [54], who evaluated the activity against white-rot fungi of 41 monoterpenes (which are part of the composition of propolis). They found that carvacrol at a concentration of 0.4 mg·mL^{-1} led to 100% growth inhibition against *Trametes hirsuta*, *Schizphylhls commune*, and *Pycnoporus sanguineus*, i.e., its MIC value was similar to that reported herein for P.

Regarding nAg treatments, just 1 μg·mL^{-1} led to 85% mycelial growth inhibition, which increased up to 100% at a concentration of 3 μg·mL^{-1} (MIC value). It should be clarified that the remarkable ability of nanosilver to inhibit fungal growth depends not only on the fungal species, but on the nanoparticles characteristics -shape, size, synthesis method, stabilization, etc.- [55], which makes direct comparisons difficult. For instance, a study was carried out to test three types of nanosilver against eighteen plant pathogenic fungi, and while no variation was detected at relatively low doses (10 μg·mL^{-1}), at higher concentrations (100 μg·mL^{-1}) the maximum inhibition for most fungi was shown by dark brown colloid nanoparticles [56]. Apropos of wood-degrading fungi, silver nanoparticles synthesized using turnip leaf extract were assayed against four fungi, showing slight to moderate growth inhibition (with 2 and 4 mg of nanosilver on paper discs) [57]. Although in both studies the nanoparticles presented a reasonably good activity, the MIC value found in the present study indicates that *T. versicolor* would be very sensitive to nAg.

Since the antifungal activity of CO was noticeably higher than that of MMWC, only composites of CO, P and nAg, in binary and ternary mixtures, were assessed. These composites presented significant differences in comparison with the same concentrations of individual products. The CO-

P binary composites inhibited 100% of mycelial growth with 2 and 0.2 mg·mL^{-1}, respectively (*vs*. MIC values of 3 mg·mL^{-1} and 0.5 mg·mL^{-1} for individual treatments with CO and P, respectively), suggesting that the mixture of both natural products would enhance the antifungal activity against *T. versicolor*. A similar behavior has also been reported by other authors, who observed that the addition of propolis to chitosan films enhanced their *in vitro* antimicrobial effect [18]. However, it is worth noting that this synergistic behavior cannot be extrapolated to all pathogens, provided that Mascheroni et al. [58] reported disparate results for different foodborne fungi, with lower MICs for propolis-only treatments than for chitosan-propolis beads in some cases. Likewise, Silva-Castro et al. [40] found no statistically significant differences between CO-P composites and propolis-only treatments against *P. alni* and *P. plurivora*.

Apropos of the binary CO-nAg composite, it resulted in 100% growth inhibition (MIC value) for a concentration of 2 mg·mL^{-1} and 2 μg·mL^{-1}, respectively (lower than the MICs of the corresponding individual products too). However, a different behavior was observed at lower doses of the mixture, provided that significant differences between CO-nAg and nAg-only treatments were not found at 0.1 μg·mL^{-1}, suggesting that the nAg dose in the composite would be crucial (as noted above, *T. versicolor* showed a much higher sensitivity to nAg than to chitosan). The results at a higher concentration would be in good agreement with other studies in which it has been demonstrated that the composites of both products have a greater antifungal activity [59], while the behavior found at lower nAg doses would be in line with the findings of Velmurugan et al. [60], who reported that chitosan with silver nano-sized particles exhibited a similar antifungal effect than the nanoparticles alone against wood staining fungi, given the remarkable antifungal activity of the latter.

The third binary mixture, P-nAg, presented a MIC value of 0.2 mg·mL^{-1} and 2 μg·mL^{-1} for P and nAg, respectively, which was also lower than MICs found for the treatments based on the individual products. However, at lower concentrations (0.1 mg·mL^{-1} and 0.1 μg·mL^{-1}) of P-nAg, again there were not significant differences in comparison with the nAg individual solutions, resulting in similar inhibition percentages (around 85%). This would reinforce the interpretation discussed above, in which nAg would play a key role in *T. versicolor* inhibition, given the high sensitivity of the fungus to this component. The dependence of the behavior on the particular pathogen is evidenced by the different results reported for the control of other forest pathogens: Silva-Castro et al. [40]found that P-nAg composites showed a similar effect to that of CO against *Fusarium circinatum*, a lower effect than that of CO against *Diplodia pinea*, and an enhanced behavior *vs*. CO-only treatments for the control of *Phytophthora cambivora*.

Finally, the ternary mixture reached full mycelial growth inhibition at a MIC value of 2 mg·mL^{-1} of CO, 0.2 mg·mL^{-1} of P and 0.2 μg mL^{-1} of nAg, so it would not present an advantageous behavior as compared to the binary composites. This same CO-P-nAg ternary composite has been tested against *Diplodia* seriata [41], *Bipolaris oryzae* [61] and eight forest phytopathogenic fungi [40], attaining a higher inhibition than the individual or binary products at concentrations of 20–25 mg·mL^{-1}, but leading to a similar effect than that of binary solutions at lower concentrations, as it happens in the present study. Paradoxically, at the lowest dose assayed herein (0.5 mg·mL^{-1} of CO, 0.05 mg·mL^{-1} of P and 0.5 μg·mL^{-1} of nAg), CO-P-nAg showed a noticeable higher antifungal effect against *T. versicolor* (79% inhibition) than the binary mixtures (51%, 64% and 47% inhibition for CO-P, CO-nAg and P-nAg, respectively).

Table 2. Effect of treatments concentration on the mycelial growth of *T. versicolor* (*in vitro*) seven days after incubation. The concentrations of MMWC, COs and P are given in mg·mL^{-1}, and that of nAg in μg·mL^{-1}.

Treatment	Concentration	Inhibition percentage (%) Mean - Confidence Interval and homogeneous groups		Shapiro Wilk p-value	Levene's test p-value	Welch's test p-value
Control	0.0	0.000 ± 0.000	C	0.000	--	--
MMWC	1.0	22.708 ± 2.027	a	0.324	0.08629	0.0000
	2.0	42.083 ± 1.429	b	0.505		
	4.0	75.375 ± 1.970	e	0.377		

	7.0	90.250 ± 1.846	i	0.988		
	10.0	95.542 ± 1.331	j k	0.001		
	1.0	78.750 ± 1.094	f	0.110		
	1.5	87.708 ± 0.857	h	0.035		
CO	2.0	92.333 ± 0.991	i j	0.433	0.0417	0.0000
	2.5	97.000 ± 0.992	k	0.680		
	3.0	99.997 ± 0.004	l	0.001		
	0.1	84.541 ± 1.135	g	0.836		
	0.2	88.333 ± 1.173	i	0.911		
P	0.3	93.333 ± 1.148	j	0.850	0.0207	0.0000
	0.4	97.500 ± 0.909	k	0.421		
	0.5	99.997 ± 0.004	l	0.001		
	1.0	85.000 ± 1.270	g	0.960		
	1.5	91.333 ± 1.221	h	0.843		
nAg	2.0	94.217 ± 1.177	j	0.473	0.0214	0.0000
	2.5	97.333 ± 0.753	k	0.725		
	3.0	99.997 ± 0.004	l	0.001		
	0.5 – 0.05	51.458 ± 2.472	c	0.540		
CO-P	1.0 – 0.1	89.167 ± 0.451	h	0.001	0.0037	0.0000
	2.0 – 0.2	99.997 ± 0.003	l	0.001		
	0.5 – 0.5	64.583 ± 0.427	d	0.001		
CO-nAg	1.0 – 1.0	84.583 ± 0.740	g	0.091	0.0281	0.0000
	2.0 – 2.0	99.996 ± 0.003	l	0.001		
	0.05 – 0.5	47.292 ± 1.673	c	0.212		
P-nAg	0.1 – 1.0	84.167 ± 0.639	g	0.001	0.1652	0.0000
	0.2 – 2.0	99.997 ± 0.004	l	0.001		
	0.5 – 0.05 – 0.5	77.500 ± 1.081	f	0.110		
CO-P-nAg	1.0 – 0.1 – 1.0	85.208 ± 1.348	g	0.804	0.0190	0.0000
	2.0 – 0.2 – 2.0	99.997 ± 0.004	l	0.001		

3.2. Wood preservation assays

3.2.1. Weight loss

Surface treatments with the chitosan-based composites were applied to mini-blocks of poplar wood in order to prevent white-rot decay. Weight loss was recorded every 5 days (see Table 3). The non-treated mini-blocks (control) and those treated with chitosan oligomers-based treatments (CO, CO-P and CO-P-nAg) started losing weight from second sampling (10 days), in contrast with those treated with MMWC. In the third sampling (after 15 days), degradation caused by *T. versicolor* increased for all treatments, although with significant differences among the control and all the other treatments. Non-covered blocks exhibited a weight loss of up to 27.3 %, followed by those treated with CO-P-nAg (22.8%), CO-P (14.7%), CO (7%) and MMWC (3%). The differences between coated and non-coated samples further increased in the fourth sampling (after 20 days), in which the weight loss only increased slightly for the treated blocks, while degradation reached 32.9% for the control. 25 days after the exposure to fungus, the wood treated with MMWC still exhibited the lowest weight loss (7.7%), while weight loss for the CO-based treatments increased, albeit still with significant differences as compared to the control (39.8 %). In the last sampling (after 30 days), the wood blocks treated with the ternary composite reached a similar degradation level to that of the control -without significant differences-, in contrast with MMWC-treated samples, for which the weight loss (10.8%) remained well-below that of the control (42.3%).

Table 3. Effect of coating treatments and time on weight loss for *Populus* spp. wood-blocks exposed to white-rot fungus *T. versicolor*.

Treatment	Time (days)	Weight loss (g)	Shapiro Wilk	Levene's test	Welch's test

		Mean - Confidence Interval and homogeneous groups		p-value	p-value	p-value
Control	5	0.138 ± 0.155	a	0.004	0.1973	0.0000
	10	5.352 ± 1.315	bc	0.079		
	15	27.261 ± 3.037	ij	0.127		
	20	32.888 ± 4.233	jk	0.073		
	25	39.774 ± 2.036	l	0.949		
	30	42.353 ± 1.866	l	0.470		
MMWC	5	0.000 ± 0.000	a	0.000	0.1132	0.0000
	10	0.000 ± 0.000	a	0.000		
	15	3.478 ± 0.541	b	0.534		
	20	7.177 ± 0.570	c	0.900		
	25	10.157 ± 1.095	d	0.955		
	30	11.455 ± 1.171	d	0.412		
CO	5	0.000 ± 0.000	a	0.000	0.0001	0.0000
	10	4.311 ± 0.883	b	0.825		
	15	6.570 ± 1.226	c	0.759		
	20	13.524 ± 0.937	e	0.999		
	25	22.190 ± 0.890	h	0.961		
	30	26.871 ± 1.164	i	0.373		
CO-P	5	0.000 ± 0.000	a	0.000	0.0254	0.0000
	10	7.567 ± 0.889	c	0.610		
	15	13.578 ± 1.084	e	0.996		
	20	17.276 ± 1.154	f	0.869		
	25	29.662 ± 1.174	j	0.340		
	30	32.856 ± 1.039	k	0.289		
CO-P-Ag	5	0.000 ± 0.000	a	0.000	0.0342	0.0000
	10	10.615 ± 1.071	d	0.138		
	15	20.150 ± 1.185	g	0.660		
	20	26.864 ± 1.154	i	0.971		
	25	35.074 ± 1.341	k	0.762		
	30	39.943 ± 2.357	l	0.882		

The poplar wood mini-blocks from the control treatment presented a higher degradation rate than those of beech wood exposed to *T. versicolor* in the study carried out by Mohebby [38]: in the latter, a weight loss of ca. 20% was recorded on the 28th day of the experiment, half of the one recorded in this study after 30 days (42%). This can be readily ascribed to the high susceptibility of *Populus* spp. wood to this fungus [32,34].

With regard to the differences in the weight loss rates for the treated samples, although most treatments resulted in a slower degradation than that of the control, MMWC was clearly the most effective protective agent (Figure 1). This should be ascribed to its higher viscosity and better adhesive properties, adhesion is directly related to the DD of chitosan: if chitosan DD increases, the number of positive charges also increases, which leads to improved adhesive properties [62], which would result in a better coating on the surface of wood blocks. This would be in agreement with Eikenes et al. [29], who demonstrated that medium MW chitosan (at a concentration of 50 mg·mL^{-1}) was able to avoid the decay caused by brown-rot fungi on Scots pine, performing better than low MW chitosan, as it also happened in this study. Low doses of this type of chitosan (lower than 10 mg·mL^{-1}) were also found to be effective against white-rot fungi, according to Nowrouzi et al. [27]. Of course, the wood preservation effect would largely depend on the concentration of chitosan, as demonstrated by El-gamal et al. [23] in relation to the growth of fungi on treated wood samples from historic artifacts (fungal growth decreased with the increase in chitosan concentration).

Concerning the effectiveness as wood protecting agents of the composites, it was lower than that of the chitosan-only treatments (especially in comparison with MMWC), in contrast with the results from the *in vitro* assays. This unexpected result may be explained by their low viscosity and poor adhesion properties [63], which may be enhanced by adding thickening agents to chitosan oligomers, such as natural gums, starches, pectins, alginate and carrageenan [64]; by preparing CO–clay based

composite materials [65]; or by using other impregnation procedures (involving vacuum and pressure).

In view of aforementioned results, MMWC would be the preferred choice for industrial applications, not only due to its higher effectiveness, but also because of its fast and facile preparation process.

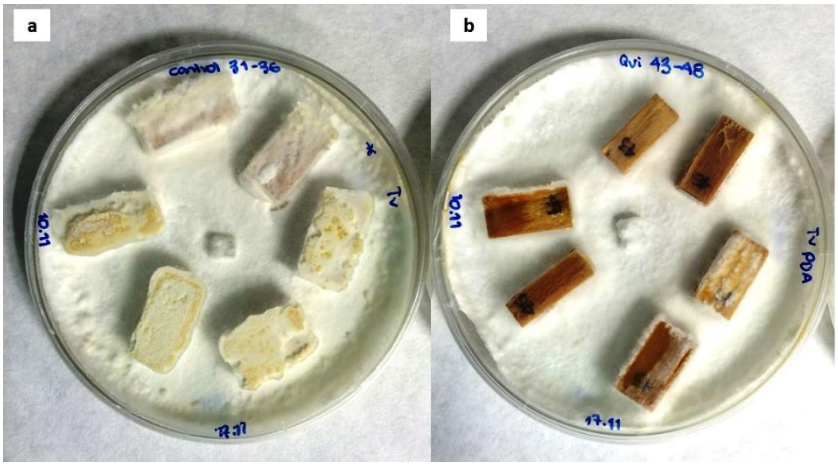

Figure 1. Poplar wood samples 30 days after exposure to *T. versicolor*: (*a*) control wood sample and (*b*) sample treated with medium molecular weight chitosan.

3.2.2. Wood degradation monitoring

The decay of the wood-blocks was tracked using microscopy techniques. In the control sample, changes such as cavities in the cell wall and a quick development of fungal hyphae were easily recognized, the change between undecayed control wood and the last sampling (after 30 days) can be observed by comparing Figure 2*a* and Figure 2*b*, respectively. The hyphae progressively increased over the 30 days, resulting in a strong structure interconnected with the wood one, as well as in holes in the cell walls and in a general degradation of the vessels and fibers of the poplar wood, confirming the high susceptibility of *Populus* spp. wood to *T. versicolor* [32]. Analogous effects were identified with SEM microscopy on uncovered blocks of the softwood from *Paulownia fortune* attacked by *T. versicolor* [66], providing evidence that the fungus can tunnel along the cellulose microfibrils, resulting in the formation of holes in transverse sections. Changes in the samples impregnated with MMWC were much less evident, even 30 days after exposure, with an associated weigh loss of 10% (Figure 2*c*).

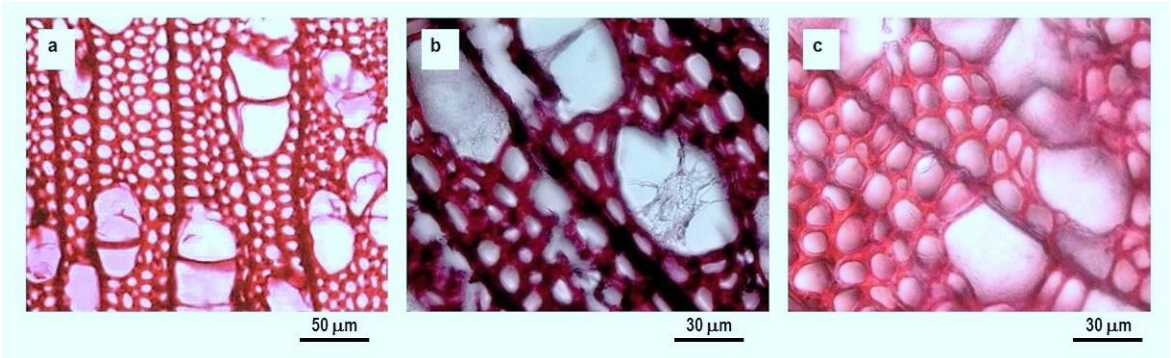

Figure 2. Optical micrographs (at 500× magnification) of the surface of poplar wood samples: (*a*) undecayed control wood sample prior to exposure to *T. versicolor*; (*b*) control wood sample 30 days after exposure to *T. versicolor*; and (*c*) sample treated with medium molecular weight chitosan 30 days after exposure to *T. versicolor*.

FTIR spectroscopy was also used to follow the decay –by tracking chemical changes– of the poplar wood mini-blocks. The infrared spectrum of undecayed control wood (Figure 3) was almost

identical to that of *Fagus sylvatica* [37], featuring a broad band at around 3400 cm^{-1} (corresponding to a convolution of O-H and N-H stretching vibration bands, at 3480 and 3270 cm^{-1}, respectively) and a band at around 2997 cm^{-1} (C–H stretching), which was also observed in the control and MMWC-coated wood samples 30 days after the exposure to *T. versicolor*. In view of the very small shifts observed in the first band for the MMWC coating, a low interaction of the solution with the wood may be inferred. When such interaction occurs, hydrogen bonds suffer a redistribution and the N-H band disappears [67].

Many well-defined bands could also be observed in the fingerprint region, between 1800 and 600 cm^{-1}: at 1740 cm^{-1} (unconjugated C=O from xylans in hemicellulose and/or C-O carbonyl band); at 1650 cm^{-1} (conjugated C-O in aromatic ring and O–H in absorbed water); at 1606 cm^{-1} and 1510 cm^{-1} (C=C aromatic skeletal vibrations in lignin); at 1465 cm^{-1} (CH_3 asymmetric bending in lignin); at 1425 cm^{-1} (CH_2 bending in cellulose); at 1380 cm^{-1} (C-H deformation in cellulose and hemicellulose); at 1327 cm^{-1} (C-O in syringyl and guaiacyl rings and/or O-H in plane bending in cellulose); at 1247 cm^{-1} (syringyl ring and/or C–O stretching in lignin and xylan); at 1165 cm^{-1} (C-O-C symmetric stretching in cellulose); at 1117 cm^{-1} (aromatic skeletal and C-O stretching vibrations); at 1044 cm^{-1} (C–O stretching in cellulose and hemicellulose); and at 903 cm^{-1} (C-H out of phase ring stretching in cellulose) [36–38,68,69].

Upon comparison of the spectra of the undecayed control sample and the control block at the end of the experiment, a decrease in the intensity of the band at 1606 and 1510 cm^{-1}, associated with lignin, and an increase in the intensity of the band at 1650 cm^{-1} (conjugated C-O from aromatic ring of xylans in hemicellulose) could be observed, suggesting a preference of the fungus for lignin [37]. In relation with the MMWC-treated wood blocks, only some slight changes in the chemical structure of the poplar wood were found, e.g., a decrease in the intensity of carbonyl band at 1,740 cm^{-1} (probably due to the opening of conjugated bonds and formation of new linkages with other groups in the hemicellulose), in good agreement with the study conducted by Nowrouzi et al. [27] on beech wood.

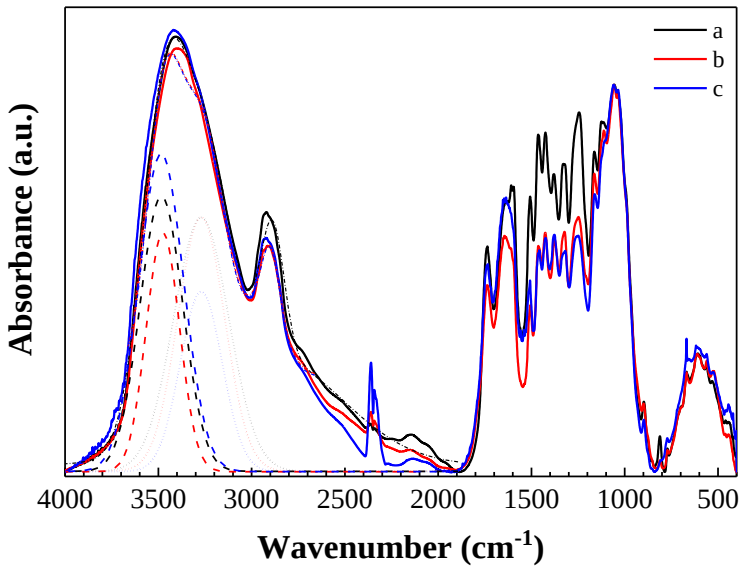

Figure 3. Normalized FTIR spectra of poplar wood samples: (*a*) undecayed control wood sample prior to exposure to *T. versicolor*; (*b*) control 30 days after exposure to *T. versicolor*; and (*c*) sample treated with medium molecular weight chitosan 30 days after exposure to *T. versicolor*. A deconvolution of O-H (dashed lines) and N-H (dotted lines) bands, together with the cumulative peak fit (short dash-dot lines), is shown.

4. Conclusions

The effectiveness of chitosan and the chitosan-based composites as wood preservatives against white-rot fungus *T. versicolor* was assessed, both *in vitro* and as surface protection treatments. The

minimum inhibitory concentration (MIC) against mycelial growth was determined for the individual components (MMWC, CO, P and nAg) and their binary (CO-P, CO-nAg, P-nAg) and ternary (CO-P-nAg) mixtures. A higher *in vitro* antifungal activity of CO *vs.* MMWC was observed, as well as a better performance of the binary mixtures in comparison with the individual products (full mycelium growth inhibition was attained at concentrations of 2 $mg \cdot mL^{-1}$ for CO, 0.2 $mg \cdot mL^{-1}$ for P and 2 $\mu g \cdot mL^{-1}$ for nAg when any two of the products were combined). Nonetheless, no significant improvements associated with the ternary composite over its binary counterparts were found for this fungus. The protective effect of the composites as coatings of poplar wood blocks was then evaluated, monitoring the wood decay over a 30-day period after exposure to the fungus in terms of weight loss and by microscopy and vibrational spectroscopy techniques. MMWC was found to be best protective agent, probably due to its higher viscosity and adhesion properties. Although a remarkable potential of the all treatments was evidenced by the *in vitro* tests, the medium molecular weight chitosan was the better considering commercial applications.

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