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Programa de Pós-Graduaçâo em Ciência e Tecnologia de Sementes

Tese

USO DE QUITOSANA, PRÓPOLIS E NANOPRATA NO TRATAMENTO DE SEMENTES DE ARROZ

Cassyo de Araújo Rufino

Pelotas, 2015

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Tese apresentada ao programa de Pós-Graduação em Ciência e Tecnologia de Sementes da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de doutor em Ciências (área do conhecimento: Ciência e Tecnologia de Sementes).

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USO DE QUITOSANA, PRÓPOLIS E NANOPRATA NO TRATAMENTO DE SEMENTES DE ARROZ

Tese aprovada, como requisito parcial, para obtenção de grau de Doutor em Ciências, Programa de Pós-Graduação em Ciência e Tecnologia de Sementes da Faculdade de Agronomia Eliseu Maciel da Universidade Federal de Pelotas.

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Oh! quão bom e quão suave é que os irmãos vivam em união.
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Resumo

RUFINO, Cassyo de Araújo. **Uso de quitosana, própolis e nanoprata no tratamento de sementes de arroz.** 2015. 45f. Tese (Doutorado em Ciência e Tecnologia de Sementes) - Programa de Pós-Graduação em Ciência e Tecnologia de Sementes, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, 2015.

A utilização de produtos naturais conjuntamente com nanopartículas de prata tem atraído interesse de pesquisadores, tanto do ponto de vista tecnológico quanto cientifico devido à ação desses produtos no controle de doenças e pragas. O objetivo desse trabalho foi testar produtos naturais (associados ou não a nanoprata) com ação antimicrobiana sobre *Bipolaris oryzae,* in vitro, e no tratamento de sementes de arroz. Para o estudo dos compostos contendo quitina e própolis adicionadas opcionalmente nanoprata foram utilizados sementes de arroz na cultivar IRGA 424 inoculadas com um isolado de *Bipolaris oryzae*. Os compostos utilizados são naturais, têm propriedades antiadesivas e atividade antimicrobiana contra microrganismos, possuem propriedades biocompatíveis e não fitotóxicas aos vegetais em geral. O processo de sínteses dos compostos contendo própolis, quitina e nanoprata, foi realizado por meio da aplicação de sonicação para a dispersão e mistura entre as substâncias. Foram testados os produtos no desenvolvimento e crescimento do fungo *Bipolaris oryzae* em placas de petri e em sementes de arroz infectadas. Os tratamentos foram: quitina, própolis, prata, quitina+própolis, quitina+própolis+nanoprata, quitina+prata, própolis+nanoprata e testemunha (T - sementes sem tratamentos), totalizando neste estudo 10 tratamentos. Logo a eficiência dos produtos e suas soluções foram testados por meio do blotter test (teste de sanidade de sementes), porcentagem de inibição e diâmetro de crescimento do fungo, germinação e comprimento de plântulas das sementes tratadas com os produtos. Os resultados demonstram que os oligômeros de quitosana e os compósitos de quitosana com extrato de própolis e nanopartículas de prata controlam o crescimento do patógeno. A importância deste achado reside no fato de que é um passo em direção ao objetivo de diminuir o uso de fungicidas químicos. A partir de testes de germinação poderá concluir-se que os melhores resultados foi o tratamento com quitosano/prata compósito NPs, com uma taxa de germinação de 91%. Os novos materiais poliméricos são considerados como muito promissores para o controle de *Bipolaris oryzae* no arroz, tornando-se assim alternativas ecológicas aos fungicidas químicos.

Palavras–chave: *Bipolaris oryzae, Oryza sativa* L., nanopartículas de prata, oligômeros de quitina e propolis.

Abstract

RUFINO, Cassyo de Araújo. **Use of chitosan, propolis and nanosilver in the treatment of rice seeds**. 2015. 45f. Tese (Doutorado em Ciência e Tecnologia de Sementes) - Programa de Pós-Graduação em Ciência e Tecnologia de Sementes, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, 2015.

The use of natural products in conjunction with silver nanoparticles has attracted interest of many researches, from both scientific and technological point of view due to their action for disease and pests control. The objective was to test natural products (with or without nanosilver) with antimicrobial action against *Bipolaris oryzae*, in vitro, and in the treatment of rice seeds. To study the compounds containing chitin and propolis optionally added nanosilver rice seeds were used in IRGA inoculated with an isolated 424 *Bipolaris oryzae*. The compounds used are natural, have anti-adhesive properties and antimicrobial activity against microorganisms, have biocompatible properties and not phytotoxic to plants in general. The process of synthesis of compounds containing propolis, chitin and nanosilver was performed by applying the sonication for the dispersion and mixing of the substances. Products were tested on the development and growth of *Bipolaris oryzae* fungus in petri dishes and infected rice seeds. The treatments were: chitin, propolis, silver, chitin + propolis, chitin + Propolis + nanosilver, chitin + silver, propolis + nanosilver and control (T - treatments seeded), totaling 10 treatments in this study. The results were conclusive in showing that chitosan oligomers and the composites of chitosan with propolis extract and silver nanoparticles led to a remarkable reduction in growth of the pathogen. The importance of this finding lies in the fact that it is a step towards the goal of decreasing the use of chemical fungicides in plant pathology. From germination tests it could be concluded that the best results was the treatment with chitosan/silver NPs composite, with a germination rate of 91%. The novel polymeric materials can thus be deemed as very promising for the control of the *Bipolaris oryzae* of rice and pave the way towards eco-friendly alternatives to chemical fungicides.

Keywords: *Bipolaris oryzae, Oryza sativa* L., silver nanoparticles, oligomers of chitosan and propolis.

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1 Introdução Geral

Há alguns anos que pesquisadores e cientistas do Brasil e do mundo discutem a respeito da presença de resíduos de agrotóxicos nos alimentos e salientam a preocupação com o meio ambiente. Aliado a isso, a exigência cada vez maior do mercado consumidor por produtos saudáveis, obtidos a partir de tecnologias de baixo impacto ambiental têm levado os pesquisadores a buscarem medidas alternativas para o controle de doenças de plantas, tais como o emprego do controle biológico e da indução de resistência (DI PIERRO e GARDA, 2008).

O uso de produtos de origem natural surge também da exigência que alguns países impõem a alguns produtos usados na agricultura, por exemplo, a União Europeia impõe restrições sobre os produtos para aplicação em plantas e sementes. Em alguns países já foram verificadas diminuição considerável nas populações de abelhas nos campos de produção de grãos e sementes, assim como também em bosques e florestas devido às aplicações de produtos químicos agressivos a organismos vivos. Portanto, surge a demanda por produtos naturais para aplicação em plantas, animais e insetos.

Nesse cenário, se faz necessário técnicas de produção de alimentos alternativas no sentido de modificar o ponto de vista de produção por caminhos mais sustentáveis, para garantir uma produção de alto nível com menor impacto. O uso de compostos naturais constitui-se como uma alternativa viável em comparação ao método químico tradicional, principalmente por não contaminarem o ambiente e não deixarem nos produtos tratados resíduos tóxicos prejudiciais ao homem e aos animais (SANG-LANG et al., 2002; FERNANDO et al., 2005). Vários autores relatam que esse método, ecologicamente viável e normalmente seguro, tem efeito fungistático, induz a resistência natural das plantas e pode prover proteção por um longo prazo para a cultura. Dessa forma, é um recurso alternativo para o biocontrole de patógenos fitopatogênicos (MAZARRO et al., 2008; RAPUZZI, 2009).

Entre os agentes que possuem propriedades antibióticas e ativadoras de mecanismos de defesa em plantas encontram-se a quitina e a quitosana. A quitina tem como principais fontes naturais às carapaças de crustáceos,

notadamente caranguejo, camarão e lagosta, que representam 15-20% do peso, e é também encontrada em insetos, moluscos e na parede celular de fungos (DI PIERRO e GARDA, 2008).

Por se tratar de polímero biodegradável, extremamente abundante e atóxico para os animais (SHAHIDI et al., 1999; RHOADES e ROLLER, 2000), a quitosana tem sido proposta como material atraente para usos diversos, principalmente em engenharia, biotecnologia e medicina. Na agricultura, a quitosana tem sido pesquisada no sentido de determinar sua habilidade no aumento da tolerância de plantas a estresses (LEE et al., 1999) e na ativação de respostas de defesa contra microrganismos fitopatogênicos (BENHAMOU, 1996). A indução de resistência com a utilização de quitosana foi demonstrada em culturas como pepino (*Cucumis sativus* L.), trigo (*Triticum aestivum* L.), ervilha (*Pisum sativum* L.), amendoim (*Arachis hypogaea* L.) e em arroz (*Oryza sativa* L.) contra *Pythium aphanidermatum* (LIN et al., 2005).

Os aditivos incorporados aos recobrimentos, para liberação de substâncias ativas com a função de proteger as sementes contra o ataque de microrganismos podem ser sintéticos ou naturais, como por exemplo, a própolis, utilizada suas propriedades biológicas (antimicrobiana, antiinflamatória, cicatrizante, antifúngicas entre outras) há milhares de anos. As pesquisas com própolis têm aumentado nas últimas décadas no sentido de caracterizar esse produto e identificar quais substâncias estão presentes na sua constituição e a partir desses resultados buscarem aplicações específicas para esse produto (CORRÊA, 2011).

Própolis é principalmente produzida dentro de colmeias de abelhas de diversas espécies, mas precisamente faz partes dos favos de mel. É considerado um produto constituído por uma mistura de diversas resinas vegetais, o qual é coletado por abelhas em plantas. As pesquisas sobre as propriedades antibióticas causam grandes descobertas sobre própolis têm sido conduzidas, sobretudo na área médica e veterinária, onde o produto tem demonstrado uma eficiente atividade bacteriostática e bactericida em relação a diversos gêneros de bactérias Gram positivas e Gram negativas. Tem sido sugerido que a atividade antibacteriana possa estar associada ao alto conteúdo de substâncias do tipo flavonóides presentes na própolis (GRANGE e DAVEY, 1990).

Também há relatos que a própolis tem ação antifúngica, porém tanto o efeito bacteriostático e antifúngico são determinados por fatores associados à técnica de extração, metodologia de condução de ensaios, local de origem da própolis e época do ano em que foi produzido podem ter influência sobre o maior ou menor grau de inibição do produto em relação às diferentes espécies bacterianas (SHUB et al., 1978; MERESTA e MERESTA, 1985; VALDES et al., 1989; FUENTES e HERNANDEZ, 1990).

Na literatura são encontrados resultados positivos sobre a própolis tanto para seres humanos, animais e vegetais, uma vez que a própolis é um produto 100% natural. Budija et al. (2008) investigaram e apresentaram a possibilidade do uso de extratos de própolis para tratamento de madeira. A atividade antimicrobiana dos extratos de própolis é influenciada pelo solvente utilizado para o seu preparo (TOSI et al., 1996). Extratos aquosos de própolis brasileira também mostraram um efeito antibiótico contra alguns patógenos de plantas, tais como: *Agrobacterium tumefaciens*, *Clavibacter michiganensis* e *Xanthomonas axonopodis. Erwinia chrysanthemi* foi em parte inibida, embora a bactéria *Pseudomonas syringae* não tenha sido inibida (BIANCHINI e BEDENDO, 1998).

A atividade antifúngica da própolis em diferentes espécies de *Candida* foi demonstrada por Uzel et al., 2005 e testes *in vitro* contra leveduras identificadas como causadores de onicomicoses também demonstram excelentes atividades fungistática e fungicida (OLIVEIRA et al., 2006; LONGHINI et al., 2007). PASTOR et. al. (2010) verificou a ação antifúngica de filmes a base de hidroxipropilmetilcelulose contendo própolis contra *Aspergillus niger* e *Penicillium sp*, sendo que a ação foi mais intensa contra *A. niger*.

Dessa forma, a utilização de recursos naturais, como própolis na confecção de biomateriais de recobrimento de sementes é uma tecnologia inovadora para o setor agrícola e ainda pouco explorada, especialmente no mercado de sementes que tem se destacado devido ao grande potencial de produção no Brasil. Além disso, discussões sobre as questões ambientais em todo mundo têm aumentado a demanda por produtos naturais a fim de reduzir os impactos causados ao meio ambiente e também à saúde humana (CORRÊA, 2011).

A quitosana é outro material que tem sido estudado por apresentar potencial para ser usado na agricultura e também em diversas outras áreas, como na, indústria de alimentos, de cosméticos e na indústria farmacêutica. O emprego da quitosana e a pesquisa por novas aplicações têm aumentado exponencialmente (SILVA et al., 2006; SHI et al., 2006; KUMAR, 2000). Após a década de 70 a produção industrial e o uso da quitosana apresentaram elevado crescimento (BEZERRA, 2011).

A quitosana é um amino polissacarídeo derivado da desacetilação da quitina, a qual se constitui na maior parte dos exoesqueletos dos insetos, crustáceos e da parede celular dos fungos. Por ser um produto natural, de baixo custo, renovável, abundante e atóxico, tem sido proposto como material potencialmente atraente para usos diversos, inclusive na agricultura (AZEVEDO et al., 2007).

Na agricultura, a quitosana tem sido pesquisada no sentido de determinar sua habilidade no aumento da tolerância de plantas a estresses (LEE et al., 1999) e na ativação de respostas de defesa contra microrganismos fitopatogênicos (BENHAMOU, 1996). A indução de resistência com a utilização de quitosana já foi demonstrada em culturas como pepino (*Cucumis sativus* L.), trigo (*Triticum aestivum* L.), ervilha (*Pisum sativum* L.), amendoim (*Arachis hypogaea* L.), arroz (*Oryza sativa* L.) e contra fungos como: *Pythium aphanidermatum* (Edson) Fitzp., *Alternaria alternata* (Fr.) Keissl., *Fusarium graminearum* Schwabe, *Fusarium solani* f.sp. pisi Snyd. & Hans., *Fusarium oxysporum* f.sp. apii Snyd. & Hans. e *Pyricularia grisea* (Cooke) (SATHIYABAMA e BALASUBRAMANIAM, 1998; BHASKARA REDDY et al., 1999; LIN et al., 2005).

Aplicada em sementes de beterraba e tomate, a quitosana teve eficiência no controle do tombamento de plântulas causadas por *Rhizoctonia solani*, além de induzir o sistema de defesa da planta, pelo aumento da atividade da enzima fenilalanina ammonia-liase (FAL) (MAZARO et al., 2009). Uma maior produção de matéria seca e de grãos de arroz cv. Suphanburi foi observada por Boonlertnirun et al. (2008) ao aplicar a solução de quitosana nas sementes e no solo antes da colheita. Segundo os autores, este fato pode estar associado ao maior período de disponibilidade da quitosana no solo.

Em estudos realizados por Pastucha (2008) foram verificados efeitos de diferentes aplicações da quitosana na proteção de plantas de soja contra patógenos do solo. De acordo com o autor, a quitosana quando aplicada nas sementes, nas mudas, e no início da antese foi mais eficiente em inibir infecções na soja em comparação com a aplicação da quitosana apenas no período de desenvolvimento da soja. O trabalho sugere que estas aplicações protegem as sementes das infecções por fungos do solo; o tratamento nas mudas prorroga o efeito protetor da quitosana e protege as folhas, e a aplicação durante a antese protege as flores e consequentemente os frutos e as sementes de infecções por fitopatógenos.

Paz-Lago et al. (2000) observaram o efeito da quitosana na proteção de plantas de tomate contra o fungo *Fusarium oxysporum* f.sp *lycopersici*, e observaram que no tratamento controle as plantas ficaram mais susceptíveis ao patógeno, e que a pulverização deste biopolímero (250 ppm ou 250 mg/mL) se mostrou eficiente na proteção do hospedeiro contra este fungo.

Além da utilização da quitosana e da própolis, outros materiais que estão cada vez mais difundidos na indústria brasileira são materiais nano estruturados. O grande interesse no uso de nanopartículas de prata (NPs Ag), por exemplo, está relacionado às suas propriedades como boa condutividade, elevado efeito catalítico, alta área superficial e excelente atividade antimicrobiana. (GUZMÁN et al., 2009). O efeito antimicrobiano da prata é reconhecido há muito anos. As nanopartículas de prata apresentam amplo espectro de ação contra bactérias, e fungos, além de ser altamente eficazes como agentes antivirais (RAI, 2003). Kim et al. (2012) avaliaram o efeito antifúngico de três diferentes nanopartículas de prata em diferentes concentrações (10; 25; 50 e 100 ppm) no controle in vitro de dezoito espécies de fungos fitopatogênicos e observaram que a inibição total do crescimento da maioria dos patógenos.

Na agricultura, esse produto representa uma importante inovação tecnológica uma vez que pode ser utilizada na produção de nanofertilizantes, nanocidas ou pesticidas encapsulados em nanopartículas para liberação controlada, além disso, esse material poderia ser usada na produção de nanocompósitos e nanobiocompósitos para revestimentos com películas

plásticas usados em embalagens de alimentos visando ao aumento de sua vida útil (FURLANETO, 2011).

Diante da incidência e severidade de doenças que acometem as culturas, torna-se necessária a adoção de estratégias de controle a fim de garantir maior sanidade e, consequentemente, produção e produtividade. De modo geral, para o controle de doenças fúngicas na agricultura, ainda predomina o uso de fungicidas sintéticos. Porém, o uso incorreto destes fungicidas na agricultura pode ocasionar prejuízos ambientais, morte de inimigos naturais e polinizadores, selecionar populações resistentes de pragas e doenças, provocar a presença de resíduos em alimentos e ainda ocasionar intoxicação em aplicadores e consumidores (ROEL et al., 2000; CARNEIRO et al., 2007; CARVALHO et al., 2008).

Desta forma, os esforços e pesquisas visando à inserção de alternativas de controle de doenças têm sido intensificados, tais como, o uso de fungicidas naturais de origem botânica (ARAÚJO e COSTA, 2013), controle biológico, além do recente emprego da nanotecnologia, que, segundo Furlaneto (2011), representa uma inovação na área da agricultura.

Os produtos citados já estão sendo usados na agricultura e podem ser alternativas viáveis no controle de fitopatógenos em plantas e também no tratamento de sementes. No entanto, ainda são escassos estudos sobre o uso destes materiais no controle de doenças em plantas e no tratamento de sementes, especialmente quando utilizados de maneira isolada, ou seja, sem adição de fungicidas. Nesse sentido, os objetivos desse trabalho foram testar produtos naturais (associados ou não a nanoprata) com ação antimicrobiana, sobre *Bipolaris oryzae,* in vitro, e no tratamento de sementes de arroz.

CAPÍTULO I- Synthesis of chitosan oligomers composite systems and study of their activity against *Bipolaris oryzae*[1]

2.1 Introduction

Rice (*Oryza sativa* L.) is a major cereal consumed by the world population, representing about 30% of world production of grains (YADAV, 2008). *Bipolaris oryzae* is the causal agent of rice brown spot disease and is responsible for significant economic losses, so brown spot can be deemed as one of the important crop diseases in the world. *Bipolaris oryzae* is classified in the subdivision Deuteromycotina (imperfect fungi), class Euteromycetes, order Moniliales, and family Dematiaceae.

Fungicide seed treatments are a must to protect the seeds and young seedlings from many seed and soil borne pathogens. Moreover, the conventional seed coating substances can be mixed with natural products, which have been reported to have protective effects on seeds (THOBUNLUEPOP, 2008). Since the European Union has placed severe restrictions to the application of conventional chemical products to plants and seeds, there is a growing interest in the development of natural (or biodegradable) products that can be used as an alternative to chemical control in order to reduce the effects of brown spot disease on young plants, provided that they would not present a menace to the health of humans, animals and environment (SHABANA et al., 2008). The use of biopolymers is ecologically viable and safe in general terms, and they are known to have antifungal effects, induce natural plant resistance and can also provide long term protection to the crop, so they constitute a particularly desirable approach for the biocontrol of phytopathogenic fungi (TAN et al., 2016).

The natural, biodegradable and biocompatible chitosan (glucosamine polymer with β-1,4 bonds), formed by the alkaline N-deacetylation of chitin, is

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one the most promising candidates to synthesize novel low-cost, environmentally friendly hybrid materials, due to its ability to form films, transparency, nontoxicity and excellent adsorption features (RAVI KUMAR, 2000). In agriculture, chitosan polymers have been used to promote plant tolerance to stress Lee et al (1999) and to activate defense responses to protect different plant species from pathogenic microorganisms Benhamou (1996), such as cucumber (*Cucumis sativus* L.), wheat (*Triticum aestivum* L.), peas (*Pisum sativum* L.), peanut (*Arachis hypogaea* L.) or rice (*Oryza sativa* L.) (LIN, et al, 2005).

On the basis of its chemical properties, apart from inducing the immunologic system to promote resistance to plant pathogens, chitosan may also stimulate plant growth and yield too (BOONLERTNIRUN et al (2008). Borges et al. (2000) reported the suitability of chitosan to protect tomato plants from *Fusarium oxysporum*. In addition, chitosan has been found to have antimicrobial properties (RHOADES, 2000; CHUNG et al, 1994) in which the size of its oligomers plays a key role (HADWIGER, 1994).

On the other hand, propolis is known to have antimicrobial properties and has been extensively used in traditional medicine (NEDJI; LOUCIF-AYAD, 2014). Propolis is a natural resinous hive product collected by honeybees from various plant sources Bankova (2005) which contains over 150 chemical species (such as coumarins, flavonoids, polyphenols, phenolic aldehydes, sesquiterpene quinines, amino acids and steroids) (GHISALBERTI, 1979). Its strong antimicrobial activity may be due to its high content in phenols and flavonoids (KALOGEROPOULOS et al, 2009). Propolis has also been found to have applications as an antioxidant and in food preservation (ELWAKIL, 2000).

Shabana et al. (2008) studied the control of brown spot pathogen of rice (*Bipolaris oryzae*) using some phenolic antioxidants (salicylic acid, benzoic acid and hydroquinone). Hydroquinone had also been reported to be a promising antioxidant for managing seed borne pathogenic fungi of peanut (GÓMEZ-ESTACA, 2010). Other research groups have conducted studies by combining chitosan with oils and/or plasticizers, so as to yield different types of composites or nanocomposites, such as gelatin chitosan-based edible films incorporated with clove essential oil, whose antimicrobial activity was tested against six selected microorganisms (namely *Pseudomonas fluorescens, Shewanella*

putrefaciens, Photobacterium phosphoreum, Listeria innocua, Escherichia coli and *Lactobacillus acidophilus*) (CHAMI et al, 2005). Thobunluepop (2008) studied the effects of various seed coating substances (chitosan lignosulphonate polymer and eugenol) on rice seed in comparison with chemical antifungal coatings. The seeds coated with biological materials were found to maintain higher sugar contents, which significantly enhanced seed storability (in contrast, under chemical fungicide stress, those compounds were lost, which directly affected seed vigor during storage).

Finally, the incorporation of nanosilver to the organic composites may also synergistically improve their antimicrobial effects, and the use of *in situ* synthesis methods allow its incorporation into the polymer matrix attaining uniform distributions and avoiding aggregation. Nevertheless, when nanoparticles (NPs) –such as nanosilver or nanozinc ones– are to be used as biocides on the surface of plants, the recommendations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) on the application of nanotechnologies in the food and agriculture sectors and their potential food safety implications should always be taken into consideration.

This work aims to study *in vitro* the effect of chitosan oligomers –and its combinations with other substances such as propolis and/or silver nanoparticles– on the growth of *B. oryzae* fungus, so as to preserve the quality of seeds and their components. These new uses often require improved application systems for better established dosages and coverage of materials Sun et al (2007) and –consequently– we herein report the synthesis and studies on the potential activity, action mechanisms and *in vitro* assays with copolymers or composites of chitosan and propolis (which may also incorporate silver NPs), with a view to their field application as seed coating substances against rice seed borne fungi.

2.2 Material and methods

Reagents and characterization equipment

Medium molar mass chitosan (CAS number 9012-76-4) was purchased from Sigma Aldrich Química SL (Madrid, Spain). Propolis came from Burgos

region (Spain), in the Duero river basin, and has a polyphenols and flavonoids content of ca. 10% w/v. Silver nitrate (CAS number 7761-88-8), malt extract agar (Reference 105398) and potato dextrose agar (Reference 110130) were supplied by Merck Millipore (Darmstadt, Germany). Potassium methoxide solution (25 wt.% in methanol, CAS number 865-33-8) and ethanol (puriss. p.a., ACS reagent, CAS number 64-17-5) were also purchased from Sigma Aldrich Química SL. The isolated (inoculum) *Bipolaris oryzae* mycelium was supplied by Universidad Federal de Pelotas (Brasil). IRGA 424 rice seeds were acquired in Pelotas, Rio Grande do Sul, Brazil.

An ultrasonic machine, model CSA 20-S500, 20 KHz was used for the sonication of the solutions.

Synthesis of solutions of chitosan oligomers, chitosan oligomers/propolis, and chitosan oligomers/propolis/Ag NPs

Chitosan oligomers preparation (A_{50})

Chitosan oligomers aqueous solutions were prepared from a solution of commercial medium molar mass chitosan with molar masses in the 190000–310000 g/mol range in AcOH 2% at pH 4–6. The hydrolysis was performed by stirring for 12 hours followed by 3–6 sonication periods (5 minutes each), at temperatures in the 30 to 60 °C range and with H_2O_2 concentrations ranging from 0.3 to 0.6 M, obtaining oligomers with molar masses in the 6000 to 2000 g/mol range, respectively, in agreement with the analogous microwave-based procedure reported by Sun et al. (2007). The molar mass of the chitosan samples was determined by measuring the viscosity, in agreement with Yang et al. (2005), in a solvent of 0.20 mol/L NaCl + 0.1 mol/L CH_3COOH at 25 °C using an Ubbelohde capillary viscometer. Molar masses were determined using the Mark-Houwink equation $[\eta] = 1.81 \times 10^{-3} M^{0.93}$ (MAGHAMI; ROBERTS, 1988). The solutions were then decanted to remove any water insoluble material, were allowed to rest till cloudiness was observed, and were centrifuged to isolate the chitosan oligomers. These were re-dissolved again in AcOH 0.5%, obtaining the solutions for the assays.

Aqueous solutions of chitosan oligomers 0.005-0.01 M or solutions at 1.25-2.5% w/v (solution A_{50}) were prepared from a solution of chitosan

oligomers with 2000 g/mol molar mass in AcOH at 0.5% and pH 4-6, adjusted with some dropplets of $KOCH_3$ 25% in methanol. The chitosan/AcOH/water mixture was sonicated for a minute and was subsequently stirred for 12 hours, resulting in a transparent and stable chitosan solution. The solution was stored in inert atmosphere at 4ºC at pH 5 till it was used. The characterization of the different products can be found in (MATEI et al, (2015).

Propolis extraction (P)

The propolis solution was prepared by grinding raw propolis to fine powder and subsequent extraction of the active ingredients by maceration in a hydroalcoholic solution 7:3 (v/v) for one week at room temperature. A hydroalcoholic medium was chosen over absolute ethanol because it results in wax-free tinctures containing higher amounts of polyphenolic substances (WOISKY; SALATINO, 1998). The resulting solution was then percolated (1 L/min) and filtrated with a stainless steel 220 mesh to remove any residue, followed by concentration at a temperature below 60 ℃ with ultrasound equipment to finally obtain a clarified propolis extract, and finally solutions with a propolis concentration of 100 mg/mL or 10% w/v were prepared (labelled as *P*).

In situ preparation of chitosan/propolis mixtures (B_{100})

A 50 mL solution of A_{50} (i.e., 2.5% w/v of chitosan in water) was mixed with 50 mL of a solution of 2.5% w/v of propolis in water/alcohol, and the resulting mixture (100 mL) was sonicated for 1 minute.

Silver NPs preparation

Silver nanoparticles were prepared by a sonication method, without resourcing to UV stabilization (used, e.g., by MONTAZER et al*,* 2012), 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 ℃. 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 ºC.

The resulting sonicated solutions had silver contents ranging from 100 to 200 ppm (nAg solution) and were characterized by UV-Vis absorbance at 420 nm with a Shimazdu UV-2450 UV-Vis spectrophotometer. The silver nanoparticles size was studied by SEM and TEM with a FEI-Quanta 200FEG and a JEOL JEM-FS2200 HRP, respectively (MATEI et al, 2015). The solutions were stable in inert atmosphere at 4 ºC.

In situ preparation of chitosan/propolis/silver NPs mixtures (C_1, C_2)

The chitosan oligomers/propolis solutions (B_{100}) were prepared according to section 0. Two different 50 mL solutions of silver nanoparticles, with a concentration of 10 ppm and 20 ppm, respectively, were also prepared. The silver NPs solutions were then added to the chitosan oligomers/propolis solutions. The resulting solutions were sonicated for a minute, so as to obtain *in situ* the two new mixtures of chitosan/propolis/silver NPs solutions, labelled as C_1 (for 10 ppm nAg) and C_2 (for 20 ppm nAg).

In situ preparation of chitosan/silver NPs mixtures (D_1, D_2)

50 mL of A_{50} chitosan oligomers solution were mixed with 50 mL of 10 ppm silver NPs solution, yielding D_1 solution, and another 50 mL of A_{50} were mixed with 50 mL of 20 ppm silver NPs solution, yielding D_2 solution. Both mixtures were sonicated for a minute.

In situ preparation of propolis/silver mixtures (F_1, F_2)

Two 50 mL hydroalcoholic propolis solutions (2.5% w/v) were mixed with 50 mL of 10 ppm silver NPs solution, yielding D_1 solution, and 50 mL of 20 ppm silver NPs solution, yielding D_2 solution, respectively. The resulting mixtures were sonicated for a minute.

Culture media and activity assays

Identification and cultivation of isolates

Conidia of *Bipolaris oryzae* isolates were induced to germinate in Petri dishes containing agar and water, followed by incubation in a growth chamber with controlled temperature (25 ℃) and photoperiod (12 h of light/12 h of darkness) for 3-4 hours. The morphology of conidia and conidiophore, spore germination, position and direction of growth of the germ tube and ontogeny of septa were checked (ALCORN, 1988). The isolates were grown in potato dextrose agar (PDA). After 14 days, the conidial length and width and the number of septa of each isolate were assessed.

Inoculum preparation

The fungal isolates were cultured in Petri dish containing PDA culture medium and incubated for 14 days in aforementioned growth chamber. 10 mL of sterile distilled water were then added to each Petri dish, and mass spores were homogenized with the aid of a sterilized brush. The suspension, after filtering (with a funnel with gauze filter), was collected in a test tube. Then the suspension was standardized at 2000 spores/mL using a Neubauer chamber. Seeds inoculation was conducted by immersion in the spore suspension in the Petri dishes for 48 hours in the growth chamber.

Seed health testing

According to ISTA (2010) standard Blotter and agar plate tests, seeds were incubated for a definite period under specific conditions. The fungicidal action of the products under study was tested *in vitro* using potato dextrose agar (PDA) as a culture medium. The nine solutions described above (A_{50}, B_{100}, C_1, C_2, D_1, D_2, F_1, F_2, P) were used as treatments against *Bipolaris orizae* fungus at three different concentrations (300 µL/mL, 600 µL/mL y 900 µL/mL) in PDA at 25 ºC and compared with the control (T). The seeds were incubated at 20±2 ℃ for seven days under a photoperiod regime of 12 hours of light/12 hours of darkness. The treatment of the seeds inoculated with *Bipolaris oryzae* was conducted by spraying at a dose of 1 mL/100 g of seeds. 2000 inoculated rice (*Oriza sativa* cv. IRGA424) seeds were validated and distributed in 8 repetitions of 25 seeds per 10 treatments (A_{50}, B_{100}, C_1, C_2, D_1, D_2, F_1, F_2, P, T).

The 25 inoculated seeds of each repetition were distributed in germitest paper rolls, previously pressed to obtain 0.5 cm × 0.5 cm capsules, and placed in 11.5 cm × 11.5 cm × 3.5 cm transparent plastic boxes with lids, previously disinfected with a sodium hypochlorite 1% solution. The germitest paper rolls were soaked with an amount distilled water equivalent to 2.5 times the weight of the paper used. After an incubation period of 14 days at 20-25ºC with 12 h of light, the seeds were individually examined for fungal growth using a stereomicroscope, and the incidence of pathogenic fungi was expressed as the percentage of infected seeds.

Determination of the inhibition percentage for *Bipolaris oryzae* fungus

Growth measurements of the diameter of the fungal mycelium were performed in triplicate (R_1, R_2 and R_3) to determine the degree of sensitivity/resistance to each of the products (A_{50}, B_{100}, C_1, C_2, D_1, D_2, F_1, F_2, P, T). The diameter of fungal growth was measured on a daily basis for 20 days, and the inhibition percentage (IP) was calculated taking the pure MEA culture (control) as a reference according to the following equation (JIANG, 2005):

$$IP\ (\%) = \frac{Dmt - Dvar}{Dmt} \cdot 100$$

Where *Dmt* is the diameter of the mycelium in the control (pure MEA) and *Dvar* is the diameter of the mycelium of the sample mixed with one of the antimicrobial composites.

Germination test

The seeds were treated with the different combinations of active products (*viz*. A_{50}, B_{100}, C_1, C_2, D_1, D_2, F_1, F_2, P) and 4 repetitions with 100 seeds were carried out for each treatment (plus control), according to ISTA (2010). The results were expressed as the percentage of normal germinated seedlings.

Seedlings stem and root dimensions

Stem and root lengths (in mm) were measured -with a graduated scale in millimeters- for 10 normal seedlings randomly chosen from 4 replications of 20 seeds per treatment, which had been placed to germinate in the germitest

paper rolls, according to the method described above. The arithmetic mean of the stem and root lengths were then calculated and the results were expressed in cm (KRZYZANOWSKI, 1999).

Statistical analysis

The trial was arranged in a completely randomized design with 4 repetitions, totaling 40 experimental units. The experimental unit was characterized by Petri plates and germitest paper rolls. Data were analyzed by ANOVA and by Tukey's HSD test at 5% significance level. Statistical analyses were performed using the statistical program SASM-Agri (CANTERI et al, 2001).

2.3 Results and discussion

Vibrational characterization

The ATR-FTIR spectra of commercial chitosan, chitosan oligomers, propolis, chitosan oligomers/propolis binary composite and chitosan oligomers/propolis/silver NPs ternary composite are depicted in Figure 1.

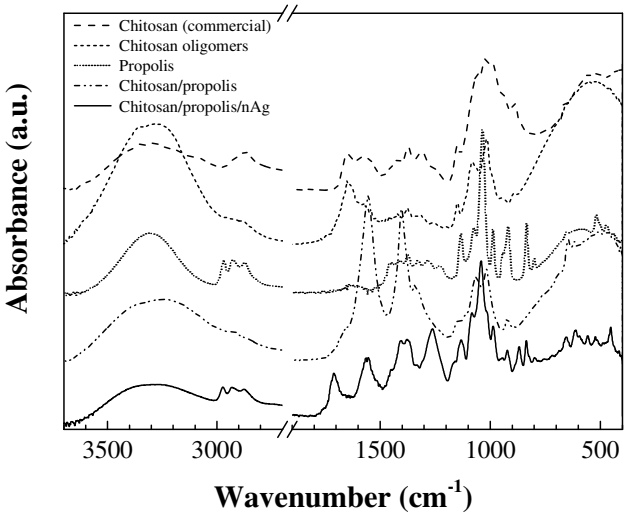


Figure 1. ATR-FTIR spectra of the chitosan- and propolis-based materials under study.

Analyses of both the binary chitosan-propolis formulation and the ternary composite showed that the first band in the ATR-FTIR spectra was

shifted to higher wavelengths in comparison to the bare chitosan spectrum (from 3285-3290 to 3300 cm^{-1}) (SILVA et al.,2012; VENKATESHAM et al., 2012) suggesting that effective hydrogen bonding occurred between chitosan and propolis. This interaction is an indicator of a synergy between the two products.

Another significant feature for the ternary composite spectrum was an obvious shift to 1700 cm^{-1} of the band located at around 1650 cm^{-1}, accompanied by an increase in the intensity, proving the responsibility of chitosan amino group for silver envelopment (VENKATESHAM et al., 2012; ABDEL-FATTAH et al., 2014; VIMALA et al., 2010; FRANCA et al., 2014). In the same way, the change in the OH band intensity in the ternary composite spectra at 1409 cm^{-1} (assigned to the OH deformation vibrations of the secondary alcohols in the pyranose monomers) revealed that the hydroxyl groups may have also participated in the stabilization of the silver nanoparticles via interaction of Ag^{+} with the electron abundant oxygen atoms of the hydroxyl groups of the chitosan. These results are in agreement with those reported by Dusan et al. (2010). Moreover, the increase in intensity of the band at 1380 cm^{-1} (attributed to CH_3 bending in $NHCOCH_3$ group (ZVEZDOVA et al., 2010) can also be attributed to interactions with silver NPs.

In the particular case of the band at 1133 cm^{-1}, characteristic of ester groups (-C-O stretching) from propolis components (BUTNARIU and GIUCHICI, 2011), a change in the intensity of the band was also observed, attributable to interactions with silver NPs. Unassigned typical bands from binary and ternary formulations have been found at around 650 and 616 cm^{-1}.

X-ray characterization

The X-ray diffraction study of the chitosan oligomers exhibits the expected broad peaks at 2θ=10º and 2θ=20º, in good agreement with Kumar et al. (2011). However, the peak observed for chitosan at 2θ=20° disappeared and the very broad peak at 2θ=10° became weak in the chitosan-propolis sample. These results suggest that chitosan has good compatibility, which leads to the formation of a composite with an amorphous form, suitable for bio-applications (KUMAR and KOH, 2012).

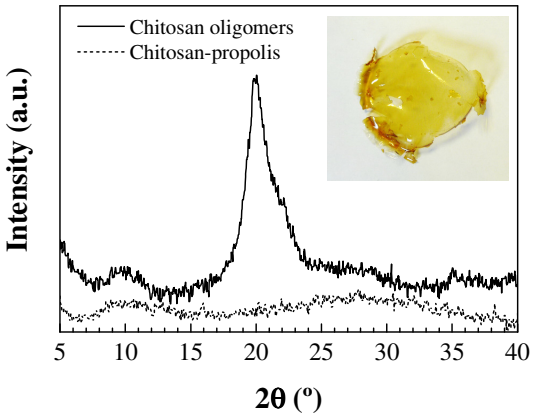


Figure 2. X-ray powder diffraction pattern for chitosan oligomers (*solid line)* and chitosan oligomers/propolis composite (*dashed line*). The inset shows a photograph of the chitosan oligomers/propolis film.

Silver NPs characterization

Silver nanoparticles were also characterized by UV-Vis absorption, XRD and TEM analysis, revealing the formation of highly pure, crystalline silver nanoparticles of ca. 30 nm (see subsection 0). The UV-Vis spectrum (Figure 3, *left*) showed the expected intense surface plasmon resonance (SPR) band at around 420 nm (VILAS et al., 2014). The X-ray powder diffraction pattern (Figure 3, *right*) matched well with the standard patterns of silver (JCPDS No. 04-0783). All the peaks of the pattern can be readily indexed to face-centered-cubic silver, where the diffraction peaks at 38.2, 44.5, 64.5 and 77.5º can be ascribed to the reflection of (1,1,1), (2,0,0), (2,2,0), (3,1,1) planes, respectively.

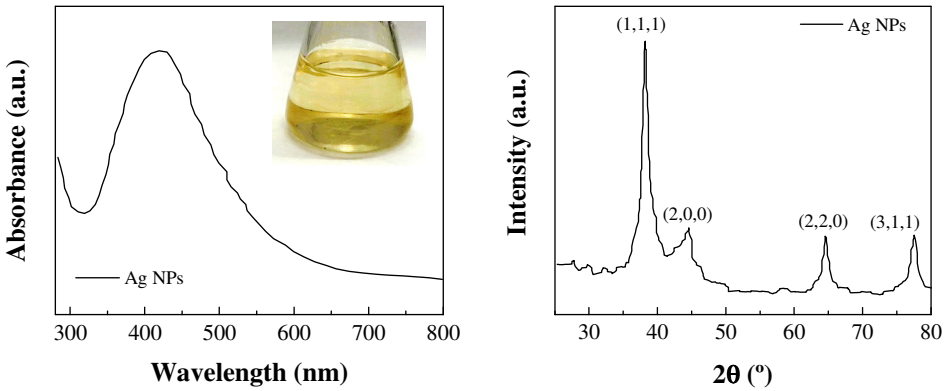


Figure 3. UV-Vis spectrum (*left*) and X-ray powder diffraction pattern, smoothed with a Savitzky–Golay 10 pt. window filter (*right*).

Textural properties

The texture of the ternary composite films has been studied by SEM and TEM. The SEM micrographs (Figure 4, *left*) –similar to those reported by Moharram et al. 2014– show the good homogeneity of the materials under study, while the TEM micrograph (Figure 4, *right*) allows to estimate the size of the silver NPs, in the 24 to 35 nm range.

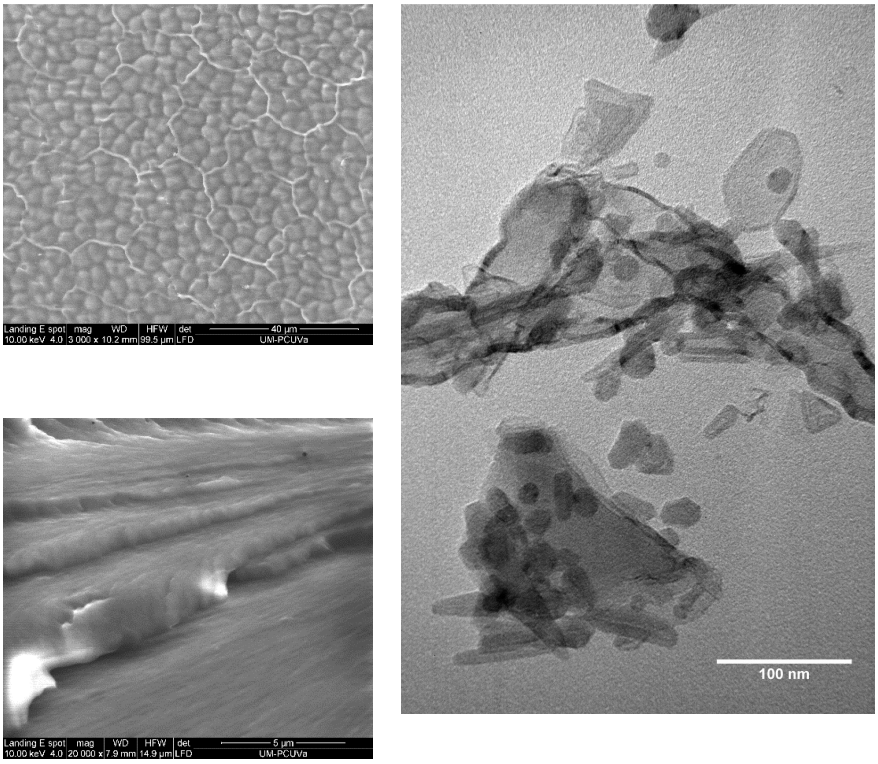


Figure 4. SEM micrographs (*left*) and TEM micrograph (*right*) of the ternary chitosan oligomers/propolis/silver NPs composite.

Treatment of rice seeds against *Bipolaris oryzae* fungus

Antifungal properties

In Table 1 it may be observed that, even at a concentration of 300 µL/mL, a complete inhibition of *Bipolaris oryzae* growth (Ø=0 cm) was attained for chitosan oligomers (A_{50}), chitosan/propolis (B_{100}), chitosan/propolis/nAg (C_1 and C_2) and chitosan/nAg (D_1 and D_2) treatments. For the propolis (P) and propolis/nAg (F_1 and F_2) treatments, higher concentrations (600 µL/mL) were required so as to achieve complete inhibition of the fungus.

Table 1. Mycelium diameter for *Bipolaris oryzae* fungus as a function of concentration for the different treatments: A_{50}, B_{100}, C_1, C_2, D_1, D_2, F_1, F_2, P and the control.

Treatment	Concentration (µL/mL)		
	300	600	900
A_{50}	0	0	0
B_{100}	0	0	0
C_1	0	0	0
C_2	0	0	0
D_1	0	0	0
D_2	0	0	0
F_1	5.7	0	0
F_2	5.7	0	0
P	6.8	0	0
Control	7.4	7.8	7.1
Coefficient of variation (%)	4.5	3.5	4.1

Figure 5 shows an example of the radial growth of the fungus in Petri dishes, depicting the control and the results obtained at different concentrations of one of the treatments (C_2). It can be readily observed that, whereas the fungus covers the entire Petri dish for the control (*right*), no mycelial growth took place for the seeds treated with the chitosan/propolis/nAg composite, regardless of the concentration.

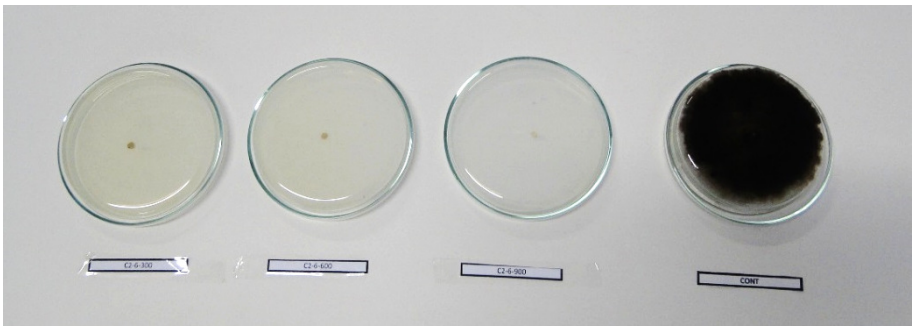
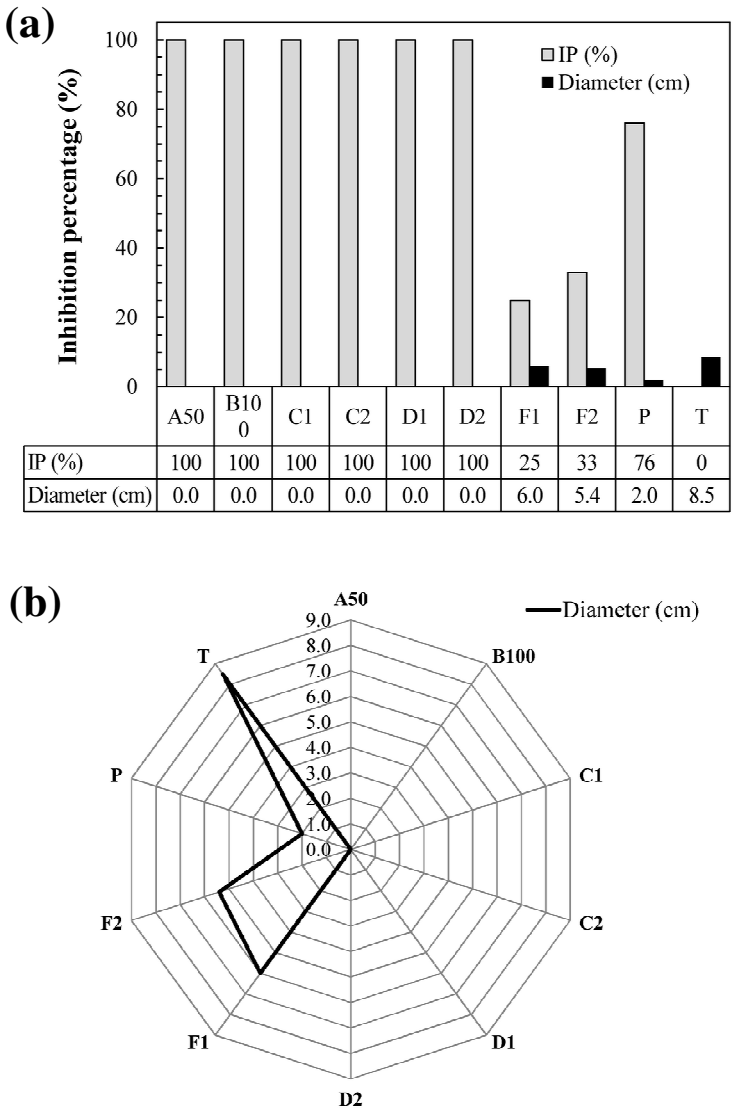


Figure 5. Growth of *Biporaris oryzae* fungus in Petri dishes. From left to right: Petri dishes with no fungal growth at concentrations of 300, 600 y 900 µL/mL for C_2 treatment and Petri dish with complete growth of the fungus for the control.

According to Figure 6, the chitosan oligomers (A_{50}), chitosan/propolis (B_{100}), chitosan/propolis/nAg (C_1 and C_2) and chitosan/nAg (D_1 and D_2) combinations would be particularly active, with maximum inhibition percentages (IP). The lower efficacy of propolis (P) and propolis/nAg (F_1 and F_2) treatments can be attributed to a decrease in solubility due to the use of hydroalcoholic solutions for propolis conveying. This suggests that subsequent studies should

focus on improving the solubility, for example by replacing the current intermolecular bonding system (cross-linkage) with the formation of graft copolymers (between chitosan and propolis oils), in line with the studies of Kumar Tiwary et al, (2011) and Gómez-Estaca et al. (2010), as it will be further discussed in section 0.



	A50	B100	C1	C2	D1	D2	F1	F2	P	T
IP (%)	100	100	100	100	100	100	25	33	76	0
Diameter (cm)	0.0	0.0	0.0	0.0	0.0	0.0	6.0	5.4	2.0	8.5

Figure 6. (*a*) Inhibition percentage (IP) and (*b*) growth diameter of *Bipolaris oryzae* fungus for the treatments with chitosan oligomers (A_{50}), chitosan/propolis (B_{100}), chitosan/propolis/nAg (C_1 and C_2), chitosan/nAg (D_1 and D_2), propolis/nAg (F_1 and F_2), propolis (P) and control (T).

Several possible mechanisms have been proposed to explain the antibacterial properties of chitosan: it is known that positively charged amine groups are capable of interacting with the negatively charged bacterial cell

membrane and, in addition, chitosan may also bind to DNA, leading to inhibition of mRNA and proteins synthesis (DUTRA et al, 2009). The increase in the antimicrobial activity of the new composites with chitosan oligomers may be due to chemical interaction of the amine and hydroxyl groups with nanosilver. It has also been demonstrated that polymeric chitosan and chitosan oligomers induce phytoalexins that help limit the spread of pathogens (KENDRA et al, 1989). Chemical synthesis of different sizes of chitosan oligomers with specific biological activity has been described by Kuyama et al. (1993).

For mixtures of pure propolis extract (*P*) or the propolis/silver NPs combinations (F_1 and F_2), their efficiency must be referred to the presence of phenolic groups (HAYAT, 1993) and to enzyme inhibition by nonspecific interactions with proteins (COWAN, 1999).

Silver nanoparticles have been deemed as one of the most promising antimicrobial species from a nanotechnologybased approach, since their activity is very broad and is well above that of raw silver. For example, silver ions can bind to negatively charged bacterial peptidoglycan walls and can diffuse into bacterial cells and bind to DNA bases, leading to bacterial death and/or inhibiting the replication and transcription processes and preventing further bacterial production (SHRIVASTAVA et al, 2007). Moreover, the generation of reactive oxygen species, which leads to nanotoxicity processes, is also a well-established antimicrobial mechanism. The main disadvantages that would limit the use of nanosilver are its ease of aggregation and the uncontrolled release of silver ions and their cytotoxicity potential (ALTIOK, 2010), which are not an issue in this case.

Phytotoxicity tests

The phytotoxicity of the different combinations (*vs*. the control) was assessed through the results of the germination tests and the measurement of stem and root lengths, summarized in Table 2. From these results it can be inferred that –for the germination of rice seeds– chitosan/silver NPs (D_2) and propolis (*P*) were the two treatments that led to the highest germination indices (91% and 90%, respectively), significantly different from that obtained for the control (85%). For the rest of the treatments, it can be concluded that the

antifungal properties are not accompanied by the stimulating properties for germination or for seedling growth that were initially expected.

Table 2. Results from the germination tests and stem and root lengths for seeds treated with different combinations of chitosan/propolis/silver NPs: A_{50}, B_{100}, C_1, C_2, D_1, D_2, F_1, F_2, P and control.

Treatment	Germination test (%)	Stem length (cm)	Root length (cm)
A_{50}	85	3.36	8.33
B_{100}	65	2.81	8.75
C_1	85	2.87	10.15
C_2	85	2.71	9.57
D_1	82	3.76	8.89
D_2	91	3.92	9.23
F_1	60	2.86	9.87
F_2	80	2.74	9.31
P	90	4.18	10.54
Control	85	4.14	10.45
Coefficient of variation (%)	2.5	3.6	3.1

Synthesis-structure-activity relationships

Chitosan oligosaccharides-based nanocomposites

Although –as noted above– chitosan is a source of potential bioactive material, it has several drawbacks for its direct utilization in biological applications, including its poor solubility under physiological conditions and a high viscosity in dilute acidic solutions (KIM, 2005). In contrast, the hydrolyzed products of chitosan, such as its oligomers, have better solubility and lower viscosity, because of their shorter chain lengths and the free amino groups in the D-glucosamine units. A weight-average molecular weight (MW) ranging from 10,000 Da to about 100,000 Da is considered for low MW chitosan, while the MW of oligochitosan is generally lower than 10,000 Da (DUY et al, 2011). The activity of chitosan is closely correlated with its structure and physicochemical properties, such as its degree of deacetylation, degree of polymerization and cationic nature, which are improved in the oligomers (XIA, 2001). Sonication has become an alternative for degrading chitosan into low-molecular-weight chitosan, chitosan oligomers and glucosamine. The degree of deacetylation tends to decrease due to the fact that the amino groups on the C-

2 of chitosan facilitate the site-specific fragmentation of the glycosidic linkage during *β*-cleavage after the sonication treatment (SAVITRI, 2014).

Grafting and crosslinking mechanisms for the formation of chitosan based nanocomposites

Different chitosan products have different structures and physicochemical properties, which may result in novel bioactivities or novel findings in known bioactive compounds (ZOU et al, 2016). Chemical modification of chitosan/chitosan oligomers can be attained by N-substitution, by O-substitution, or by N,O-substitution, and also *via* chitosan association with small molecules or macromolecules. In this regard, chitosan can be deemed as a promising material, since it contains three types of reactive functional groups: an amino/acetamide group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions. The amino contents are the main reason for the differences between their structures and physicochemical properties, which are correlated with their chelation, flocculation and biological functions (XIA, 2011) (Figure 7).

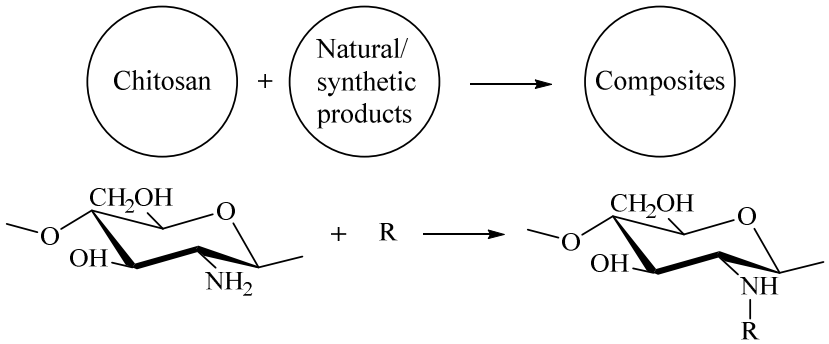


Figure 7. Composite formation from the D-glucosamine structure by addition of a functional group.

Chitosan carries free amine functionalities on the deacetylated units and hydroxyl groups on the acetylated as well as deacetylated units. The modification of chitosan by introduction of small functional groups such as alkyl or carboxymethyl groups can increase its solubility at neutral and alkaline pH without affecting its cationic character. Thus, chitosan can be grafted with other molecules through covalent binding (Figure 8a). The amino groups can be used for acetylation, quaternization, reactions with aldehydes and ketones, chelation

of metals, etc., while the hydroxyl groups can lend to o-acetylation, H-bonding with polar atoms, etc. (KUNUR, 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.

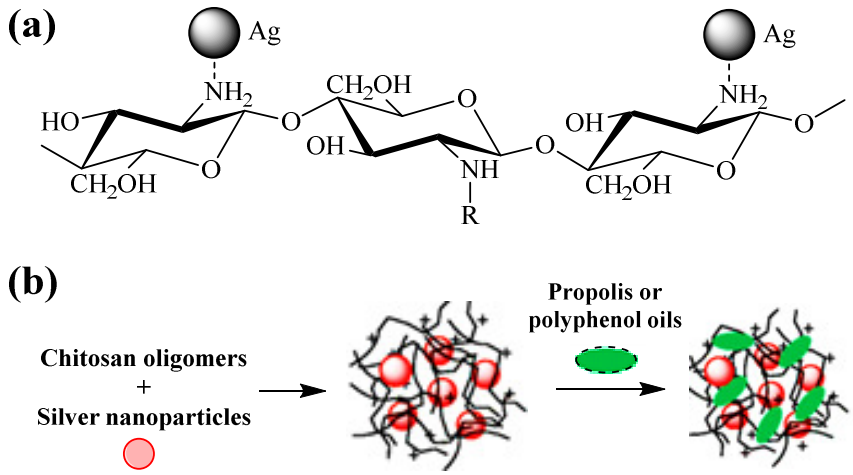


Figure 8. Mechanisms for the formation of chitosan based nanocomposites: (*a*) Composite formation from chitosan oligomers grafted to propolis or polyphenol oils (R) and silver nanoparticles (Ag); (*b*) Composite formation by electrostatic interactions and intermolecular hydrogen bonding between chitosan, propolis and silver nanoparticles.

Chitosan may also be cross-linked with natural extracts by electrostatic interactions and intermolecular hydrogen bonding (Figure 2b). In recent years, the unparalleled and functional properties of essential oils have been extensively reported, but the sensitivity of essential oils 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 evaluated by FTIR and thermal analysis. The results indicated 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 and ASGARI, 2015). Thyme oil has also been mixed with chitosan in order to make biofilms, for potential applications of wound dressing.

The antimicrobial and the antioxidant activities of the films were also investigated. The results revealed that thyme oil had a good potential to be incorporated into chitosan to make antibacterial and permeable films for wound healing applications. The FTIR spectra of chitosan films incorporated with different amounts of thyme oil showed the same pattern on their informative peaks as the control chitosan films, thus indicating that there was no interaction between active groups of thyme oil with functional groups of chitosan (ALTIOK et al., 2010) 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 that case, and also for the treatments assayed in the study reported herein, the ATR-FTIR vibrational characterization suggested the existence of hydrogen bonding between chitosan and propolis.

Our results and those related in the literature suggest that chitosan-propolis graft copolymers would feature an enhanced stability and solubility in comparison to the copolymers based in crosslinking or intermolecular hydrogen bonds. Therefore, future lines of research should place emphasis on the development of the former composites.

2.4 Conclusion

The effect of chitosan oligomers and their combination with propolis and/or silver nanoparticles was studied *in vitro* against a seed borne pathogenic fungus: *B. oryzae*. The results were conclusive in showing that chitosan oligomers and the composites of chitosan with propolis extract and silver nanoparticles led to a remarkable increase of the plant resistance against microbial pathogens.

The importance of this finding lies in the fact that it is a step towards the goal of decreasing the use of chemical fungicides in plant pathology. From germination tests it could be concluded that the best results (in comparison to the other treatments) corresponded to the treatment with chitosan/silver NPs

composite, with a germination rate of 91%. The novel polymeric materials can thus be deemed as very promising for the control of the brown spot disease of rice and pave the way towards eco-friendly alternatives to chemical fungicides. Other further research aimed at the preparation of chitosan/propolis graft copolymers and to assess the shelf-life of the different seed coating substances during storage is under way.

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