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SYNTHESIS OF CHITOSAN OLIGOMERS COMPOSITE SYSTEMS AND STUDY OF THEIR ACTIVITY AGAINST BIPOLARIS ORYZAE

CASSYO ARAUJO-RUFINO[1], JUCILAYNE FERNANDES-VIEIRA[1], PABLO MARTÍN-RAMOS[2,3], IOSODY SILVA-CASTRO[3], MARCIABELA FERNANDES-CÔRREA[3], PETRUTA MIHAELA MATEI[4], MERCEDES SÁNCHEZ-BÁSCONES[4], M. CARMEN RAMOS-SÁNCHEZ[5] and JESÚS MARTÍN-GIL[3]

[1]Seed Science and Technology Laboratory
Federal University of Pelotas
96001-970 Pelotas, RS
Brazil

[2]Department of Agricultural and Environmental Sciences
Higher Polytechnic School of Huesca
University of Zaragoza
Carretera de Cuarte s/n
22071 Huesca
Spain
e-mail: pmr@unizar.es

[3]Department of Agricultural and Forestry Engineering
ETSIIAA, University of Valladolid
Avenida de Madrid 44
34004 Palencia
Spain

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[4]Department of Agriculture and Forestry Science
ETSIIAA, Universiity of Valladolid
Avenida de Madrid 57
34004 Palencia
Spain

[5]Río Hortega University Hospital
Calle Dulzaina 2
47012 Valladolid
Spain

Abstract

The synthesis of composite systems of oligomeric chitosan with propolis extract which allow the incorporation of a third component (silver nanoparticles) is reported, together with their application in aqueous solutions with a view to the formation of adhesive substances or nanofilms for the protection of agricultural crops. The antimicrobial properties resulting from the association of the two biological products or from the incorporation of silver nanoparticles have been studied *in vitro* for rice seeds inoculated with harmful *Bipolaris oryzae* fungus, proposing the field application of the novel composites as biofungicides to control the brown spot disease. Several synthesis-structure-activity models have been proposed to justify the synergies and to design new polymeric materials which may represent an eco-friendly alternative to traditional chemical fungicides due to their solubility, safety and effectiveness.

1. Introduction

Rice (*Oryza sativa* L.) is a major cereal consumed by the world population, representing about 30% of worldwide grain production [1]. *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 most 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

[2]. 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 [3]. 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 [4].

The natural, biodegradable and biocompatible chitosan (glucosamine polymer with β-1,4 bonds), formed by the alkaline N-deacetylation of chitin, is 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 [5]. In agriculture, chitosan polymers have been used to promote plant tolerance to stress [6] and to activate defense responses to protect different plant species from pathogenic microorganisms [7], such as cucumber (*Cucumis sativus* L.), wheat (*Triticum aestivum* L.), peas (*Pisum sativum* L.), peanut (*Arachis hypogaea* L.) or rice (*Oryza sativa* L.) [8].

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 [9]. Borges et al. [10] reported the suitability of chitosan to protect tomato plants from *Fusarium oxysporum*. In addition, chitosan has been found to have antimicrobial properties [11, 12], in which the size of its oligomers plays a key role [13].

On the other hand, propolis is known to have antimicrobial properties and has been extensively used in traditional medicine [14, 15]. Propolis is a natural resinous hive product collected by honeybees from various plant sources [16] which contains over 150 chemical species (such as coumarins, flavonoids, polyphenols, phenolic aldehydes, sesquiterpene

quinines, amino acids, and steroids) [17]. Its strong antimicrobial activity may be due to its high content in phenols and flavonoids [14]. Propolis has also been found to have applications as an antioxidant and in food preservation [18].

Shabana et al. [3] studied the control of brown spot pathogen of rice (*Bipolaris oryzae*) using some phenolic antioxidants (salicylic acid, benzoic acid and hydroquinone). Hydroquinone has also been reported to be a promising antioxidant for managing seed borne pathogenic fungi of peanut [19]. 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*) [20]. Thobunluepop [2] 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 *Bipolaris 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 [21] 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. Material and Methods

2.1. 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 (25wt.% 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, 20KHz was used for the sonication of the solutions.

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

2.2.1. 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 190000-310000g/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 60ºC range and with H_2O_2 concentrations ranging from 0.3 to 0.6M, obtaining oligomers with molar masses in the 6000 to 2000g/mol range, respectively, in agreement with the analogous microwave-based procedure reported by Sun et al. [22]. The molar mass of the chitosan samples was determined by measuring the viscosity, in agreement with Yang et al. [23], in a solvent of 0.20mol/L NaCl + 0.1mol/L CH_3COOH at 25ºC using an Ubbelohde capillary viscometer. Molar masses were determined using the Mark-Houwink equation $[\eta] = 1.81 \times 10^{-3} M^{0.93}$ [24]. 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 0.005-0.01M or solutions at 1.25-2.5% w/v (solution A_{50}) were prepared from a solution of chitosan oligomers with 2000g/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 [25].

2.2.2. 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 [26]. The resulting solution was then percolated (1 L/min) and filtrated with a stainless steel mesh to remove any residue, followed by concentration at a temperature below 60°C with ultrasound equipment to finally obtain a clarified propolis extract, and finally solutions with a propolis concentration of 100mg/mL or 10% w/v were prepared (labelled as *P*).

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

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

2.2.4. Silver NPs preparation

Silver nanoparticles were prepared by a sonication method, without resourcing to UV stabilization (used, e.g., by Montazer et al. [27]) as follows: an aqueous solution of $AgNO_3$ (50mM) was treated with sodium citrate (30mM) 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. Polyvinylpyrrolid one was added to prevent the silver nanoparticles aggregation. A 10mM solution of $NaBH_4$ (reducing agent) was then added dropwise; the first droplet made the solution turn from colourless to yellowish and successive droplets led to an intensification of the yellow colour (care had to be taken so as to avoid an excess of reducing agent, which would lead to a brownish colour). 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.

The resulting sonicated solutions had silver contents ranging from 100ppm to 200ppm (nAg solution) and were characterized by UV-Vis absorbance at 420nm 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 [25]. The solutions were stable in inert atmosphere at 4°C.

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

The chitosan oligomers/propolis solutions (B_{100}) were prepared according to Subsection 2.2.3. Two different 50mL solutions of silver nanoparticles, with a concentration of 10ppm and 20ppm, 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 10ppm nAg) and C_2 (for 20ppm nAg).

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

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

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

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

2.3. Culture media and activity assays

2.3.1. 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°C) and photoperiod (12h of light/12h 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 [28]. 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.

2.3.2. Inoculum preparation

The fungal isolates were cultured in Petri dish containing PDA culture medium and incubated for 14 days in aforementioned growth chamber. 10mL 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 by 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.

2.3.3. Seed health testing

According to ISTA [29] 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 oryzae* fungus at three different concentrations (300µL/mL, 600µL/mL, and 900µL/mL) in PDA at 25°C and compared with the control (T). The seeds were incubated at 20 ± 2°C 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 1mL/100g of seeds. 2000 inoculated rice (*Oryza*

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 × 0.5cm capsules, and placed in 11.5cm × 11.5cm × 3.5cm 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 12h 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.

2.3.4. 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 [30]:

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

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.

2.3.5. 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 [29]. The results were expressed as the percentage of normal germinated seedlings.

2.3.6. 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 [31].

2.3.7. 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 by using the statistical program SASM-Agri [32].

3. Results and Discussion

3.1. Treatment of rice seeds against *Bipolaris oryzae* fungus

3.1.1. Antifungal properties

In Table 1, it may be observed that, even at a concentration of 300μL/mL, a complete inhibition of *Bipolaris oryzae* growth (∅ = 0cm) 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 (600μL/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 1 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*), nomycelial growth took place for the seeds treated with the chitosan/propolis/nAg composite, regardless of the concentration.

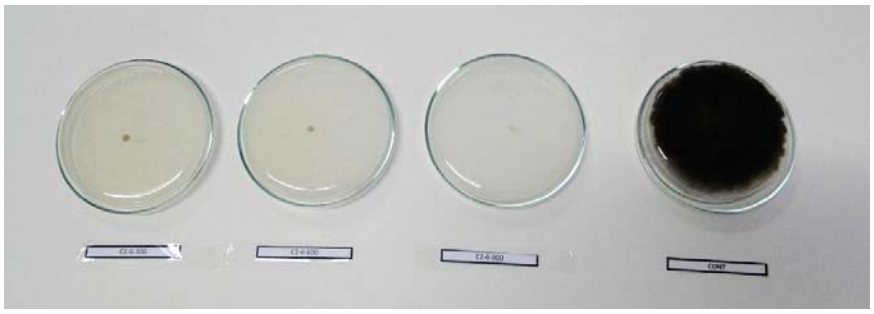
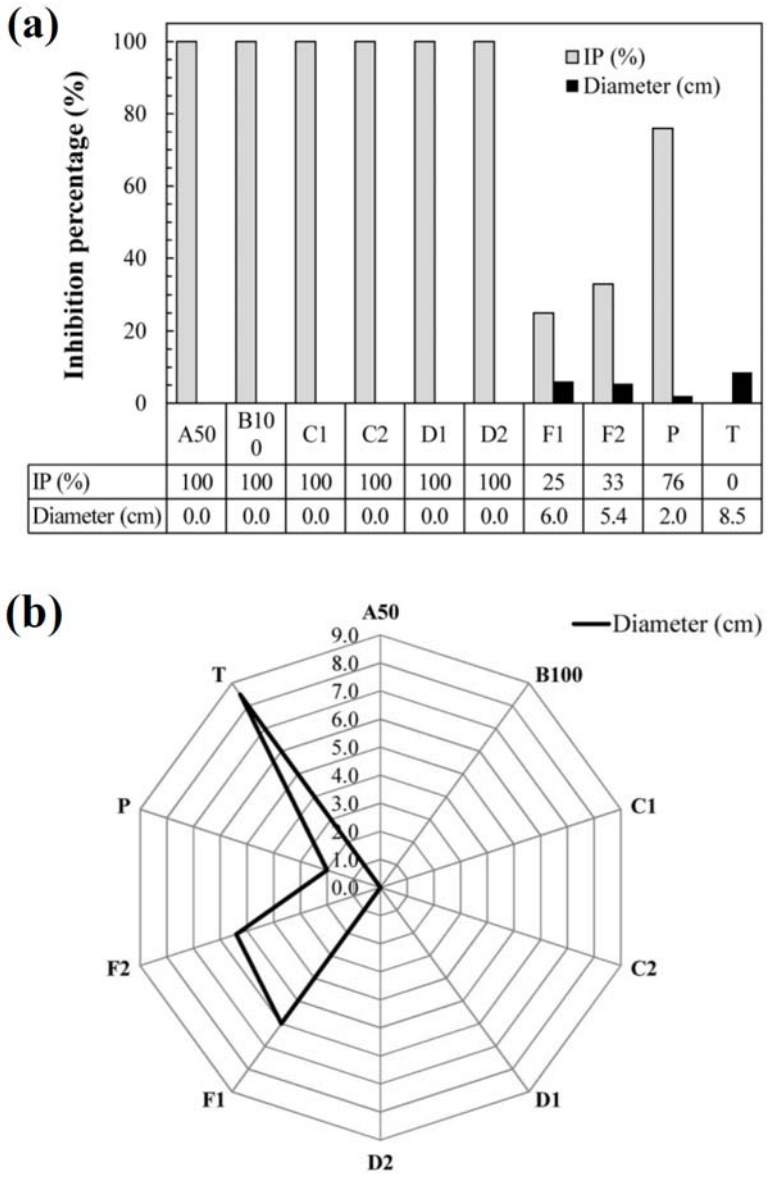


Figure 1. Growth of *Bipolaris oryzae* fungus in Petri dishes. *From left to right*: Petri dishes with no fungal growth at concentrations of 300, 600, and 900μL/mL for C_2 treatment and Petri dish with complete growth of the fungus for the control.

According to Figure 2, 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 Tiwary et al. [33] and Gómez-Estaca et al. [20], as it will be further discussed in Subsection 3.2.



	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 2. (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 [34]. 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 [35]. Chemical synthesis of different sizes of chitosan oligomers with specific biological activity has been described by Kuyama et al. [36].

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 [37] and to enzyme inhibition by nonspecific interactions with proteins [38].

Silver nanoparticles have been deemed as one of the most promising antimicrobial species from a nanotechnology based 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 [39]. 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 [40], which are not an issue in this case.

3.1.2. 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

3.2. Synthesis-structure-activity relationships

3.2.1. 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 [41]. 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 [42]. 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 [43]. 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 [44].

3.2.2. 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 [45]. Chemical modification of chitosan/chitosan oligomers can be attained by N-substitution, by O-substitution, 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 [43] (Figure 3).

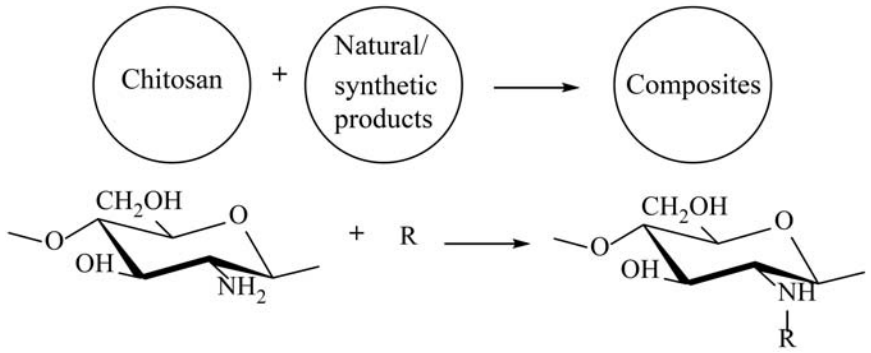


Figure 3. Composite formation from the D-glucosamine structure by addition of a functional group.

Chitosan carries free amine functionalities on the deacetylated units and hydroxyl groups on 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 4(a)). 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. [33].

Chitosan is a cationic polysaccharide due to the presence of the amino group, which conferms more reactivity and the ability to bind functional groups that appear in natural or synthetic extracts, resulting in the formation of novel compounds.

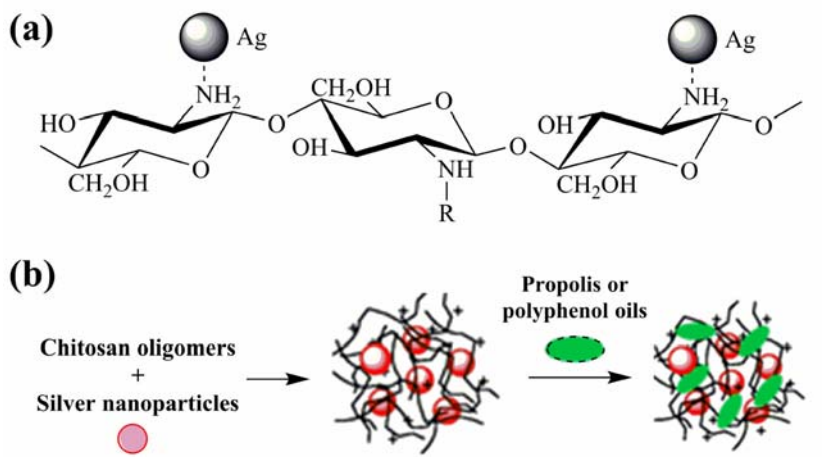


Figure 4. Mechanisms for the formation of chitosan based nano-composites: (a) composite formation from chitosan oligomers grafted to propolis or polyphenol oils (R) and silver nanoparticles (Ag); (b) composite formation by electrostatic interactions and intermolecular hydrogen bonding between chitosan, propolis and silver nanoparticles.

Chitosan may also be cross-linked with natural extracts by electrostatic interactions and intermolecular hydrogen bonding (Figure 4(b)). 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 [46]. 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 [47]. Matei et al. [25] 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.

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: *Bipolaris 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. 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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