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Biopolymer films to control *fusarium* dry rot and their application to preserve potato tubers

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ABSTRACT: Films were cast using sodium alginate (NaAlg), high molecular weight (HMW) chitosan, and low molecular weight (LMW) chitosan as film forming biopolymers. Fludioxonil (Fl) at 1% concentration was used as fungicide. Thermal stability, mechanical, and water sorption properties of the films were examined. The effects of films on the *Fusarium solani* colony radial growth were evaluated *in vitro* and in potato tubers. Results showed that chitosan films were more thermally stable and less hydrophilic than alginate films. Addition of fluodioxonil to the films significantly reduced the film strength and increased the elongation at break as well as the film stiffness. *In vitro* studies showed that when fludioxonil was added to the formulation, NaAlg and Chitosan-LMW films had significantly higher antifungal activity (Fungistatic index = 56%) than Chitosan-HMW films (Fungistatic index = 50%). *In vivo* studies showed that Chitosan-LMW-1%Fl films delay the mycelial growth of *F. solani* in tubers kept at 25 °C for 2 weeks. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 44017.

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INTRODUCTION

Fusarium solani causes Fusarium dry rot, one of the most important potato diseases affecting tubers in storage and seed pieces after planting.[1,2] As *F. solani* cannot penetrate the periderm of the tubers, infection can only occur through wounds or breaks in the periderm. Therefore, *Fusarium* infection is generally controlled by limiting wounding and coating the potato tubers with fungicide during storage. Some of these fungicides are coated in powder form, which leads to exposure by workers applying the coatings.[3] This creates possible health concerns for the workers. Also, direct coating of the tubers might not be the most efficient method for fungicide application since sufficient amounts need to be applied on the tuber surface to ensure effectiveness. Consequently, over application might occur and results in some fungicide loss that can cause potential harm for the environment.

One method to reduce fungicide exposure by the worker as well as fungicide loss is by incorporating it in a biopolymer film. The total fungicide amount incorporated in the films would be lower than those used in direct applications. The fungicide could then be control-released from the film over time to prevent fungal infections. Biopolymer films had been used as coatings in food products to prevent bacteria and fungi contamination.[4–9] Two biopolymers that could be used to form these films were sodium alginate and chitosan. Sodium alginate is the sodium salt of alginic acid, a polysaccharide extracted from brown algae. Alginic acid is a copolymer consisting of D-mannuronic and L-guluronic acid monomers. Another biopolymer is chitosan, which is prepared by deacetylation of chitin, a polysaccharide found in crustacean shells. One advantage of chitosan over sodium alginate was that chitosan films had been shown to have antibacterial[5,10–16] and antifungal[4,9,14,17–23] properties. Chitosan's antimicrobial properties derived from its cationic amino groups. These positively charged groups interfered with negatively charged portions of microbial cell membranes, causing leaks in the membranes. The antimicrobial properties of these films could also be enhanced by incorporating antibacterial and antifungal agents. These agents were released from the film and diffused onto the food surface.

There had been few studies involving the use of antimicrobial coatings on potato tubers. One study by Rabea and Badawy[8] involved coating tubers by dipping them in chitosan solutions. The authors found that an increase in chitosan solution concentration resulted in a decrease in decay of the tubers. However, the authors did not incorporate any additional antimicrobial agents into the chitosan solutions.

In this study, we incorporated a common potato fungicide, fludioxonil, into sodium alginate and chitosan films. Two different chitosans, low molecular weight (LMW) and high molecular weight (HMW) chitosan, were used to make the films. The objectives of this study were to characterize the physicochemical properties, such as water affinity, thermal stability, and mechanical properties, of the biopolymer films, to determine *in vitro* the antifungal activity of the films against *F. solani*, and to evaluate the control effect of the films on dry rot in potato tubers.

EXPERIMENTAL

Preparation of Films

Sodium alginate from brown algae, LMW chitosan, HMW chitosan, glycerol, and fludioxonil were purchased from Sigma-Aldrich (St. Louis, MO). Three different film forming solutions were prepared: sodium alginate, LMW chitosan (50–190 kDa) and HMW chitosan (310–375 kDa). Sodium alginate solutions were prepared by slowly adding 1% (w/w) sodium alginate to distilled water. Glycerol [60% (w/w) of alginate] was also added to the solution as a plasticizer to improve the flexibility and workability of the film. Solutions for each type of chitosan were prepared by dispersing 1.5%, (w/v) chitosan in aqueous solutions of lactic acid [0.7% (v/v)]. Glycerol [25% (w/w) of chitosan] was added to the solution. The film forming solutions were magnetically stirred for 1 h. Fludioxonil was first dissolved in methanol (10 mg/mL) and then added at 1% (w/w) of total solids. Control solutions were prepared without the addition of fludioxonil. The film solutions were degassed by applying vacuum to prevent micro-bubble formation in the films. Glass casting plates (30 × 30 cm) with Mylar (Dupont, Hopewell, VA) covers were used for film casting. The solutions were cast to a thickness of 1.15 mm onto plates using casting bars and the plates were allowed to dry at room temperature (22 °C) for 24 h. After drying, the films were removed from the Mylar sheet and cut into 14 mm diameter discs using a sterilized cork borer. The weight of the films was measured with an analytical balance. The films were sterilized by exposure to ultraviolet light for 5 min on each side.

Dynamic Vapor Sorption

The moisture sorption isotherm is a means to characterize the water absorption properties of the biofilms to predict film integrity during storage of potato tubers and film solubility during planting and growing.

A dynamic vapor sorption analyzer DVS-1 (Surface Measurement Systems, Allentown, PA) was used to measure the water sorption isotherms of the films. Each 4 mg sample was hydrated at a specific relative humidity until the sample reached equilibrium. The sample was exposed to a humidity range of 0–98% and then 98% down to 0% again. All measurements were performed at 25 °C.

The isotherm data were fit to the Guggenheim–Anderson–De Boer (GAB) model. The model is:

$$\frac{M}{M_o} = \frac{CKa_w}{(1-Ka_w)(1-Ka_w+CKa_w)} \quad (1)$$

where M is the equilibrium moisture content (g water/100 g dry film), M_o is the water content in the monolayer (g water/100 g dry film), a_w is the water activity, C is a constant associated with the monolayer enthalpy of sorption, and K is a constant associated with the multilayer enthalpy of sorption. Three replicates were tested for each sample.

Thermogravimetric Analysis

A Perkin–Elmer (New Castle, DE) thermogravimetric analyzer (TGA) Pyris 1 was used to characterize the thermal stability of the films. The films were conditioned in a 50% relative humidity chamber for at least 48 h prior to each test. Each 9–11 mg sample was heated from 30 to 800 °C at a rate of 10 °C/min. The sample chamber was purged with nitrogen gas at a flow rate of 40 cm^3/min. Three replicates were tested for each sample.

Mechanical Properties

The films were cut to have a rectangular midsection of 15 mm wide by 100 mm long, flaring to 25 mm by 35 mm square sections on each end. Ten replicates of each film were tested. The cut films were then conditioned at 50% RH for 72 h. An Instron Universal Testing Machine (model 1122, Instron Corp., Canton, MA) was used to determine Young's modulus (E), maximum tensile strength (TS), and maximum percentage elongation at break (EL). The instrument was operated with self-alignment grips that consist of one fixed and one free end. The free end moves easily into alignment when load was applied. The mechanical properties were determined at 21 °C according to ASTM D882-97. The ends of the cut films were clamped with grips, and films were stretched using a speed of 50 mm/min. The tensile strength was the maximum stress a film could withstand against applied tensile stress before the film tears. It was calculated by dividing the maximum load at break by the cross-sectional area of the film. Elongation at break was the percentage change in the original film length between the grips. The final length was measured when the film broke.

In Vitro Antifungal Activity

The fungus cultures of *Fusarium solani* (22678) used in this study were obtained from ATCC, the American Type Culture Collection (Manassas, VA). The isolates were activated in potato dextrose agar (PDA) media (Becton Dickinson, Franklin Lakes, NJ) and incubated at 30 °C for 10 days. Spore stocks were prepared by transferring the spores from the PDA plates into a sterile solution (5.9 mL phosphate buffered saline and 2.1 mL 80% glycerol solution). The spores were stored at −80 °C. For the antifungal experiments, spores were harvested in a sterile solution of 0.05% (v/v) Tween 80. The spore concentration of the suspension was determined using a hemacytometer and adjusted to a final concentration of 1×10^6 spores/mL. To evaluate the effect of the films on the fungi growth area, each agar plate was divided into three equal sectors. In each sector, one sterile 14 mm diameter film disc was deposited over the agar. The weight of the three film discs was 25 ± 4 mg. The plates were inoculated by placing 10 μL of the solution containing 1×10^6 spores/mL in the center of the agar plate. The inoculated plates were incubated at 25 °C. Photos were taken and the area extension growth of the fungi colony was measured from the photographs using the ADOBE PHOTOSHOP program (Microsoft Corporation, 1997). For this purpose, a tool included in

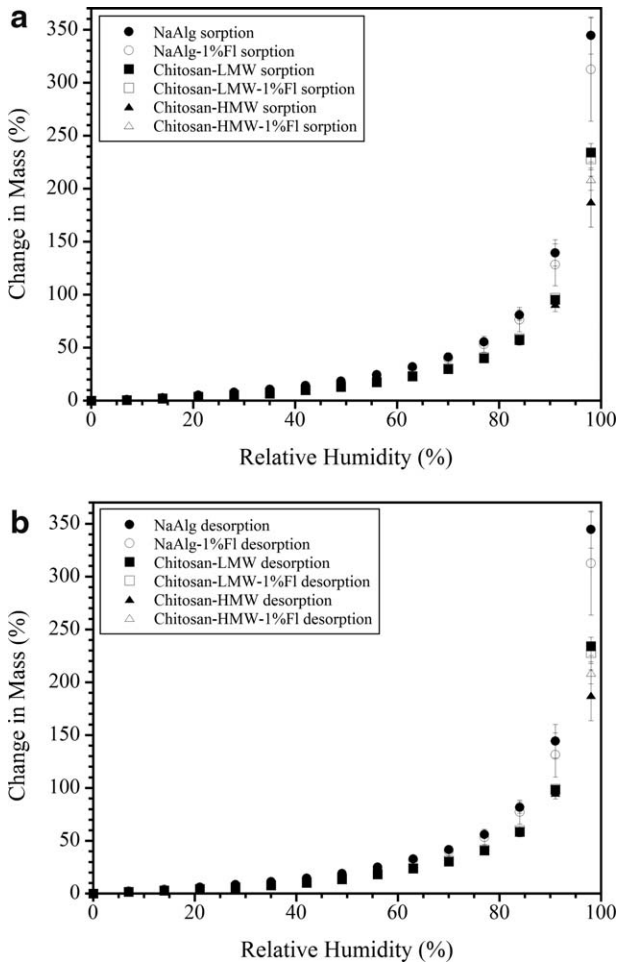

Figure 1. Isotherm curves for (a) water sorption of biopolymer films and (b) water desorption of biopolymer films.

the program, which is able to identify and count the pixels of the image with a certain color, was used. The colony area growth was calculated as the percentage of the agar plate covered by fungi. Four replicates were taken for each film formulation.

Evaluation of Antifungal Activity of Biopolymer Films in Potato Slices

Potato tubers (cv. Russet Burbank) were cut into slices weighing 28.0 ± 4.6 g. The potato slices were plated separately in petri dishes. Each potato slice was completely covered on one side by placing on its surface a film the same size as the potato slice. In the center of the slice, 10 μL of *F. solani* spore suspension (1×10^5 spores/mL) was inoculated. Potato slices without films were also inoculated and used as control samples. Following the inoculation, tubers were incubated up to 21 days at two different temperatures: 4 °C (optimal conditions for seed potato storage) and 25 °C. High relative humidity (RH 95%) and darkness were maintained in the two treatments. Disease presence was visually assessed. Three replicates were taken for each film formulation and storage temperature.

Statistical Analysis of Data

Statistical tests were performed using Minitab 14.2 (Minitab Inc., State College, PA) to study the significant effects of the film composition on the mechanical properties of the films and on the growth of *F. solani* with time.

RESULTS AND DISCUSSION

Water Sorption Isotherms

The sodium alginate films had higher water sorption values throughout the entire relative humidity range than those of chitosan films. This is shown in Figure 1. This indicated that sodium alginate films were more hydrophilic than chitosan films and had higher equilibrium moisture contents. Also glycerol, due to its hydrophilic nature, helps retaining water in the film matrix. The LMW and HMW chitosan films had comparable water sorption values, indicating molecular weight had little effect on chitosan sorption. In addition, the addition of fludioxonil had little effect on sorption values for both alginate and chitosan films. This might be due to the low concentration of fludioxonil incorporated into the films. All films also had little hysteresis, with comparable sorption and desorption isotherms. This is shown in Figure 1(b), which shows the desorption isotherms for all films.

The alginate films had higher monolayer water content, Mo, than chitosan films from the GAB model fit [eq. (1)] to the isotherms though these differences were not significant different at 95% CI. The GAB model parameters are shown in Table I. The GAB parameters were in the same range as those found for other biopolymer films.[24] Previous studies on alginate[25] and chitosan films[26] showed lower Mo values than those obtained in this study. This might be due to the different degree of deacetylation of chitosan, 95% in Bajdai study as compared with 82.5 and 86.6% for HMW and LMW chitosan, respectively. Gámiz-González *et al.*[27] found that equilibrium water content increases

Table I. GAB Model Parameters for Biopolymer Films with and without Fludioxonil

	GAB parameters		
Sample	Mo (g water/100g solid)	C	K
NaAlg	18.53 ± 3.20^{a}	1.35 ± 0.64^{a}	0.968 ± 0.008^{b}
NaAlg-1%Fl	16.69 ± 2.82^{a}	1.54 ± 0.16^{a}	0.968 ± 0.004^{a}
Chitosan-LMW	12.70 ± 1.01^{c}	1.38 ± 0.31^{a}	0.967 ± 0.003^{a}
Chitosan-LMW-1%Fl	13.33 ± 0.95^{bc}	1.34 ± 0.31^{a}	0.963 ± 0.004^{b}
Chitosan-HMW	14.84 ± 0.95^{ab}	1.07 ± 0.44^{a}	0.945 ± 0.010^{b}
Chitosan-HMW-1%Fl	13.33 ± 0.27^{c}	1.46 ± 0.02^{a}	0.958 ± 0.004^{b}

Different letters mean significant differences between films.

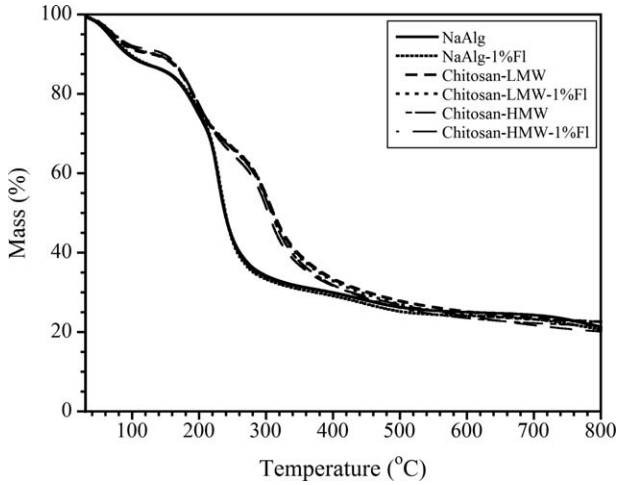

Figure 2. TGA curves of biopolymer films.

as DD decreases, because the instability of crystals during the swelling process increases with decreasing deacetylation degree, explaining the high equilibrium water content of low deacetylation chitosans.

Thermogravimetric Analysis

Chitosan films were more thermally stable than alginate films. This is shown in Figure 2, where we plot mass percent as a function of temperature for all samples. All TGA curves showed three different stages of mass loss. The initial decrease in mass for all samples indicated loss of moisture. The alginate films had greater decreases in mass at 100 °C compared with the chitosan films, indicating higher equilibrium moisture contents. This was consistent with the sorption isotherm results (see Figure 1). The second stage of mass loss for the films occurred at 190 °C, which corresponded to the volatilization of glycerol. The alginate films then began to have large decreases in mass at 230 °C, which corresponded to the degradation of alginate. In comparison, the chitosan films did not have large mass losses until 310 °C. At this point, the chitosan in the films began to degrade at a rapid rate.

All chitosan films had comparable TGA curves, indicating little difference in thermal stability between LMW and HMW samples. Also, the addition of fludioxonil did not affect the thermal stability of the films. This was due to the low concentration of fludioxonil incorporated in the films.

Mechanical Properties

No significant differences at 95% CI were observed in *TS*, *EL*, and *E* among the different film matrices without the addition of fluodioxonil. This is shown in Figure 3.

The TS of the Chitosan-HMW was higher (6.1 ± 1.3 MPa) than that of chitosan-LMW films (4.6 ± 1.3 MPa). Similar values of TS for chitosan films were found by Fundo *et al.*[28] Also, similar effect of the chitosan M_w on the TS values was observed in β-chitosan films prepared with different acids.[29] This might be explained by the high chitosan M_w samples forming entanglement networks during the film forming process, thus increasing the mechanical strength of the films.[29]

Results also showed that addition of 1% fluodioxonil to biopolymer films resulted in significant losses of strength and significant increases of EL (Figure 3). Chitosan-HMW lost 75% of strength and increased 15% of EL, Chitosan-LMW lost 78% of strength and increased 25% of EL, and Na-Alg films lost 92% of strength and increased 23% of EL. This might be due to the small molecule of fluodioxonil acting as a plasticizer. Also addition of 1% fluodioxonil resulted in significant lower values of Young's modulus (*E*). *E* values for Chitosan-HMW, Chitosan-LMW, and Na-Alg films were 90, 84, and 86% lower, respectively, than the corresponding films without fludioxonil.

Antifungal Properties

Effects of Films on *F. solani* Growth. The alginate films without fludioxonil showed no antifungal behavior over the entire time period since it had comparable colony growth area to those of the control plates (Figure 4). Alginate films had been shown in previous studies to have no antimicrobial properties.[30,31] However, LMW and HMW chitosan films showed some fungistatic activity. A 4% reduction in the area growth with respect to the control films was observed after 17 days of incubation, once the fungi colony reached the films placed on the agar plates (Figure 5).

Chitosan coatings had been shown to have antifungal properties when used on pizza dough,[4] cut sweet potato,[7] red table grapes,[14] and cut honey melons.[14] Also neat chitosan films

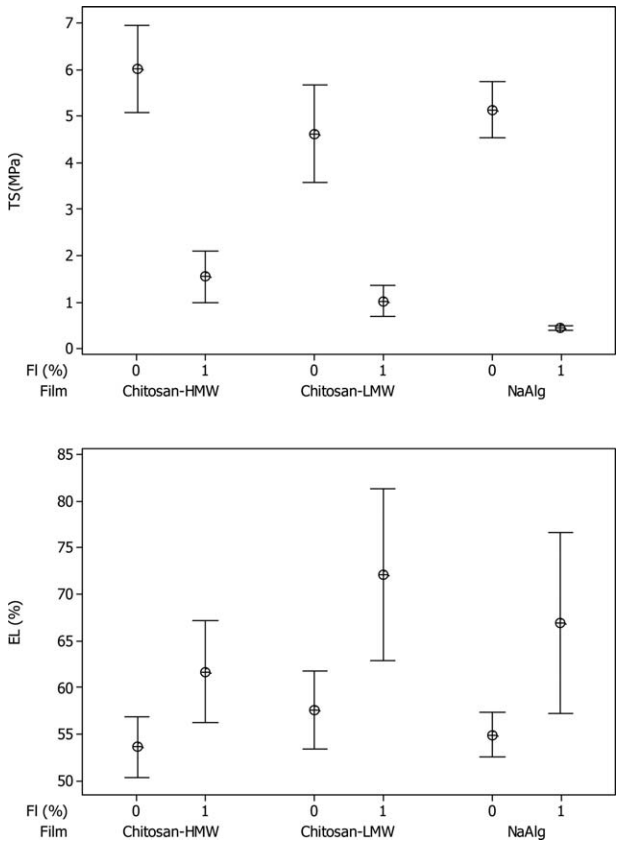

Figure 3. Interval plot (95% CI) of (a) tensile strength (*TS*), (b) Elongation at break (*EL*), and (c) Young's modulus (*E*) for each biopolymer film.

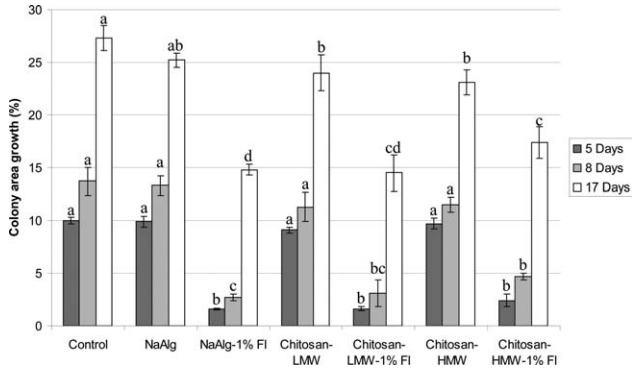

Figure 4. *In vitro F. solani* growth area in the presence of biopolymer films.

Table II. Presence (+) or Absence (−) of Dry Rot Disease on Potato Tubers Inoculated with *F. solani*

	25 °C			
	Incubation time (days)			
	4	7	15	21
Control	−	+	+	+
NaAlg	−	+	+	+
NaAlg-1% Fl	−	−	+	+
Chitosan-LMW	−	+	+	+
Chitosan-LMW-1% Fl	−	−	−	+
Chitosan-HMW	−	+	+	+
Chitosan-HMW-1% Fl	−	−	+	+

showed antifungal properties.[14,17–23] However, there had been other studies that showed chitosan coatings and films had no antifungal properties.[16,32–34] In these cases, chitosan molecules might not have diffused into the growth media in sufficient amounts to have antimicrobial effects.

Films containing fludioxonil had higher ($P \leq 0.05$) inhibitory effect on the average fungal growth area than films without fludioxonil. This is shown if Figure 4. This indicated that sufficient fludioxonil was released into the growth media to delay fungal growth. However, none of the films completely inhibited the fungus, so the effect was fungistatic and not fungicidal. Application of fludioxonil as seed tuber treatment was shown to reduce the inoculum potential of soil surrounding the progeny tubers by affecting the spread of the pathogen from infected seed tubers.[35] Fludioxonil alone or in combination with mancozeb as seed tuber treatment was also reported effective against dry rot.[36] The colony area growth of Chitosan-LMW-1%Fl films was lower than that of Chitosan-HMW-1%Fl. In this study, chitosan-LMW has slightly higher DD (86.6%) than chitosan-HMW (82.5%) which showed the proportion of free amino groups in the polymer that were directly related to the antimicrobial activity of chitosan. Consequently, the higher colony growth values for Chitosan-HMW-1%Fl films might be due to lower DD. Similar colony growth area values as those found for Chitosan-LMW-1%Fl were observed for NaAlg-1% Fl. In this case, higher amounts of fludioxonil molecules might have been released into the growth media. The higher content of glycerol and therefore the less dense biopolymer films might have contributed to this effect.

Effect of Films on the Development of Dry Rot on Potato Tubers Inoculated with *F. solani*. No lesions due to *F. solani* were observed in any of the potato slices kept at 4 °C during three weeks. However, the tuber slices kept at 25 °C were vulnerable to dry rot disease. Figure 5 shows photographs of the inoculated potato slices after 2 weeks at 25 °C. Table II shows the presence (+) or absence (−) of dry rot disease on the potato tubers. Absence of disease was considered when none of the three replicates showed signs of *F. solani* growth. None of the films without fludioxonil showed antifungal activity against

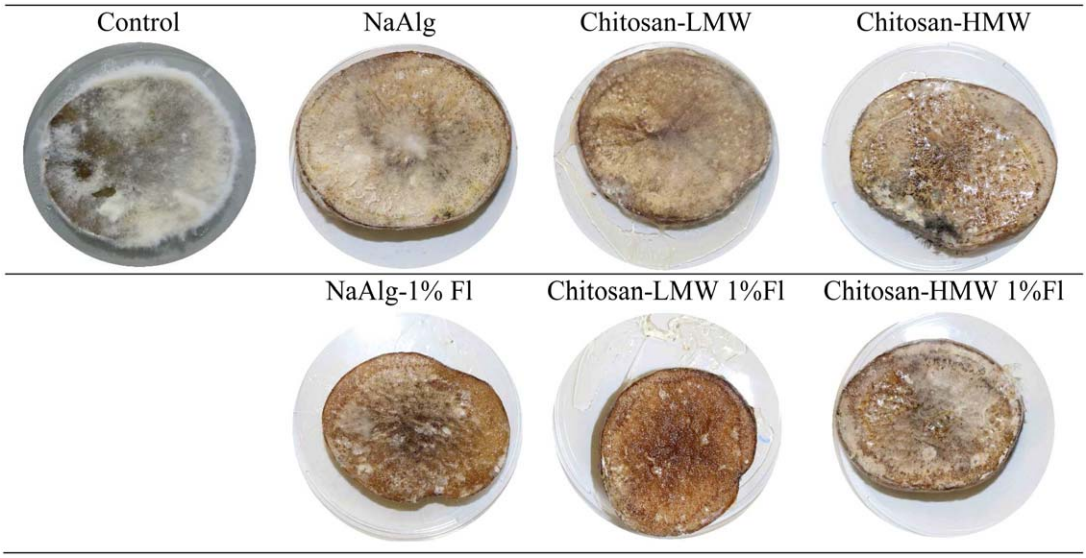

Figure 5. PDA plates showing *F. solani* growth areas in the presence of biopolymer films after 2 weeks incubated at 25 °C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

spore germination. Fluodioxonil delayed the development of fungi from 7 to 15 days in NaAlg and Chitosan-HMW films and from 7 to 21 days in Chitosan-LMW films. This might be due to fludioxonil molecules being easier to release from LMW chitosan films.

CONCLUSIONS

We evaluated sodium alginate and chitosan films containing 1% fludioxonil as possible coatings for potato seeds to prevent dry rot infections. The physicochemical properties showed that sodium alginate films were more hydrophilic than chitosan films. All the films were thermally stable up to 190 °C when glycerol volatilization started to occur. Up to this temperature only little water evaporation occurred in the films. No differences were observed in the mechanical properties among different native films. However, addition of 1% fluodioxonil to the films resulted in weaker, more elastic, and less ductile films than native films. In addition, films containing fludioxonil showed *in vitro* antifungal activity against *F. solani*. The colony area growth was reduced by 12% after 17 days of incubation. Also, tubers coated with Chitosan-LMW film containing only 1% fludioxonil showed no growth of *F. solani* for 2 weeks at 25 °C, indicating its effectiveness in controlling dry rot of potato tuber inoculated with the pathogen.

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