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Antifungal films based on starch-gelatin blend, containing essential oils.

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Abstract

The antimicrobial activity of essential oils (EO) is very well-known and it has been reported that incorporating them into edible films based on biopolymers extends the food's shelf-life. In this study, cinnamon, clove and oregano EO, at 25% with respect to the polymer, were incorporated into glycerol plasticized starch-gelatin blend films (ratio 1:1) in order to elucidate their effect on the physical (barrier, mechanical and optical), structural and antifungal properties of the films. Whereas EOs exhibited no significant effect on tensile behavior in the case of casting films conditioned at 53% relative humidity and 25º C, the EO compounds did significantly reduce the water vapor and oxygen permeability of the films. Likewise, the EOs increased the films' transparency but reduced their gloss. Despite the fact that about 60 % of the incorporated EOs were lost during the film drying step, they exhibited antifungal activity against the two tested fungal species, *Colletotrichum gloesporoides* (CG) and *Fusarium oxysporum* (FOG), as revealed by the in vitro agar diffusion method.

Keywords: *Colletotrichum gloesporoides*, *Fusarium oxysporum*, antifungal, tensile, microstructural, barrier, oregano, clove, cinnamon.

1. Introduction

Starch is widely available from renewable sources and has a great ability to form edible films with reasonable mechanical resistance, which can be used to coat food products in order to prevent moisture loss or to protect them from oxidation. Starch films are transparent, tasteless, odorless and have very good oxygen barrier properties. Nevertheless, some drawbacks must be improved, such as the water sensitivity and retrogradation phenomena, both giving rise to changes in the film barrier and mechanical properties during storage (Cano et al., 2014; Jiménez et al., 2012; Jiménez et al., 2013). Blending starch with other compatible (such as chitosan and caseinate) or non-compatible polymers (such as poly-caprolactona) is a commonly-used strategy by which to improve the properties and stability of starch-based films (Averous & Boquillon, 2004).

Starch-gelatin blend films have been studied by different authors (Al-Hassan & Norziah, 2012; Fakhouri et al., 2013) who report that these blends offer advantages in terms of oxygen and water vapor barrier properties, mechanical behavior and optical parameters (Wang et al., 2003). Even though gelatin may improve the performance of starch-based materials, both polymers are highly hydrophilic, which limits the water vapor barrier capacity of the blend films.

Recently, Acosta et al. 2015, have reported the benefits of the addition of lipids on the starch-gelatin blend films with different polymer ratios. They report that cassava starch films with 50 % gelatin exhibited highly adequate properties for the purposes of food coating or packaging. Gelatin blending gave rise to more resistant, harder and more extensible films than pure starch material. In this blend, although lipid incorporation improved the film's stretchability and water barrier capacity, it enhanced oxygen permeability. In this sense, essential oils (EOs) are interesting lipids that can be used in

54 starch-gelatin blend films, due to their antimicrobial and antioxidant capacity which
55 adds functionality to the film materials. Likewise, some essential oils have been
56 effective at reducing the oxygen permeability of different polymer films in line with
57 their oxygen scavenging activity (Bonilla et al. 2013).

58 The antibacterial activity of EOs has been reported by many authors (Burt, 2004; Alves-
59 Silva et al., 2013), but few studies analyse the antifungal effect of these compounds
60 (Avila-Sosa et al., 2012 Sánchez-González et al. 2010; Saggiorato et al., 2012; Avila-
61 Sosa et al., 2012, Perdones et al., 2012 and 2014; Alves Silva et al., 2013; Roselló et al.,
62 2015). The EOs affect the microbial cells via different mechanisms, including
63 interactions with the phospholipid bilayer of the cell membrane, the disruption of
64 enzyme systems and compromising the genetic material of bacteria (Burt et al., 2007).
65 The use of EOs as food preservatives is limited by their strong flavour, but embedding
66 them into a polymer matrix represents an alternative means of reducing their sensory
67 impact while the diffusion of the antimicrobials to the product may be modulated (Ruiz-
68 Navajas et al., 2013). Nevertheless, the non-polar nature of the EO components requires
69 the homogenization techniques in order to incorporate them into the aqueous film
70 forming dispersions (FFD) of hydrocolloids, such as starch or gelatin. During the drying
71 of the FFD cast to obtain the films, a part of the EO is lost as the water evaporates , the
72 amount depending on the stability of the initial emulsion and the extension of the
73 coalescence and creaming phenomena that occur in this step (Sánchez-González et al.
74 2011b; Perdones, et al., 2014), which affects the final bioactivity of the film. Likewise,
75 the presence of a lipid dispersed phase and the potential interactions of lipid molecules
76 with the polymer also affect the film's functional properties (Chiralt et al. 2015).

77 The aim of this study was to analyze the effect of three different essential oils
78 (cinnamon bark, clove and oregano) on the functional properties of cassava starch-

bovine gelatin (ratio 1:1) blend films, in terms of the tensile, barrier, optical and structural properties, as well as their antifungal effect against two fruit fungal pathogens (*Colletotrichum gloesporoides:* CG and *Fusarium oxysporum f.sp. gladiolo*: FOG) through in vitro tests.

2. Materials and methods

2.1. Raw materials

Cassava starch, with 9.28 wt. % amylose and an amylose:amylopectin ratio of 1:9.8, was produced by Asia CO, LDT (Kalasin, Thailand) and purchased by Quimidroga SA (Barcelona, Spain). Bovin skin type A gelatin (Bloom 220-240°), used in combination with starch, was supplied by Sancho de Borja, S.L. (Zaragoza, Spain), and glycerol, used as plasticizer, was provided by Panreac Química, SA (Barcelona, Spain). Essential oils (oregano, clove and cinnamon bark) were acquired from Herbes del Moli (Benimarfull, Alicante, Spain). Table 1 shows the main components of EO used in this study and their boiling point. Stock culture of *Colletotrichum gloesporoides* (CG), isolated from citrus trees from Valencia (Spain), was provided by the Mediterranean Agroforest Institute (Universitat Politécnica of Valencia, Spain). *Fusarium oxysporum f.sp. gladiolo* (FOG) CECT 2868 was supplied by the Colección Española de Cultivo Tipo (CECT, Burjassot, Spain). These were preserved frozen in Agar Potato Dextrose (PDA, Scharlab, Barcelona, Spain), then incubated at 25°C until sporulation, and were used after 7 days of active growth.

2.2. Preparation of film-forming dispersions

The film forming dispersions were prepared from a 2% (w/w) polymer aqueous solution, using the same proportion of cassava starch (S) and gelatin (G). Every

formulation contained glycerol as plasticizer (polymer:glycerol ratio of 1:0.25). Furthermore, three more formulations were prepared by adding essential oils as antimicrobial agents (polymer:essential oil ratio of 1:0.25). So, four formulations were considered with the following codes: SG for the control starch-gelatin blend films, SG-C, SG-Cl and SG-O, respectively for films containing cinnamon, clove, and oregano essential oils.

Firstly, starch and gelatin dispersions were prepared separately. Starch aqueous dispersions were maintained at 95ºC for 30 min to induce starch gelatinization. Meanwhile, gelatin was dissolved in water at 40 ºC. Then, the glycerol was added to the dispersion blend and homogenized by using a rotor-stator homogenizer (Ultraturrax D125, Janke and Kunkel, Germany) at 13,500 rpm for 1 min and 20,500 rpm for 3 min at 95 ºC under vacuum, in agreement with previous studies (Jiménez et al., 2012). For formulations containing essential oils, these were added prior to the homogenization step.

Controlled volumes of film-forming aqueous dispersions (equivalent to 1.5 g of solids) were cast into leveled Teflon ® casting plates (15 cm diameter) and dried at 25ºC and 45% RH for 48 h. Then, they were peeled intact from the plates and conditioned at 53% RH and 25ºC in a chamber with magnesium nitrate-6-hydrate saturated solution (Panreac Química, S.A., Barcelona, Spain).

2.3. Characterization of the films

2.3.1. Microstructure

The microstructural analysis of the films was carried out by scanning electron microscopy (SEM), using a JEOL microscope model JSM-5410 (Japan). Prior to testing, films were equilibrated in desiccators with P_2O_5 in order to remove any water present in the samples. SEM observations were carried out by considering the surfaces and cross sections of film samples. Films were frozen in liquid nitrogen, and then cryofractured to observe the cross section. Samples were fixed on copper stubs, gold coated to make them conductive (for 1.5 minutes) and observed directly with an accelerating voltage of 10 KV.

2.3.2. Water vapor permeability

The water vapor permeability (WVP) of films was determined following the gravimetric method ASTM E96-95 (1995) for a 53-100% relative humidity gradient at 25ºC by using Payne permeability cups (Payne, Elcometer SPRL, Hermelle/s Argenteau, Belgium) of 3.5 cm diameter. The RH gradient of 53-100% of the cabinet was obtained using oversaturated solutions of $Mg(NO_3)_2$ and distilled water, respectively. The weight of the cup was measured every 2 h using an analytical balance (± 0.00001 g). The water vapor transmission (WVTR) was determined from the slope obtained from the regression analysis of weight loss data versus time, once the steady state had been reached, divided by the film area. Water vapor permeability (WVP) values were obtained, in quadruplicate, according to previous studies (Jiménez et al. 2012).

2.3.3. Oxygen permeability

The oxygen permeability (OP) of the films was analyzed in film samples (50 cm^2) by using an Oxtran system (Mocon, Minneapolis, USA), following the standard method (ASTM D3985-05, 2002) at 53% RH and 25ºC. Films were exposed to pure nitrogen flow on one side and pure oxygen flow on the other side. OP was calculated by dividing

149 the oxygen transmission rate by the difference in oxygen partial pressure between the
150 two sides of the film, and multiplying by the average film thickness. At least two
151 replicates per formulation were taken.

152 2.3.4. Tensile behavior

153 The tensile properties were measured using a universal test machine (TA.XT plus
154 model, Stable Micro Systems, Haslemere, England) and following the ASTM standard
155 method D882 (ASTM, 2001). Equilibrated samples (8 per formulation) were mounted
156 in the film-extension grips (model A/TG, Stable Micro System, Haslemere, England) of
157 the testing machine and stretched at a rate of 50 $mm \cdot min^{-1}$ until breaking. The relative
158 humidity of the environment was held at nearly 53% during the tests, which were
159 performed at 25 ºC.

160 The force-distance data obtained in the test were transformed into true stress-Henky
161 strain curves, which allow the mechanical parameters to be calculated: elastic modulus
162 (EM), tensile strength at break (TS) and percentage of elongation at break (E).

163 2.3.5. Optical properties

164 The transparency of the films was determined by applying the Kubelka-Munk theory
165 (Hutchings, 1999) for multiple scattering to the reflection spectra. The surface
166 reflectance spectra of the films were determined from 400 to 700 nm with a
167 spectrocolorimeter CM-3600d (Minolta Co., Tokyo, Japan) on both a white and a black
168 background. Internal transmittance (Ti) of the films was quantified using eq 1. In this
169 equation, R_0 is the reflectance of the film on an ideal black background. Parameters a
170 and b were calculated by eqs 2 and 3, where R is the reflectance of the sample layer
171 backed by a known reflectance Rg. Measurements were taken in triplicate for each
172 sample on the free film surface during its drying.

$$T_i = \sqrt{(a - R_0)^2 - b^2} \quad (1)$$

$$a = \frac{1}{2} \cdot \left(R + \frac{R_0 - R + R_g}{R_0 R_g} \right) \quad (2)$$

$$b = (a^2 - 1)^{1/2} \quad (3)$$

The gloss of the films was measured at a 60º incidence angle, using a flat surface gloss meter (Multi.Gloss 268, Minolta, Germany), according to the ASTM standard D-523 (ASTM, 1999). Three films of each formulation were measured over a black matte standard plate. Results were expressed as gloss units, relative to a highly polished surface of standard black glass with a gloss value close to 100.

2.3.6. Antifungal properties. In vitro assays

Antifungal properties of films against FOG and CG were determined by the agar diffusion method using film discs of 2.4 cm diameter, which were placed on the plate with the fungus spores. To this end, for each fungus, a suspension of $5x10^6$ CFU/mL spores in water with Tween 80 (0.1%) was prepared from a colony of 7 days' active growth in PDA medium. This suspension was distributed in plates and left to solidify. Then, the film discs were deposited on the different plates and they were incubated under the optimal conditions for each fungal species. The diameter of the growth inhibition halo was measured at 48 and 72 hours in all cases. Six replicates per treatment and control sample (without film) were made.

2.3.8. Statistical analysis

The analysis of data was performed through the analysis of variance (ANOVA) using the Statgraphics Plus 5.1. software (ManugisticsCorp., Rockville, MD). Fisher′s least significant difference (LSD) procedure was used at the 95% confidence level.

196

197 **3. Results and discussion**

198 3.1. Film microstructure and losses of essential oils during film formation.

199 The microstructure of films, which depends on the interactions between film
200 components and drying conditions, seriously affects the physical properties of the final
201 materials, such as barrier, mechanical and optical properties. In films containing lipids,
202 such as essential oils, the final structure is also affected by the structural characteristics
203 and stability of the film-forming emulsion. The different emulsion destabilization
204 mechanisms (flocculation, creaming and coalescence) that occur during the film drying
205 step determine the final distribution and size of lipid droplets in the matrix (Villalobos,
206 et al. 2005; Fabra et al. 2009).

207 Figure 1 shows the SEM micrographs corresponding to the surfaces and cross-sections
208 of the studied films. SEM observations show that starch-gelatin composite films
209 exhibited heterogeneous structures and irregularities in both surface and cross-section
210 images. Round formations are observed on the surface of these films, while in film
211 cross-sections, fibrous regions appear. These formations have been related with the lack
212 of total miscibility of starch and gelatin and the polymer phase separation, giving rise to
213 a starch rich phase interpenetrated with a gelatin rich phase (Acosta et al. 2015). In the
214 gelatin phase, helical chain conformation and association (triple helix) occur in a similar
215 way to in the original collagen structure, as observed by Hassan et al. (2009) and Al
216 Hassan and Norziah, (2012) in gels of starch-gelatin. Gelatin association domains
217 appear as fibrous zones differentiated in the amorphous starch matrix. On the surface of
218 the film, the rounded formations (droplets in non-dried film) reflect the migration of
219 gelatin-rich liquid phase to the film surface during the film drying step due to the phase

220 separation of polymer solution and the lower density of the protein phase with respect to
221 the starch-rich solution (Acosta et al. 2015).

222 The addition of EOs also promoted irregularities on the film surface due to the low
223 miscibility of these lipid compounds in the polymer matrix. EOs are dispersed in the
224 film-forming formulations and, during the film drying process, flocculation,
225 coalescence and creaming phenomena occur, leading part of the oil droplets to the film
226 surface, where the EO evaporates. The footprints of oil droplets are clearly appreciated
227 in the film containing CL oil, whereas wider traces can be seen for films with O
228 essential oil. Oil could be evaporated during the film drying and conditioning steps or
229 under the microscopic observation at high vacuum. However, for cinnamon oil,
230 although the lipid footprint at the surface level is not evident, a different surface
231 topography can be observed, as compared with control films without essential oils,
232 which suggests the presence of oil on the surface at some time during the film
233 preparation. The cross-sections of films containing EOs also reveal the presence of
234 numerous holes that correspond to the location of oil droplets. These could evaporate
235 partially on the cryo-fractured surface during SEM observations due to the high
236 vacuum. They appeared elongated in shape, which can be justified by their deformation
237 produced during the film drying and subsequent packing of the polymer chains, as has
238 been previously described (Sánchez-González et al., 2010; Bonilla et al. 2013). Films
239 with O essential oil exhibit a porous structure, but without well differentiated drops,
240 which suggests their coalescence during film drying and the formation of essential oil
241 channels that become entrapped in the polymer matrix. The proportion of porous zones
242 is higher near the free surface of the film during drying, in agreement with the oil
243 creaming to the top of the film. These results highlight a greater instability of oregano
244 oil emulsion during film drying.

The appearance of films with essential oils is similar to that previously described for hydrocolloid matrices with essential oils (Perdones et al., 2012). Differences in the distribution of essential oils in the polymer matrix are related with the different composition, volatility and affinity (interactions) between them and the polymers of matrix, which, in turn, affect the stability of the initial emulsion and its development during the film drying step. Notable differences in the structure and distribution of oil droplets in the film matrix have previously been observed for gelatin films with essential oils (Ahmad et al., 2012), starch films with fatty acids (Jiménez et al., 2012) or chitosan and HPMC films with essential oils (Sánchez-González et al., 2010). In this sense, the quantification of the total EO present in the film after the drying step is relevant, not only because of its effect on the functional film properties, such as mechanical or barrier performance, but also due to its active properties, such as antimicrobial or antioxidant activity.

The mean percentages of EO lost during the film preparation processes are estimated for the obtained films. This was carried out from the difference between the weights of the dried film (without moisture) and the total solids of the cast film-forming dispersions with respect to the corresponding initial amount of EO. The losses of EO were about 56-59 % for C and CL, whereas they were significantly higher (66%) for the O EO. This could be due to the greater instability of the initial emulsion of this EO, as deduced from the SEM micrographs of the films, which leads to the coalescence and creaming of the oil droplets, which evaporate at the film's surface during the drying step. The greater volatility of the main compounds of oregano EO (50% carvacrol and 21% thymol) in comparison with cinnamon (60% eugenol) and clove (90% eugenol) (Table 1) could also enhance the oil compounds lost. During the film's drying period, water and EO compounds evaporate together at a lower temperature than pure water

(immiscible blends) from the film surface until a certain level of concentration of the film solids was reached, when the oil droplets remain entrapped in the highly viscous aqueous system and do not cream to the surface. From this point, the oil droplets become encapsulated in the film matrix and the release of the oil components from the dried films due to evaporation takes place very slowly, as deduced by their very small weight loss throughout time.

3.2. Barrier properties

In Table 2, the average values of water vapor and oxygen permeabilities of starch-gelatin based films are also shown. The addition of EO to starch-gelatin films caused a decrease in the water vapor permeability, as can be expected from the increase in their hydrophobicity and the interruptions in the matrix continuity for mass transfer. Water molecules should diffuse mainly through the continuous polymeric phase, where the presence of lipid droplets represents discontinuities that cause an effective increase in the tortuosity factor for water transfer in the matrix. No significant differences between the WVP values of films with different EOs were obtained.

The effect of EO incorporation on the water barrier properties of the films has been analyzed in previous studies and different results have been reported. This effect depends on different structural factors, such as the kind of matrix, the composition and amount of oil added and the interactions with the matrix (Sánchez-González et al., 2011b). The process of water vapor transfer in films depends on the hydrophilic-hydrophobic balance in the matrix and the final film microstructure. Sánchez-González et al. (2011b) found a reduction in the WVP in chitosan and HPMC matrices when 0.5, 1 and 2% of bergamot, tea tree, lemon and bergamot EO were added. In general, the higher the EO concentration, the lower the WVP values in both matrices. The kind of

294 EO significantly affected this property in HPMC matrices. The films containing citrus
295 EO exhibited a greater WVP reduction, as compared with films based on tea tree EO.
296 This was explained by the more hydrophobic nature of the main compounds of citrus
297 EO (limonene) with respect to tea tree oil (terpineol). The kind of EO affected the WVP
298 values to a lesser extent in the chitosan matrix due to its greater hydrophilic nature.
299 Kechichian et al (2010) found a reduction in the WVP in cassava starch films when
300 cinnamon and clove powder (0,2 g/100 g of film) were added to the matrix. At higher
301 concentrations, the WVP values increased.
302 Ahmad et al (2012) obtained a different effect in gelatin-based films with bergamot and
303 lemon EO. At EO concentrations higher of over 5%, the WVP slightly increased in the
304 films with bergamot oil, while those containing lemon EO showed a WVP reduction.
305 This was related with the different hydrophobic nature of these oils. Interactions
306 between the EO compounds and the hydrophobic parts of proteins could promote an
307 overall decrease in the hydrophobic nature of the matrix (Perez-Gago y Krochta, 2001).
308 The oxygen permeability values (Table 2) also showed a significant decrease when EOs
309 were added to the films, as compared to the values of the control films, but using
310 different oils did not lead to any significant differences between the films. Lipid
311 incorporation usually promotes higher oxygen permeability of the films due to the
312 greater gas solubility in the lipid phase (Fabra et al., 2012). However, essential oils
313 (from thyme and basil) and other antioxidants notably reduced the oxygen permeability
314 of starch-chitosan blend films (Bonilla et al., 2013). The poorer oxygen solubility in
315 these more polar oils and the presence of antioxidant compounds that can play an
316 oxygen scavenging role are the reasons for the positive effect of EO on the oxygen
317 barrier properties of films.
318

3.3. Mechanical and optical properties

The mechanical and optical properties of different films, with and without essential oils, are shown in Table 3. In general, the mechanical parameter exhibited few changes as a result of the EO incorporation. The values of elasticity modulus (EM), tensile strength (TS) and deformation at fracture point (%E) of the control and EO-containing films are not significantly different.

Previous studies have described different effects of the incorporation of EO into polymeric matrices. Sanchez-Gonzalez et al. (2011b) found similar tendencies in films based on HPMC and chitosan after additions of different EO concentrations (0.5- 1 and 2%). The addition of EO caused a decrease in EM, TS and %E. The kind of EO only had a significant effect on chitosan films. Kechichian et al. (2010) also found that there was a decrease in the mechanical properties after incorporating powder of cinnamon and clove into starch films. However, Bonilla et al. (2013) found an increase in the film's rigidity and tensile strength in starch-chitosan blend films with basil essential oil; with thyme oil, meanwhile, the films only were slightly less extensible. In this study, authors found that in pure chitosan films, the incorporation of thyme and basil EO caused a significant decrease in the rigidity and tensile strength of the films (the higher the essential oil concentration, the more intense the effect), while the films gained extensibility.

All of the described results highlight that the effect of EO on the film's mechanical properties depends on the type and concentration of EO, the kind of polymer matrix and the specific interactions between components (Ahmad et al. 2012), which determine the effective adhesion forces at the polymer-oil interphase. In general, if essential oils, or other immiscible, non-polymeric components, are added in excessive concentrations, the films support a great quantity of dispersed phase and the structure becomes too

heterogeneous, with a great number of discontinuities, which imparts a highly fragile nature to the matrix. Nevertheless, if the optimal level of essential oil is incorporated, the film's properties can be enhanced depending on the specific interactions between components.

The few changes obtained in the mechanical properties of starch-gelatin films as a result of the incorporation of essential oils reveal that the concentrations used are suitable for preserving the mechanical behavior of the films based on starch:gelatin, while imparting other positive effects, such as the improvement in the barrier properties commented on above and potential active properties.

Table 3 also shows the values of the optical properties of the films. Film gloss is related with surface morphology (Villalobos et al. 2005) and, in general, the smoother, less irregular the film surface, the glossier the film. The gloss values revealed that the incorporation of EO caused a reduction in this parameter in comparison with the control films. As previously commented on, EOs introduce structural irregularities at the surface level of the films, which directly reduce film gloss. The kind of EO affected the gloss of the films; the oregano EO produced the glossiest films and the cinnamon oil the least glossy. The surface topography of the films, influenced by the oil creaming during the drying period, and possible oil exudation to the surface in the dried film, will be determining factors in the film's final gloss.

The control films exhibited lower transparency values, evaluated through Ti, than those that contain EO, despite the presence of an oil dispersed phase that could contribute to light dispersion and an increase in opacity. In fact, the incorporation of EO at different concentrations in chitosan and HPMC matrices caused a decrease in the transparency of all the films (Sánchez-González et al. 2011b). Nevertheless, in starch-gelatin films, Ti values significantly increased as the EO was incorporated, regardless of the kind of oil .

This could be due to the less compact nature of the polymer matrix in the presence of the EOs and the possible inhibition of starch and gelatin crystallization phenomena. In fact, Jiménez et al. (2012), observed a decrease in the transparency and gloss of corn starch films during storage attributable to the progress of amylose crystallization. The presence of oils could help to limit these crystallization phenomena, leading to more transparent films.

3.4. Antifungal activity in vitro tests

Table 4 shows the results of the antifungal effectiveness of the films containing EO through the diameter of inhibition of the fungal growth for *Fusarium oxysporum* (FOG) and *Colletotrichum Gloeosporiodes* (CG), after 48 and 72 hours of incubation. The control plates (without film) were used to compare the fungal growth. The results obtained in the control plates showed a growth equivalent to 2/3 of the total plate surface after 24 h, whereas at 72 h, the entire surface was covered by the fungus.

The three films containing EOs were effective against the fungal growth. In general, this effect was somewhat more marked in films containing cinnamon essential oil against the *Fusarium oxysporum* (FOG) fungus, while films with clove and cinnamon essential oils were more active against the *Colletotrichum Gloeosporiodes* (CG) fungus. The films with oregano essential oil were always less effective than the other two. These differences may be related, among other factors, with the nature and composition of the EOs, the ratio of EO:polymer in the film and possible interactions between polymers and active compounds which can affect their diffusion in the medium (Sánchez-González et al., 2011a). In these essential oils, eugenol, carvacrol and thymol are the main phenols. Eugenol is abundant in clove (90%) and cinnamon (60%), and is the main volatile compound of the oils; while in oregano oil, high percentages of carvacrol (50%) and thymol (21%) are usually found (Roselló, J.; Sempere, F.; Sanz-Berzosa, I.;

Chiralt, A.; Santamarin M. P. (2015). Moreover, the antimicrobial activity of essential oils is not only attributable to their main constituents. Different studies concluded that essential oils are more effective than isolated active compounds or mixtures of these, probably due to the development of synergistic effects (Mourey and Canillac, 2002; Ranasinghe et al., 2002).

The greater effectiveness of films containing cinnamon EO is coherent with previous studies about the in vitro analysis of the antimicrobial effectiveness of films based on Arabic gum against pathogens causing antracnosis (*C. musae and C. Gloeosporiodes*) (Maqbool et al., 2011). Films containing cinnamon EO were also reported to exert a good rot control when applied to papaya (Acosta, 2015-TESIS)

4. Conclusions

The incorporation of cinnamon bark, clove and oregano essential oils (25% with respect to polymer) into cassava starch-bovine gelatin films in a 1:1 ratio was effective at providing the films with antifungal activity. 35-45 % of the essential oil initially incorporated into the film-forming dispersion was retained in the film matrix of the films obtained by casting. The remaining essential oils were dispersed in the polymer matrix due to their lack of miscibility. Their presence in the film led to a significant reduction in the water vapor permeability of starch-gelatin films, a 17-30% reduction in oxygen permeability (depending on the essential oil) and an increase in transparency, without causing any negative effects in the film's mechanical behavior, apart from the fact that it does reduce the film's gloss. Films with EOs exhibited a notable antifungal activity against the two tested fungus species; this was particularly true in the cases of

418 those containing cinnamon EO against *Fusarium oxysporum f.sp. gladiolo* and those
419 containing clove EO against *Colletotrichum gloesporoides*.

420

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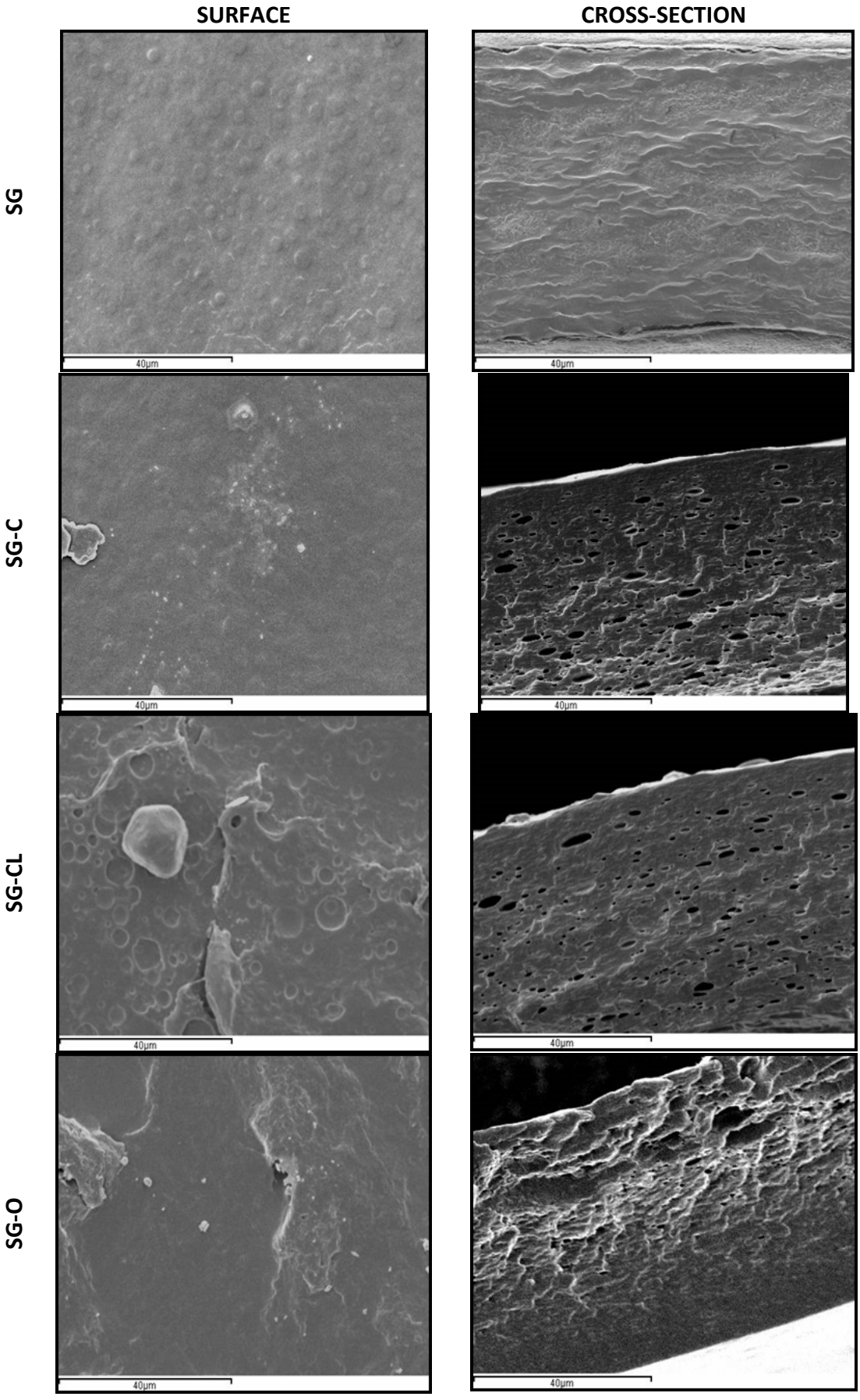
FIGURE CAPTIONS

Figure 1. SEM images of the surfaces (left column) and the cross-sections (right column) of starch-gelatin films, containing or not different essential oils (cinnamon (C), clove (CL) and (O) oregano).


SURFACE
CROSS-SECTION
SG
SG-C
SG-CL
SG-O
40µm

557 Table 1. Average value (*) (wt. %) of the main EO components and their melting point
558 (ºC) between brackets (adapted from Rosello, et al 2015).

Components (melting point, ºC)	Cinnamon EO (%)	Clove EO (%)	Oregano EO (%)
Eugenol (256°C)	60	90	-
Acetato de cinamilo (265°C)	5	-	-
Acetato de eugenilo (268°C)	18	-	-
Benzoato de benzilo (323°C)	4	-	-
Beta-Cariofileno (280°C)	-	7	-
Timol (233°C)	-	-	21
Carvacrol (238°C)	-	-	50
Gamma-Terpineno (183°C)	-	-	9
Para-Cimeno (177°C)	-	-	11

559 *values depend on several factors as method of extraction, geographical location, genetic and
560 environmental conditions, among others,

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570 Table 2. Barrier properties (WVP: water vapour permeability and OP: oxygen
571 permeability), and loss of essential oil after films drying. C: cinnamon; Cl: clove; O:
572 oregano. Mean values, standard deviation and ANOVA results.

FILMS	WVP ($g.mm/kPa.h.m^2$)	$OPx10^{-13}$ ($cm^3/m.sec.Pa$)	Loss of essential oil (%) (1)
SG	$5,9 \pm 0,4^b$	$0,52 \pm 0,10^b$	-
SG-C	$4,2 \pm 0,2^a$	$0,39 \pm 0,02^a$	59 ± 4^a
SG-Cl	$4,2 \pm 0,2^a$	$0,43 \pm 0,02^a$	56 ± 2^a
SG-O	$4,6 \pm 0,4^a$	$0,358 \pm 0,005^a$	66 ± 4^b

573 (1)Percentage of lost throughout film drying respect initial quantity of film forming dispersion

574 a,b, Different superscripts within the same column indicate significant differences among films ($p <$
575 0.05).

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589 Table 3. Tensile (Elastic modulus: EM, tensile strength: TS and deformation: E at
590 break) and optical (internal transmittance: Ti and gloss) properties of starch-gelatin
591 based films, containing or not essential oils. C: cinnamon; Cl: clove; O: oregano. Mean
592 values, standard deviation and ANOVA results.

FILMS	EM(MPa)	TS(MPa)	E (%)	Ti (480nm)	Gloss (60°)
SG	627 ± 54^{ab}	24 ± 2^{ab}	14 ± 4^{ab}	$81 \pm 0{,}04^{a}$	37 ± 4^{d}
SG-C	775 ± 119^{b}	25 ± 2^{b}	8 ± 3^{a}	$91 \pm 0{,}5^{b}$	12 ± 2^{a}
SG-Cl	642 ± 71^{ab}	25 ± 3^{b}	17 ± 3^{b}	$90 \pm 0{,}4^{b}$	17 ± 3^{b}
SG-O	612 ± 213^{a}	22 ± 2^{a}	12 ± 3^{ab}	$92 \pm 0{,}4^{b}$	23 ± 4^{c}

593 a,b, Different superscripts within the same column indicate significant differences among films ($p <$
594 0.05).

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608 Table 4. Halo (diameter) of the growth inhibition of Fusarium oxysporum (FOG) and
609 Colletotrichum Gloeosporiodes (CG) at 48 and 72 hours of incubation on plaque
610 containing discs with different films. C: cinnamon; Cl: clove; O: oregano.

	Halo of fungus growth inhibition (mm)			
FILMS	FOG at 48 h	FOG at 72 h	CG at 48 h	CG at 72 h
SG-C	57 ± 4^{c}	48 ± 2^{b}	49 ± 3^{b}	38 ± 3^{c}
SG-Cl	49 ± 2^{b}	46 ± 2^{b}	52 ± 2^{b}	45 ± 2^{b}
SG-O	42 ± 3^{a}	36 ± 2^{a}	41 ± 7^{a}	30 ± 7^{a}

611 a,b, Different superscripts within the same column indicate significant differences among films ($p <$
612 0.05).

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