POLYMER MEMBRANES FROM BIODEGRADABLE POLYMER AND CHEMICAL FUNGICIDE PREPARED BY ELECTROSPINNING

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ABSTRACT

Esca is a grapevine disease caused by several different fungi such as Phaeomoniella chlamydospora (P. chlamydospora) and Phaeoacremonium aleophilum (P. aleophilum). In the last years this disease has become a worldwide problem. Membranes from cellulose acetate (CA) and cellulose acetate/polyethylene glycol (CA/PEG) containing chemical fungicide - 5-chloro-8-hydroxyquinolinol (5-Cl8Q) were prepared by electrospinning and were studied as suitable candidates for plant protection against fungi. Several methods including scanning electron microscopy (SEM), IR spectroscopy, water contact angle measurements and UV-VIS spectroscopy were utilized to characterize the obtained fibrous materials. The antifungal activities of the obtained materials against P. chlamydospora and P. aleophilum were studied as well. The present study reveals the possibility to use electrospun polymer membranes containing 5-Cl8Q to impede the penetration and growth of P. chlamydospora and P. aleophilum.

Keywords: *electrospinning; cellulose acetate, chemical fungicide, Phaeomoniella chlamydospora, Phaeoacremonium aleophilum*

1. Introduction

Esca is a grapevine disease that seriously affects vine yield and longevity causing dark red or yellow stripes on leaves, trunk damages and sudden wilting of the entire plant [1]. It is known that this disease is caused mainly by species Phaeomoniella chlamydospora and Phaeoacremonium aleophilum [2]. Over the last three decades the impact of esca has become a dramatic global threat to all vineries. The disease spreads rapidly and is a real threat to all vineyards in Europe.

In recent years, electrospinning has proven to be a promising technique for the fabrication of polymeric materials for medicine, pharmacy and agriculture [3]. This is due to the fact that when the diameters of the polymer fibers are in the nano-scale, some interesting size-related properties of the materials are observed, e.g. high-surface-area-to-volume ratio, flexible surface modification and modulation of the release profile of incorporated biologically active compounds, etc.

8-Hydroxyquinoline and its derivatives are of low toxicity to humans [4], manifest antibacterial, antifungal, anti-inflammatory, antineuro-degenerative, anticancer, antioxidant and antidiabetic activities [5]. Recently we have shown that the combination of water-soluble polymers and 8-hydroxyquinoline derivatives results in obtaining of stable solutions with antifungal

activity suitable for applications in agriculture [6].

The aim of this work was to fabricate and characterize electrospun membranes of cellulose acetate (CA) and polyethylene glycol (PEG) containing 5-chloro-8-hydroxyquinolinol (5-Cl8Q). The composition of the polymer matrix was selected so as to improve the release of 5-Cl8Q. The effect of the fibrous membranes on the biological behavior upon contact with P. chlamydospora and P. aleophilum was investigated as well.

2. Materials and Methods

2.1. Materials

Cellulose acetate (CA, Aldrich) with $\overline{M}_n$ = 30 000 g/mol and DS 39.8%, polyethylene glycol (PEG, Fluka) with Mr = 1 900-2 200 g/mol and 5-chloro-8-hydroxyquinolinol (5-Cl8Q, Sigma-Aldrich) were used. Acetone (Sigma-Aldrich) of analytical grade of purity was used. Potato dextrose agar medium was purchased from Merck, Germany. The disposable consumables were supplied by Orange Scientific, Belgium.

2.2. Preparation by electrospinning

Four types of fibrous membranes were prepared by electrospinning: CA, CA/5-Cl8Q, CA,PEG and CA,PEG/5-Cl8Q. For the preparation of membranes the following spinning solutions were prepared in acetone/water 80/20 v/v: (i) CA; (ii) CA/5-Cl8Q; (iii) CA,PEG and (iv) CA,PEG/5-Cl8Q with total polymer concentration of 10 wt% and 5-Cl8Q 10 wt.% in respect to total polymer weight.

The electrospinning set-up was composed of a custom-made high voltage power supply (up to 30 kV), a grounded rotating drum collector, an infusion pump (NE-300 Just InfusionTM Syringe Pump, New Era Pump Systems Inc., USA) for delivering the spinning solution at a constant rate and a syringe equipped with a metal needle (gauge: 20GX1½″). Electrospinning was performed under the following conditions: flow rate of 3.0 ml/h, voltage of 25 kV, needle tip-to-collector distance 15 cm, collector rotating speed of 1000 rpm, room temperature - 21 ° C, relative humidity - 50%.

2.3. Characterization

The dynamic viscosity of the spinning solutions was measured using a Brookfield DV-II+ Pro programmable viscometer for cone/plate option equipped with a sample thermostated cup and a cone spindle, at 25 ± 0.1 °C.

The morphology of the fibrous membranes was analyzed by SEM. The samples were vacuum-coated with gold and observed by a Jeol JSM-5510 SEM (Japan). The average fiber diameter was estimated by ImageJ software [7] by measuring at least 20 fibers from three different SEM micrographs for a total of 60 measurements and their morphology was assessed applying the criteria for overall evaluation of electrospun materials as described in details in [8].

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectra were recorded using an IRAffinity-1 spectrophotometer (Shimadzu Co., Kyoto, Japan) equipped with a MIRacle™ATR (diamond crystal, depth of penetration of the IR beam into the sample - about 2 μm) accessory (PIKE Technologies, USA) in the range of 600-4000 cm^{-1} with a resolution of 4 cm^{-1}. All spectra were corrected for H_2O and CO_2 using an IRsolution software program.

Static contact angle measurements of the membranes were performed using an Easy Drop DSA20E Krűss GmbH drop shape analysis system (Germany) at 20 ± 0.2 °C. A sessile drop of deionized water with a volume of 10 μL controlled by a computer dosing system was deposited onto the membranes (2 cm × 7 cm; cut in the direction of rotation of the collector). The contact angles were calculated by computer analysis of the acquired images of the droplet. The data are average from 20 measurements for each sample.

The 5-Cl8Q release was studied in vitro at 37 °C in acetate buffer (CH_3COONa/CH_3COOH) containing lactic acid (acetate buffer/lactic acid = 96/4 v/v) at pH 3 and ionic strength of 0.1. 5-Cl8Q-containing nanofibrous membranes (4 mg) were immersed in 100 ml of buffer solution under stirring in water bath (Julabo, Germany). The release kinetics was determined by withdrawing aliquots (2 ml) from the solution at determined time intervals, adding back the same amount of fresh buffer and recording the absorbance of the aliquots by a DU 800 UV-vis spectrophotometer (Beckman Coulter) at a wavelength of 255 nm. The amount of released 5-Cl8Q was calculated using calibration curves (correlation coefficient R = 0.999) for the membranes in acetate buffer/lactic acid = 96/4 v/v, pH = 3, ionic strength 0.1. The data are average values from three measurements.

2.4. In vitro antifungal assay

The antifungal activity of membranes was monitored against the fungi P. *chlamydospora* CBS 239.74 and *P. aleophilum* CBS 631.94. *P. chlamydospora* CBS 239.74 and P. *aleophilum* CBS 631.94 were purchased from Westerdijk Fungal Biodiversity Institute, The Netherlands. In order to measure the zones of inhibition, in vitro studies were performed using potato dextrose agar medium (PDA, Merck, Germany) for the fungal strains. The surface of the solid agar was inoculated with a suspension of fungi culture with a fungi concentration of 1 × 105 cells/ml and on the surface of the agar in each Petri dish one membrane was placed. The Petri dishes were incubated for 96 h for the *P. chlamydospora* and *P. aleophilum* at 28 °C and subsequently the zones of inhibition around the disks were measured. The average diameters of the zones of inhibition were determined using the ImageJ software based on 15 measurements in 15 different directions for each zone.

3. Results and discussion

The dynamic viscosity of the prepared four solutions was measured. The viscosity of 10 wt.% solutions of CA, CA/5-Cl8Q, CA,PEG and CA,PEG/5-Cl8Q was 184, 190, 91, and 108 cP, respectively. The incorporation of PEG into the solutions resulted in viscosity decrease which was due to the low molecular weight of this polymer.

Four types of fibrous materials based on biocompatible and biodegradable CA, non-toxic and biocompatible PEG and the antifungal drug - 5-Cl8Q were fabricated by electrospinning (**Figure 1**).

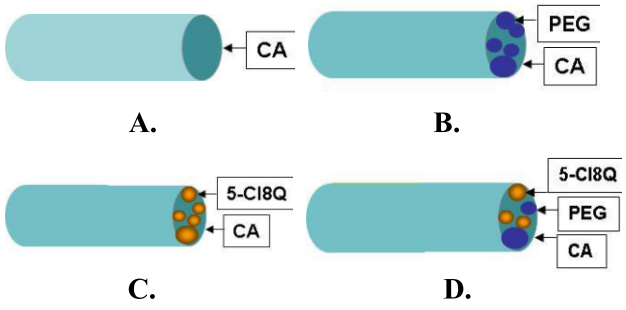
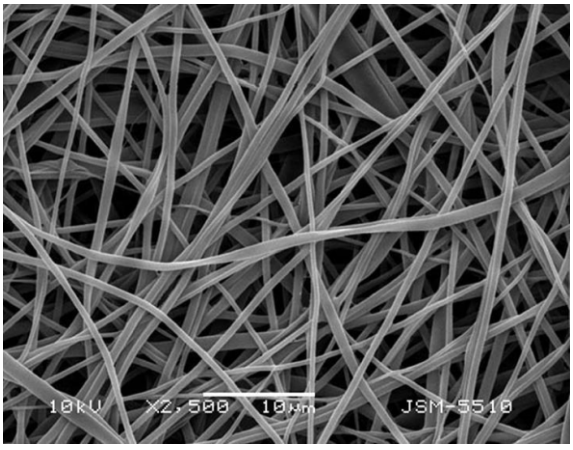
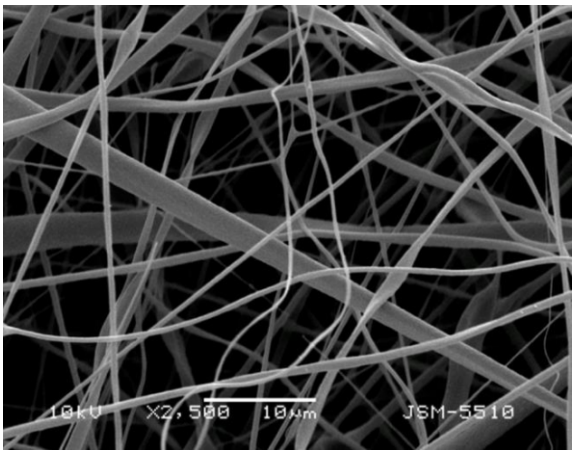


Figure 1 Schematic representation of fibers: A. CA, B. CA,PEG, C. CA/5-Cl8Q and D. CA,PEG/5-Cl8Q.

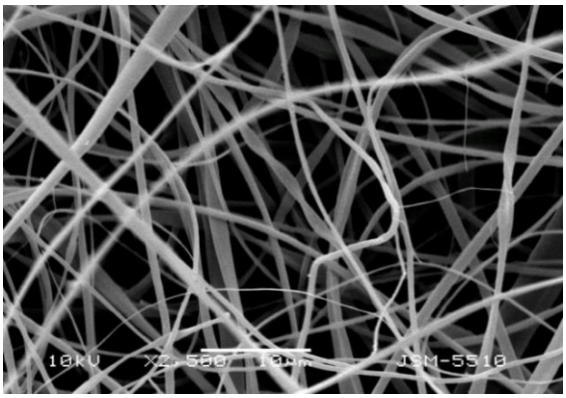
SEM micrographs of the obtained CA, CA/5-Cl8Q, CA,PEG and CA,PEG/5-Cl8Q membranes are shown in **Figure 2.** Electrospinning of CA solution under the selected conditions reproducibly resulted in obtaining continuous defect-free fibers with mean fiber diameter of 780 ± 100 nm (**Figure 2(A)**). The addition of PEG into the spinning solutions resulted in decrease of the dynamic viscosity and decrease of the average fiber diameter. The average diameter of the CA/PEG fibers (531 ± 80 nm) was smaller (**Figure 2B**) as that of the CA fibers at constant total polymer concentration and spinning conditions. Representative SEM images of the obtained CA and CA,PEG membranes, containing 5-Cl8Q are shown in **Figure 2(C)** and **(D)**. The addition of 5-Cl8Q (10 wt%) to the spinning solutions led to the preparation of fibers with smaller diameters 750 ± 90 nm for CA/5-Cl8Q fibrous membrane and 446 ± 60 nm for the CA,PEG/5-Cl8Q membrane.



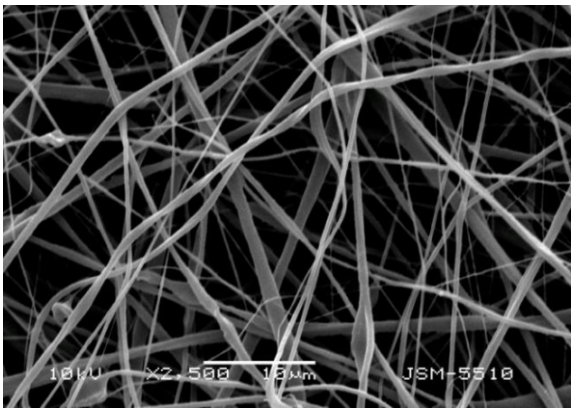
A.



B.



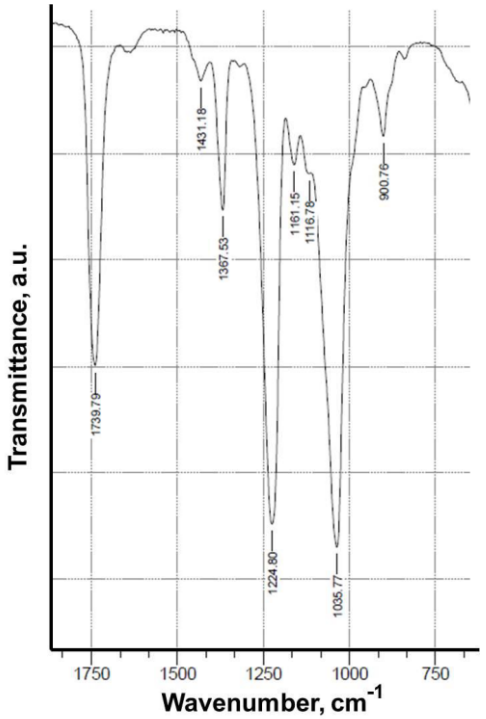
C.

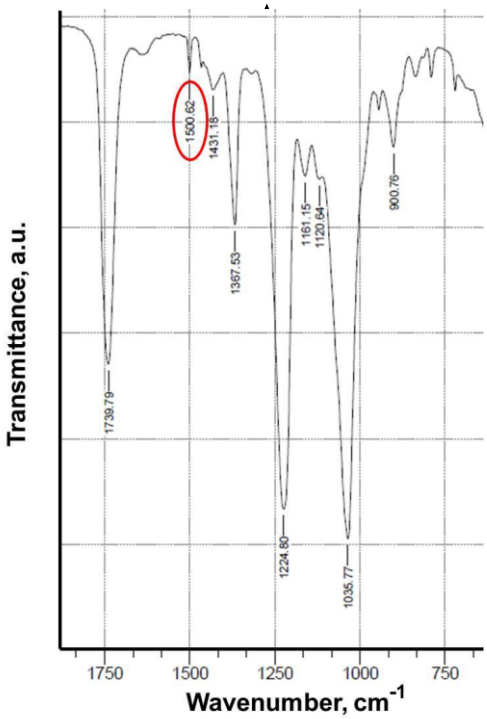


D.

Figure 2 SEM micrographs of fibrous materials: A. CA, B. CA,PEG C. CA/5-Cl8Q and D. CA,PEG/5-Cl8Q.

All the prepared membranes were characterized by FTIR spectroscopy. The IR spectra of CA and CA/5-Cl8Q fibrous materials were shown in **Figure 3**. In the IR spectrum of CA membrane (**Figure 3A**) bands characteristic for CA appeared at 1740 cm^{-1} for the C=O groups, at 1369 and 1226 cm^{-1} for the CH_3 groups, and at 1037 cm^{-1} for the ether C–O–C groups were detected in accordance with literature [9]. In the FTIR spectra of CA/5-Cl8Q (**Figure 3B**) fibrous materials in addition to the characteristic bands of CA new band appeared at 1500 cm^{-1} characteristic for quinoline ring [10], proving the presence of the bioactive compound in the electrospun membrane. In the spectra of the CA,PEG and CA,PEG/5-Cl8Q new characteristic bands were observed at 1 100 cm^{-1}, assigned to C-O-C ether groups of PEG, as well as at 2875 cm^{-1} characteristic for the vC–H vibrations. In the FTIR spectra of CA,PEG/5-Cl8Q in addition to the bands characteristic of CA and PEG new bands appeared at 1500 cm^{-1} characteristic for the aromatic ring of the bioactive compound demonstrating the successful incorporation of the 5-Cl8Q in the CA/PEG membrane.

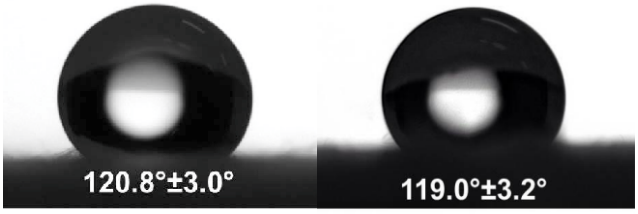




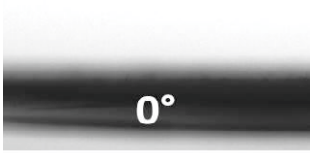
B.

Figure 3 FTIR spectra of electrospun membranes of: A. CA and B. CA/5-Cl8Q.

It was found that the contact angle values depended on the composition of the prepared in these study membranes. Neat CA fibrous membranes were hydrophobic with water contact angle of 120.8°±3.0° (**Figure 4A**). The measured contact angle values of CA/5-Cl8Q membranes and were close to those measured for the CA membranes (119.0 ± 3.2°) (**Figure 4B**). The incorporation of water-soluble polymer - PEG resulted in significant decrease of the measured contact angle value. The values of the water contact angle of CA,PEG and CA,PEG/5-Cl8Q membranes were 0° thus indicating complete wetting (**Figure 4C**).



A. **B.**

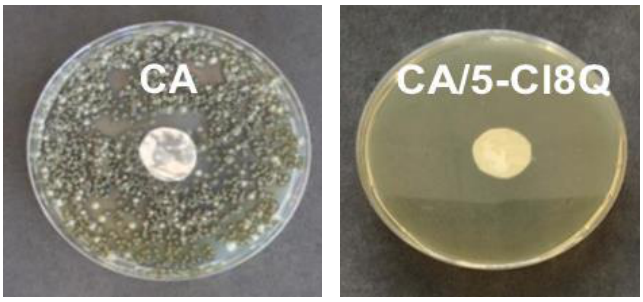


C.

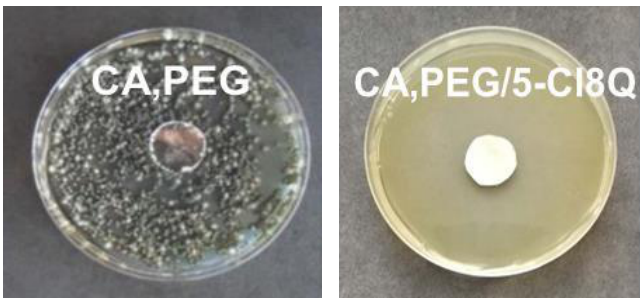
Figure 4 Digital images of water droplets deposited on the surface of the fibrous mats and contact angle values for membranes from: A. CA; B. CA/5-Cl8Q and C. CA,PEG and CA,PEG/5-Cl8Q.

The release of the chemical fungicide from CA/5-Cl8Q and CA,PEG/5-Cl8Q fibrous membranes was studied spectrophotometrically. For all membranes burst release was initially observed followed by a second stage of gradual release. It was found, that 5-Cl8Q was released slowly from CA/5-Cl8Q membrane. This observation may be explained with the hydrophobicity of this membrane. The released chemical fungicide from the hydrophobic CA/5-Cl8Q fibrous mat was ca. 78% for 175 min. 5-Cl8Q was released more rapidly and in the greatest amount from CA,PEG/5-Cl8Q membrane. In the case of CA,PEG/5-Cl8Q membrane ca. 83% of the loaded amount was released within 30 min. This rapid release of bioactive compound is most likely due to higher wettability of CA,PEG/5-Cl8Q membrane due to the presence of water-soluble polymer PEG in the membrane. Thus, the penetration of the aqueous medium in the membrane and the release of 5-Cl8Q were favored.

In the present study, the antifungal activity of the electrospun membranes was assessed by performing tests against P. chlamydospora and P. aleophilum. The results obtained by determination of the zones of inhibition after contact of the fibrous materials with P. chlamydospora fungal are shown in Figure 5. It was found that the incorporation of 5-Cl8Q in the membranes that were placed in contact with fungal cells resulted in complete inhibition for all fungi. In contrast, as expected, the neat CA and CA,PEG membranes did not alter the fungal growth and did not exhibit any antifungal activity. The observation of wide zones of inhibition around all membranes containing 5-Cl8Q is evidence that the incorporated bioactive compound impart antifungal activity to the prepared novel fibrous membranes.



A. **B.**



C. **D.**

Figure 5 Digital images of the zones of inhibition against P. *chlamydospora* after contact of the membranes with fungi cells.

4. Conclusions

Fibrous materials of biocompatible and non-toxic polymers - cellulose acetate and cellulose acetate/PEG containing chemical fungicide 5-chloro-8-hydroxyquinolinol (5-Cl8Q) were successfully prepared by electrospinning. The addition of PEG resulted in hydrophilization of the prepared materials. The incorporation of 5-Cl8Q in the membranes imparted a considerable antifungal effect against P. chlamydospora and P. aleophilum. Thus prepared fibrous membranes are suitable candidates for plant protection against pathogenic fungi.

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