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DESIGN OF ECO-FRIENDLY ELECTROSPUN MATERIALS WITH FUNGICIDAL ACTIVITY AGAINST P. *CHLAMYDOSPORA*

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ABSTRACT

Novel electrospun materials based on poly(3-hydroxybutyrate) (PHB) and TiO_2 were fabricated by electrospinning or combining the electrospinning and electrospraying. These eco-friendly materials were purposely designed as type "in" and type "on" in order to protect mechanically damaged vines from entering the spores of Phaeomoniella chlamydospora. The morphology of the fabricated fibrous materials was observed by scanning electron microscopy, while the distribution of TiO_2 nanoparticles in/on the PHB fibers depending on the materials type was observed by transmission electron microscopy. The crystallinity was assessed by X-ray diffraction analysis. The antifungal activity of the fibrous materials against the P. chlamydospora was evaluated. It was demonstrated that the obtained composite TiO_2/PHB fibrous materials are promising for application in agriculture as eco-friendly active dressings for plant protection against penetration and growth of main fungi causing esca disease.

Keywords: *electrospun membranes, esca, Phaeomoniella chlamydospora, antifungal activity, TiO_2, PHB.*

1. Introduction

Esca is one of the earliest described diseases in grapevines that cause trunk damages and sudden wilting of the entire plant. It is known that the esca disease is caused mainly by species Phaeomoniella chlamydospora and Phaeoacremonium aleophilum [1,2]. Over the last three decades the impact of esca disease has become a dramatic global threat to all vineries. The disease spreads rapidly and is a real threat to all vineyards in Europe. Regarding the curative approaches to fight with esca the existing data in literature on evaluation the possibility to treat this disease are scarce [3].

For the modern agriculture, it is necessary not only to create new plant protection products with low toxicity to non-pathogenic soil micro-organisms, but also to seek rational solutions and approaches to improve the existing ones. Modern plant protection products should be designed to achieve the desired biological effect without harmful impact and side effects. An alternative to the used chemical agents for plant protection is the use of natural substances and/or microorganisms with their own biological activity against phytopathogenic microorganisms. Therefore, finding the conditions and methods for creating an "active dressing" for vines is a promising approach to incorporate selected antifungal agents in appropriate polymers with imparted biological activity. Furthermore, the created material will not only protect the plants against the penetration of Phaeomoniella chlamydospora and Phaeoacre-monium aleophilum, but can also help in their destruction. This will enable the creation and development of new and effective agents to fight fungi-caused disease in grapevine.

Currently, the electrospinning is a cutting-edge nanotechnology that allows facile fabrication of fibrous materials with unique features [4,5]. In our previous studies we have succeeded purposefully to create novel environmentally friendly fibrous agro-pharmaceuticals in which the biological agent is incorporated in chitosan or in polymer complexes of chitosan and synthetic water-soluble polymers [6-8]. Moreover, facile covering of the plant sprouts with biohybrids by direct electrospinning was demonstrated [6].

In this respect, the present study is focused on designing innovative polymer composites with imparted fungicidal activity against P. chlamydospora - associated with esca. The suitable combination of biocompatible and biodegradable poly(3-hydroxybutyrate) (PHB) with nanosized TiO_2-anatase capable to generate singlet oxygen by using electrospinning alone or in conjunction with electrospraying, is expected to enable the creation of innovative polymer composites with varied design able to protect mechanically damaged vines from entering the spores of P. chlamydospora, i.e. to protect vineyards from esca - the most devastating disease of grapevines.

2. Experimental part

Poly(3-hydroxybutyrate) (PHB, 330000 g/mol), titanium (IV) oxide (TiO_2, 99.7% anatase nanopowder, <25 nm), chitosan oligomers (COS, 3000-5000 g/mol), chloroform ($CHCl_3$) and N,N-dimethylformamide (DMF) were of analytical grade and used without further purification.

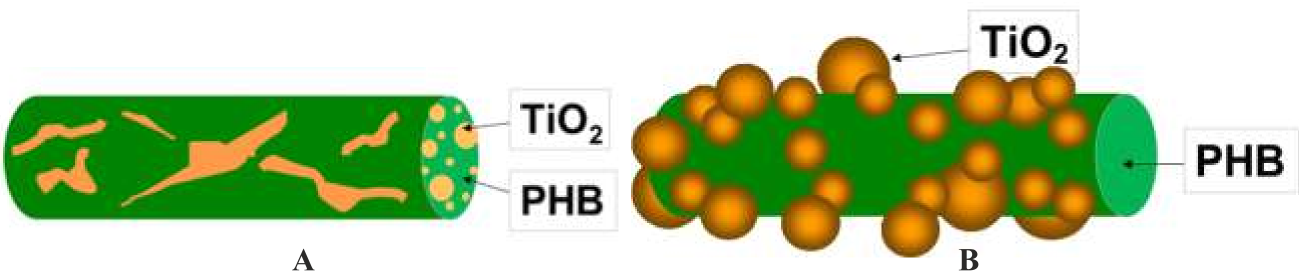
Two types of fibrous materials based on PHB and nanosized TiO_2-anatase were prepared by electrospinning alone or in conjunction with electrospraying. Fibrous TiO_2-in-PHB materials were fabricated by electrospinning of a mixture of PHB solution with TiO_2 (7% w/v). Fibrous TiO_2-on-PHB materials were obtained by using a PHB spinning solution (14% w/v) for electrospinning and TiO_2-COS dispersion for electrospraying. For this purpose, an aqueous COS solution (0.5%) was added to TiO_2 (10% w/v) dispersion in ethanol. Electrospinning was performed at 25 kV voltage and 1500 rpm collector rotation speed. The tip-to-collector distances were 25 and 10 cm for electrospinning and electrospraying, respectively. The feeding rate of the PHB/TiO_2 mixture was 5 ml/h, whereas that of PHB and TiO_2-COS dispersion – 3ml/h and 2 ml/h, respectively.

The structure and morphology of the fibrous materials were observed using scanning (Philips 515 SEM) and transmission (JEM 2100, Jeol) electron microscopy. For the characterization of the fabricated fibrous materials, Fourier-transform infrared (FT-IR) spectroscopy (Shimadzu Co. spectrophotometer supplied with a MIRacle ATR device) was used. The hydrophilic-hydrophobic properties on the surface of the prepared composite materials were studied by contact angle measurements (Easy Drop DSA20E Krűss GmbH).

The antifungal activity of fibrous materials was monitored against the fungi P. chlamydospora CBS 239.74 (Westerdijk Fungal Biodiversity Institute). The fibrous materials were cut in disks with diameters of 4.5 cm and thickness ~1 μm. For the preparation of the conidia suspension test microorganisms were grown on Potato Dextrose Agar (PDA) medium for 14 days. In order to study the antifungal activity against P. chlamydospora of the fibrous materials, 20 ml conidia suspension (with final concentration 10^7 conidia/ml) was passed through each fibrous material by using a filtration device.

3. Results and discussion

One-pot electrospinning of a suspension of TiO_2 in PHB solution resulted in materials in which TiO_2 was incorporated within the fibers (design type "in", ***Scheme 1A***). Simultaneous electrospinning of PHB solution and electrospraying of TiO_2-COS dispersion enabled the preparation of materials consisting of PHB fibers on which TiO_2 was deposited on the fibers' surface (design type "on", ***Scheme 1B***). In this case, biodegradable and water-soluble COS play the roles of both stabilizing the TiO_2 dispersions and serving as a sticking agent for the TiO_2 particles onto PHB fibers.



Scheme 1 Representation of design types of fibrous materials: (A) TiO2-in-PHB and (B) TiO2-on-PHB.

The morphology of the prepared TiO_2-in-PHB and TiO_2-on-PHB fibrous materials was studied by SEM (***Figure 1***) and compared to that of the electrospun PHB. As seen, electrospinning of PHB (***Figure 1A***) results in uniform and defect-free fibers. In contrast, electrospinning of PHB/TiO_2 mixture leads to the formation of fibers with rough surface (***Figure 1B***). This result was expected, because some particle aggregation was observed during the electrospinning process. As seen, in the case of TiO_2-on-PHB materials (***Figure 1C***), electrospraying of the TiO_2-COS dispersion ensures enrichment of the surface of the PHB fibers with TiO_2 particles. Moreover, due to the addition of COS to the dispersion, TiO_2 particles were sticked onto PHB fibers (***Figure 1D***). The obtained SEM results demonstrated that purposely designed fibrous materials can be successfully achieved by electrospinning and by its conjunction with electrospraying.

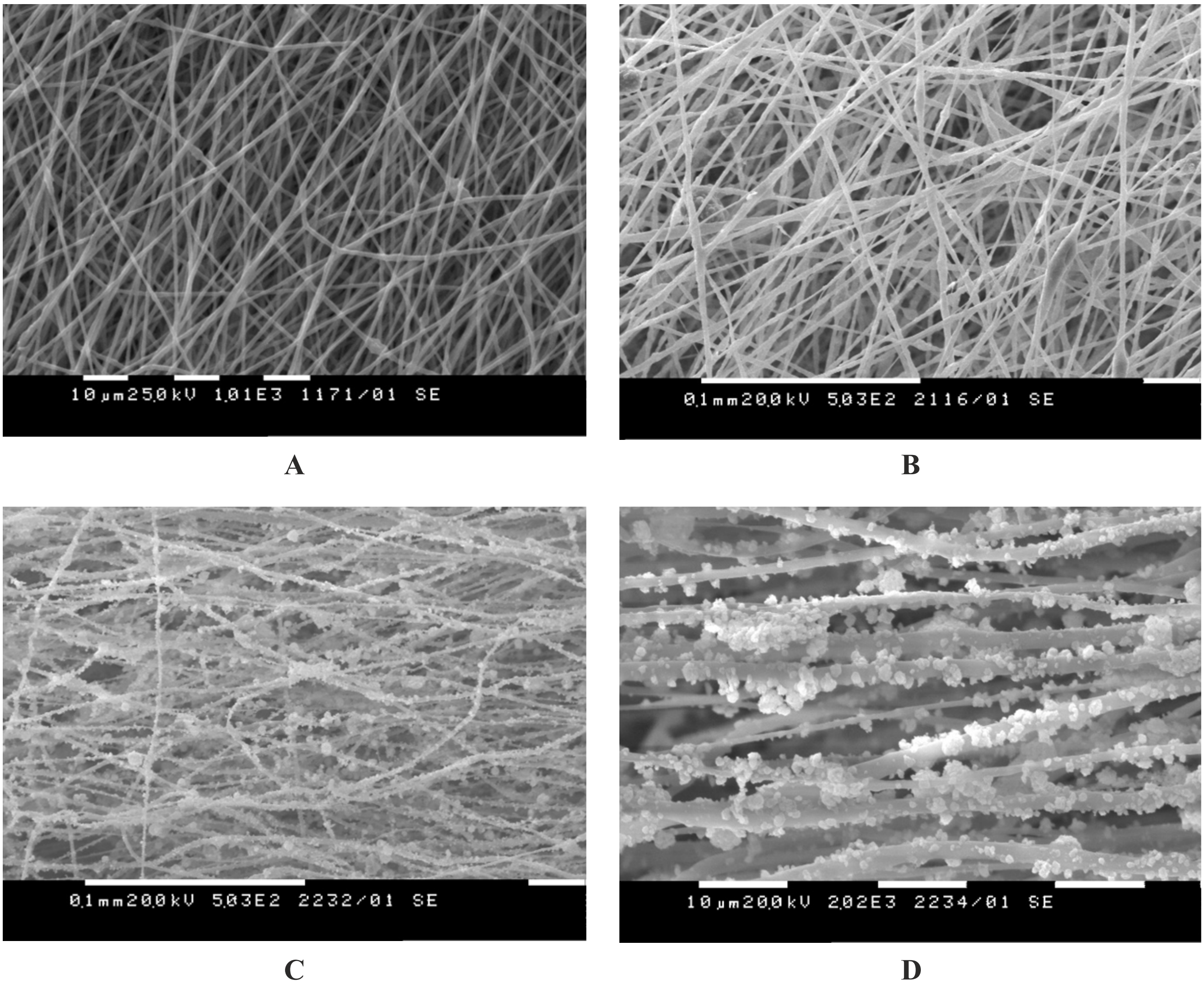


Figure 1 SEM micrographs of fibrous materials:
(A) PHB (×1000), (B) TiO_2-in-PHB (×500), (C) TiO_2-on-PHB (×500) and (D) TiO_2-on-PHB (×2000).

TEM analyses (***Figure 2***) provided additional information for the distribution of the TiO_2 particles. In conformity to the SEM analysis, as seen in the TiO_2-in-PHB materials particles were incorporated in the PHB fibers and formed agglomerates (***Figure 2A***). Apparently, electrospraying results in distribution of the TiO_2 particles on the surface of the PHB fibers (***Figure 2B***). Obviously, adding of COS leads to aggregation of the particles but also ensures their adhesion to PHB fibers.

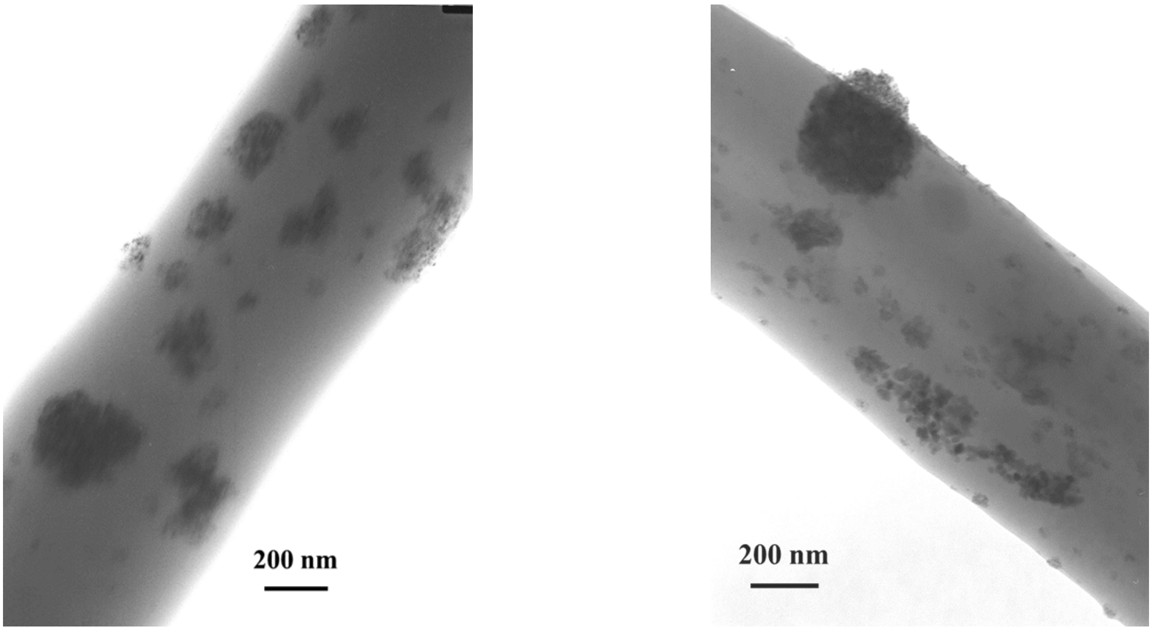


Figure 2 TEM images of fibrous materials: (A) TiO_2-in-PHB and (B) TiO_2-on-PHB.The FT-IR spectra of the TiO_2-in-PHB and TiO_2-on-PHB fibrous materials were compared with the spectra of the PHB mat (***Figure 3***). All spectra showed the following characteristic bands: at 1721 cm^{-1} (C=O stretching vibrations) and 1055 cm^{-1} (C–O stretching). The bands for PHB were in accordance with the literature [9]. The results clearly showed that there is no interaction between PHB and TiO_2 particles.

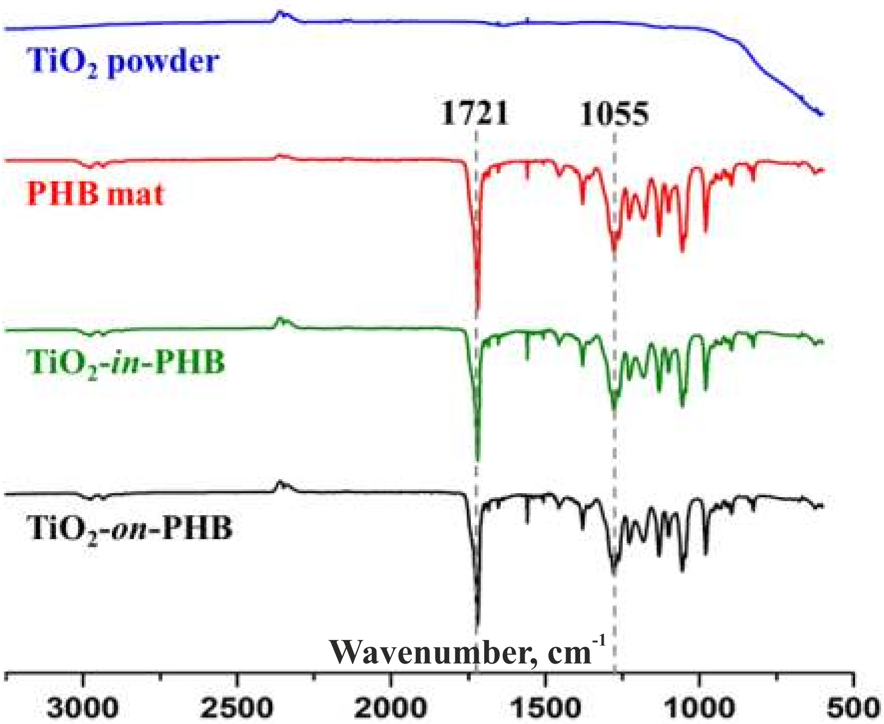


Figure 3 FT-IR spectra of fibrous materials and TiO_2 powder.

In order to evaluate hydrophilic/hydrophobic balance, the water contact angle of the obtained fibrous materials was measured. It could be seen that PHB mats were hydrophobic, with a water contact angle of 108° ± 3.3° and the water droplet has spherical shape (***Figure 4A***). The addition of the TiO_2 particles resulted in an increase of the measured contact angle values - the measured contact angle values for TiO_2-in-PHB and TiO_2-on-PHB fibrous materials were 124 ± 2.3° and 127 ± 3.5°, respectively (***Figure 4B*** and ***C***). This probably is due to the rough surface of the fibrous materials, resembling the lotus leaf architecture.

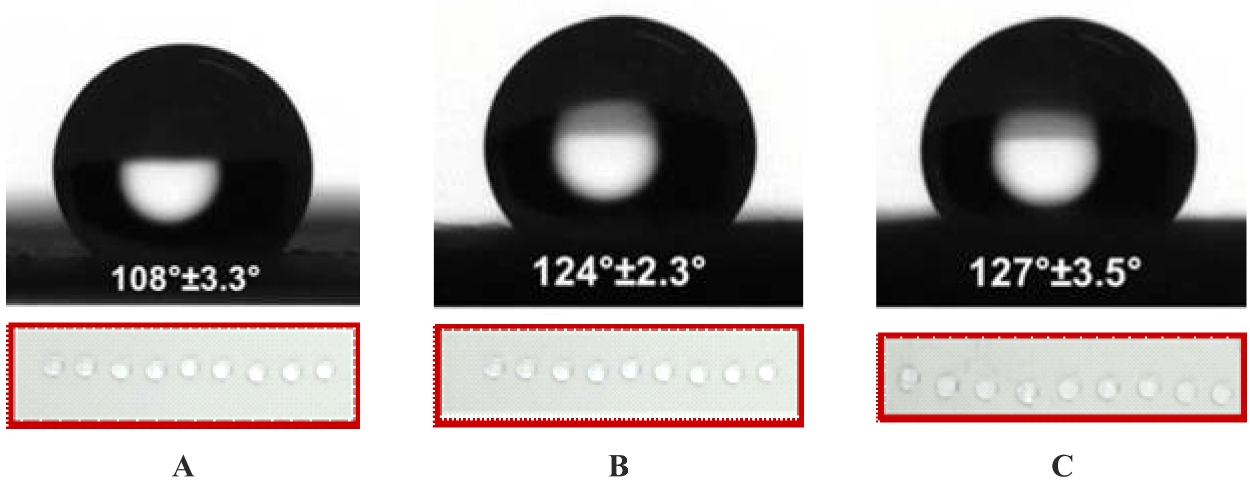


Figure 4 Water contact angle of fibrous materials: (A) PHB mat; (B) TiO_2-in-PHB and (C) TiO_2-on-PHB

The obtained results indicate that TiO_2 particles provided a rough surface of the TiO_2-in-PHB and TiO_2-on-PHB fibrous materials. In addition, COS ensure the fixation of TiO_2 onto PHB fibers upon electrospraying. Moreover, it is known that TiO_2 successfully degraded pesticides, used for plant germination and growth, crop disease control, water purification, pesticide residue detection [10,11]. For these reasons, the next step was to assess the antifungal activity of the fabricated materials against P. chlamydospora and therefore, to study their barrier efficacy. In ***Figure 5A***, a schematic presentation of the filtering device that was used in the antifungal tests, is shown. In a typical experiment, the fibrous membranes were cut in disks (4.5 cm diameter) with thickness ~1 μm. Then, fibrous disks were sterilized for 30 min under UV light, and after that 20 mL of conidia suspension was passed through each fibrous material by using a filtration device. As seen (***Figure 5A***), the conidia suspension of the P. chlamydospora contacted with the central part of the fibrous filter. Finally, every used fibrous disk was placed on a surface of a solid agar in a Petri dish, incubated for 96 h at 28°C, and subsequently the fungal growth was assessed. Clearly, compared to the TiO_2-in-PHB, TiO_2-on-PHB fibrous materials showed complete inhibition of fungal growth with wide zone of inhibition around the fibrous disk (***Figure 5C, B***). Therefore, TiO_2 deposited onto the surface of the PHB fibers imparts antifungal activity.

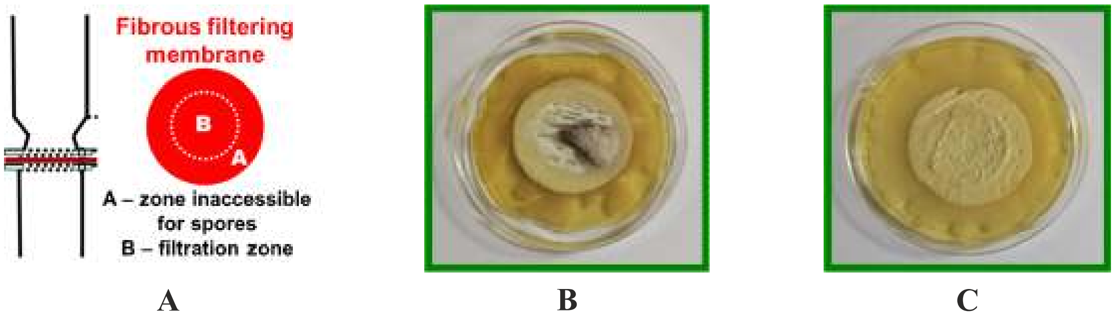


Figure 5 (A) Schematic presentation of the filtering device used in the antifungal tests;
(B, C) Digital images of the growth P. chlamydospora on the TiO_2-in-PHB and TiO_2-on-PHB, respectively.

4. Conclusions

The incorporation of TiO_2 in PHB fibers or the decoration of PHB fibers with TiO_2-COS resulted in increased roughness and hydrophobicity of the obtained fibrous materials. The incorporation of TiO_2 imparted a considerable antifungal activity against P. chlamydospora and the complete suppression of the fungal growth was obtained in the case of TiO_2-on-PHB materials. Therefore, the obtained composite TiO_2/PHB fibrous materials are promising for application in agriculture as eco-friendly active dressings for vines protection against penetration and growth of main fungi causing esca disease.

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