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New biodegradable polylactide material with antimicrobial properties

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Abstract. This study aimed to investigate the bactericidal, fungicidal and non-phytotoxic properties of vapor permeable polylactide films containing five different concentrations (in the range of 0.2–1.0%) of thiabendazole. All films showed bactericidal properties on *Staphylococcus aureus* and *Escherichia coli*. Thiabendazole introduced into polylactide affected the antifungal activity of the materials containing 0.8 and 1.0% thiabendazole. The films containing thiabendazole were characterized by increased permeability. The vapor permeability of the film increased with the increase of the biocide content in the composites. The new materials had no a negative effect on the growth and development of monocotyledonous and dicotyledonous plants. It has been shown that the presence of thiabendazole increases the water vapor permeability of polylactide films. The obtained materials are biodegradable and can be used in horticulture and agriculture to protect plants against pathogens. The use of films with biocide properties will reduce the use of plant protection products. This is particularly important due to the need to protect biodiversity in the ecosystem of agricultural soils.

Keywords: agricultural foil, polymer material, mintezol, PLA, biocide.

1. Introduction

Recently, biodegradable polymers have been intensively studied as an alternative to the conventional, slow-degrading synthetic polymers that have a negative effect on the living organisms (Adams et al., 1995; Seretoudi et al., 2002; Richert et al., 2017). Among the commercially available biodegradable polymers, the polyhydroxyalkanoates (PHAs), polylactide (PLA), polybutyleneadipate-co-terepthalate (PBAT) or starch-basedpolymers are more and more popular, gaining popularity and acceptance among consumers (Auras et al., 2011; Sabu et al., 2014; Urtuvia et al., 2014). The main advantage of using these materials is no toxicological effect of degradation products after disposal, but it must be noted that only very few publications deal with the eco-toxicity of degradation products of bio-based polymers (Fritz et al., 2003; Rychter et al., 2006, 2010; Tuominen et al., 2002).

About 2.5% of all plastics produced in Europe (40 million tons per year) are used in agriculture. They are employed for greenhouses or small tunnels to protect

plants against atmospheric factors and to maintaining an optimal temperature for growth (Bahroun & Belgacen, 2019; http://www.whatischemistry.unina.it/en/agriplast.html). Numerous efforts are being undertaken towards analysis of typical biodegradable polymers as well as classic polymers, their mixtures with biodegradable polymers and their composites (Nair & Laurencin, 2007; Singh & Sharma, 2008; Nampoothiri, 2010; Woodruff & Hutmacher, 2010; Liu et al., 2012; Janczak et al., 2018; 2020; Richert & Dąbrowska, 2021).

Thiabendazole (INN, BAN) (2-(4-Thiazolyl)benzimidazole), also known as thiabendazole (AAN, USAN) or TBZ and the trade names mintezol, Tresaderm, and Arbotect, is a preservative, an antifungal agent, and an antiparasitic agent (Caumes, 2000). Thiabendazole is used primarily to control mold, blight, and other fungal diseases in fruits (e.g. oranges) and vegetables; it is also used as a prophylactic treatment for Dutch elm disease. It is also used in anti-fungal wallboards as a mixture with azoxystrobin. Thiabendazole is also used as a food additive, a preservative with E number E233 (INS number 233). For example, it is applied to bananas to ensure freshness, and is a common ingredient in the waxes applied to the skins of citrus fruits (Loos et al., 2010). Rakotonirainy et al. (1999) shown that a solution of thiabendazole applied 10% by thermal fogging, at a rate of 5 mL/m^3, makes it possible to obtain the effective sanitation of the atmosphere while acting on the spores deposited on surfaces at library.

The aim of this study was to evaluate the possibility of using thiabendazole in polylactide film, produced for the needs of the agricultural and horticultural industry. Thiabendazole acts as a factor limiting the growth of pathogenic bacteria and fungi in agricultural crops. After the process of using tangible film, it would undergo a biodegradation process and is therefore extremely desirable for environmental protection and ecological aspects.

2. Materials and methods

2.1. Preparation of polymer films

The films of PLA type 2003D (Nature Works) were prepared based on the solvent method using the same procedure as (Uchida et al., 2000). 1.5 g of polymer material was weighed and dissolved in 50 ml of chloroform, adding the appropriate amount of thiabendazole (Sigma Aldrich, Poland), to get a final concentration of 0.2, 0.4, 0.6, 0.8, 1.0%; successively marked with symbols: A1, A2, A3, A4, A5. The control was PLA

foil without thiabendazole (A0). Then the foil was poured into a glass laboratory vessel in such a way as to obtain a foil not exceeding 1 mm in thickness. Allow 24 hours for the film to solidify and research work to be undertaken. Designations and composition of individual samples are shown in Table 1.

Table 1. Symbols and composition of individual samples.

Symbol sample	Sample composition	
	PLA (A) [g]	thiabendazole [g]
A	100.0	-
A1	99.8	0.2
A2	99.6	0.4
A3	99.4	0.6
A4	99.2	0.8
A5	99.0	1.0

2.2. Antibacterial properties

Determination of antibacterial activity was carried out based on the standard: ISO 20645 (2006) "Flat textile products. Determination of antibacterial activity. Diffusion method on an agar plate." Two bacterial reference strains were used in the study: *Escherichia coli* (ATCC 8739) and *Staphyloccocus aureus* (ATCC 6538P).

Agar medium containing the composition [g/l]: tryptone peptone - 15, phyton peptone - 5, sodium chloride - 5, agar - agar - 15 was poured onto each petri dish and allowed to gel. The medium was then inoculated with a bacterial culture at a concentration of $1.5x10^8$ cfu/ml (0.5 McFarlanda). Centrally tested samples and control samples in the shape of a circle with a diameter of 25 ± 5 mm (four replicates) were placed on the dishes prepared in this way. Plates were incubated 20 h at 37 ± 1°C. After the end of the incubation time, the presence or absence of zones inhibiting the growth of microorganisms was determined. The width of the braking zone, i.e. the zone without bacteria near the edge of the sample, was calculated using the following formula:

$$H=D-d/2 \quad (1)$$

where:

H - braking zone width [mm];

D - total diameter of the working sample and width of the braking zone [mm];

d - diameter of the working sample [mm].

After measuring the zones of inhibition of growth, the occurrence of the growth of microorganisms in the contact zone between the ground was assessed.
The scale shown in Table 2 was used to assess the potency of antibacterial activity.

The antibacterial properties of the materials obtained were evaluated according to ISO 22196 (2011): Measurement of antibacterial activity on plastic and non-porous surfaces. The analysis was carried out in three repetitions for each of the studied samples. Table 2 described antimicrobial efficacy criteria.

Table 2. Antibacterial effect of antibacterial treatment (ISO 20645, 2006).

Braking zone [mm] The average value of rise	Growth[a)]	Description	Rating
>1	lack	Inhibition zone above 1, no increase [b)]	
1 - 0	lack	Growth inhibition zone up to 1, no growth [b)]	Good effect
0	lack	No braking zone, no increase [c)]	
0	weak	Lack of braking zones, only some colonies limited growth almost completely stopped [d)]	Limited Efficiency
0	average	No braking zone, height reduced to half compared to control [e)]	
0	powerful	Lack of braking zones, the absence of a reduction in growth compared to the control, or only a slight reduction in growth	Insufficient effect

[a)]Bacterial growth on the medium under the working sample.
[b)]The dynamometer range should only be partially taken into account in the calculations. An increase in the braking zone may be due to excess active substance or unevenness of the substance in the article.
[c)]Lack of growth with a simultaneous lack of braking zone can be considered a good effect. A braking zone may not be possible due to limited diffusion.
[d)]"Almost as good as lack of growth" - an indication of limited efficiency.
[e)]Limited bacterial growth density means both the number of colonies and the diameter of the colonies.

Specified amounts of bacterial cells were applied onto control films PLA (A0) and test films (A1, A2, A3, A4, A5). After 0h (for the reference sample) and 24 h for the reference sample and the test samples, bacteria were retrieved from the surface of the films and placed in a neutralising solution. Afterwards, the number of culturable cells was determined by placing them in a PCA medium (Plate Count Agar, Oxoid) used to determine the total bacterial growth of a sample. Incubation of the microorganisms on the plates containing the medium was carried out for 48 hours at 35°C.

Antibacterial activity (R) was determined using the following equation:

$$R = (Ut - Uo) - (At - Uo) = Ut - At \quad (2)$$

where:

R ($\log_{10}$ reduction) – difference between the logarithm of the average cfu number (colony-forming unit) on reference samples after 24 h, and the logarithm of the average cfu on the test samples; R is antibacterial activity.

Uo – $\log_{10}$ average of the number of living bacteria (cells/cm^2) recovered from the reference sample immediately after inoculation (0 h).

Ut – $\log_{10}$ average of the number of living bacteria (cells/cm^2) recovered from the reference sample after 24 h from inoculation.

At – $\log_{10}$ average of the number of living bacteria (cells/cm^2) recovered from the test sample after 24 h from inoculation.

Table 3. Antimicrobial efficacy criteria (ISO 22196, 2011).

Antibacterial activity R, log	Decrease in the number of microorganisms	Antimicrobial efficacy
<1.0	<90.0	poor
1.0 - 2.0	>90.0-99.0	satisfactory
2.0-3.0	>99.0-99.9	good
>3.0	>99.9	very good

2.3. Antifungal properties

The evaluation of the action of microorganisms on film samples was based on the ISO 846 (2002), Plastics standard. Evaluation of the action of microorganisms. Method A of the standard "Mycelial growth test", regarding the determination of plastic resistance to fungi. Standard strains of fungi were used: *Aspergillus niger* (ATCC 6275),

Chaetomium globosum (ATCC 6205), *Gliocladium virens* (ATCC 9645) and *Paecilomyces variotti* (ATCC 18502). The samples were exposed to a suspension (10^6 mixtures of fungal spores) in the presence of defective medium of composition [g/l]: $NaNO_3$ - 2.0, KH_2PO_4 - 0.7, KCl - 0.5, $MgSO_4 x7H_2O$ - 0.5, agar - 20. Fungi can grow only at the expense of the material. If the samples do not contain nutrients, fungal growth cannot occur and, as a result, the plastic properties will not be affected.

According to the standard, a division into 3 batches of samples was used:

— lot 0 – control samples, stored at standardized temperature and relative humidity;

— lot I – samples inoculated with microorganisms and incubated at 24 ± 1°C;

— lot S – unvaccinated samples, stored under the same conditions as lot I.

The visual assessment was carried out in accordance with the scale contained in the standard (Table 4).

Table 4. Assessment of microorganism growth in accordance with ISO 846 (2002).

Increase intensity	Rating
0	No visible growth under the microscope.
1	Growth invisible to the naked eye, but clearly visible under a microscope
2	Growth noticeable by the unaided eye covering up to 25% of the test area.
3	Growth noticeable by the unaided eye covering up to 25% of the test area.
4	Significant increase covering more than 50% of the test area.
5	Intense growth covering the entire test surface.

For visual assessment, photos taken with a SCAN® 500 automatic colony counter (Interscience, France) were used. Material structure analysis was performed using a scanning electron microscope (HITACHI SU 8010, Hitachi High-Technologies Co.). The tests were performed to determine changes in the morphology of the film surface from PLA (A0) and PLA with the addition of thiabendazole (A1, A2, A3, A4, A5) after the incubation of film samples in the presence of microscopic fungi. In order, to achieve the best quality photos, the film samples were previously sprayed with Au / Pd alloy. Pictures taken at a magnification of 1.000 x.

2.4. Plant growth assessment

The interaction of new materials with thiabendazole on plants was analyzed. Phytotoxicity studies were carried out with the use of radish (*Raphanus sativus* var. *sativus*) and wheat (*Triticum aestivum* L.) seeds (Torseed, Poland), which were sown in pots, in triplicate (n=3) on 50 seeds for each test variant. During the growth of plants, it was checked whether there was any chlorosis or necrosis. Plant growth test was undertaken according to OECD 208 (2006) guidelines for the PS samples A0, A1, A2, A3, A4, and A5, using the concentrations 1000 mg of each sample per kg of dry weight soil.

2.5. Water vapor permeability test

Determination of water vapor permeability was carried out in accordance with the standard (ISO 15106-1, 2007), using a L80-5000 type apparatus (PBI Dansensor). This test consists in determining the amount [g] of water vapor permeating a given surface of the sample per unit of time and at a constant temperature.

The samples prepared for measurement were stabilized at 23 ± 2°C and 50 ± 5% relative humidity. Five measurements were made for each sample, and the arithmetic mean of these measurements was taken as the test result. The study was conducted at 38°C.

3. Results and Discussion

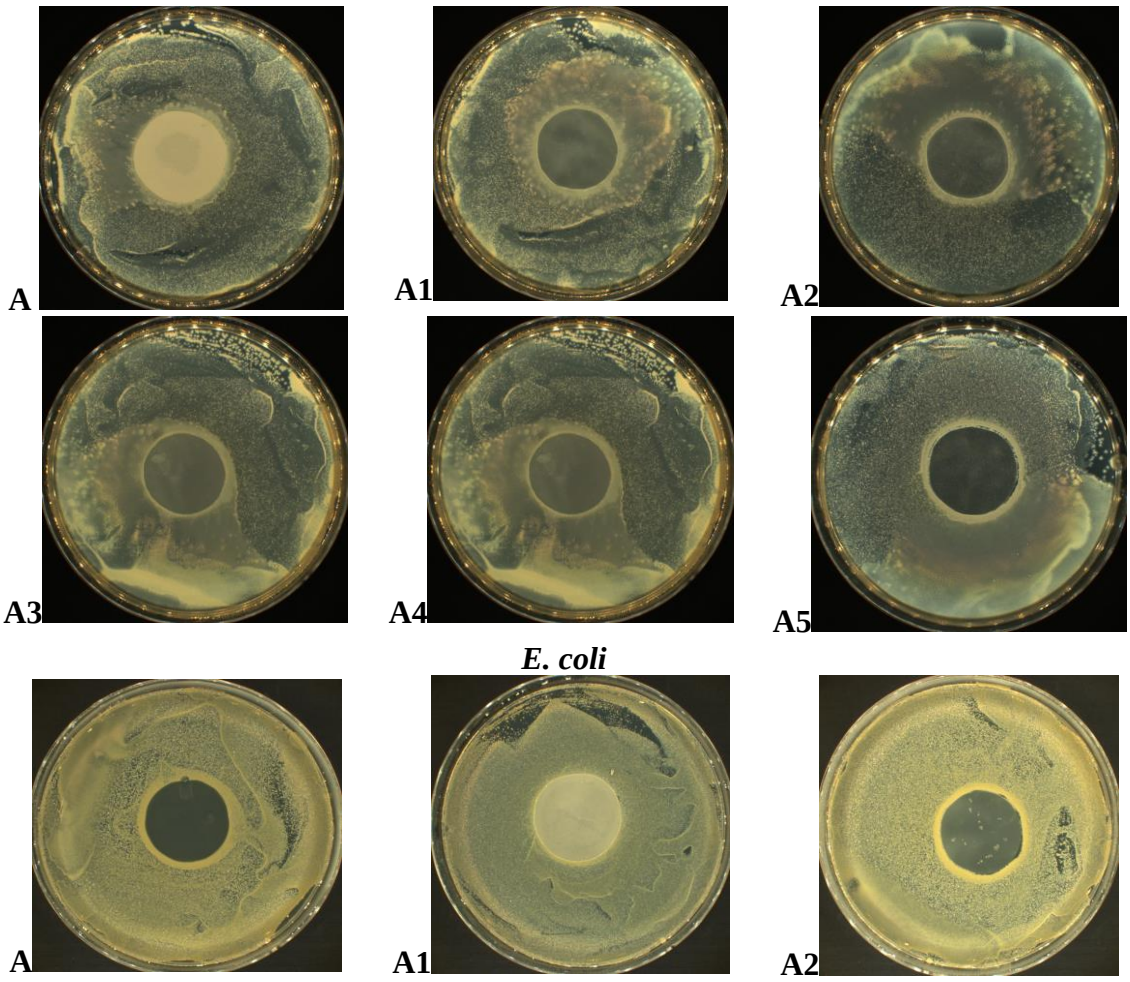
Chemical pesticides and mineral fertilizers are commonly used in plant cultivation. Intensive modern agricultural production requires the use of large amounts of plant protection products. As much as 90% of chemicals are used that may have a negative impact on human health, even after the grace period. FAO (Food and Agriculture Organization of the United Nations) data show that the use of pesticides in Poland and in the world is still not decreasing (Bjørling-Poulsen et al., 2008).

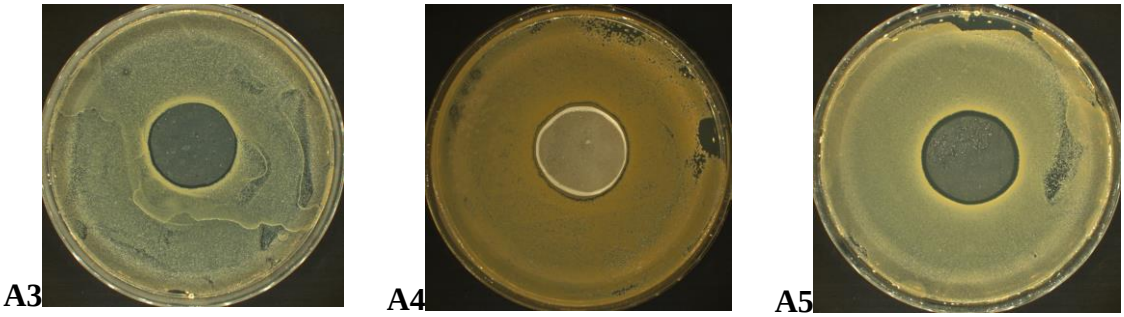
Pesticides are also used in food storage. Humans and animals are almost constantly exposed to contamination by pesticides. Residues of these compounds are present in food products and drinking water (Struciński et al., 2007; http://ec.europa.eu/food/fvo/specialreports/ pesticide_residues/report_2005_en.pdf). It has been confirmed many times that even trace amounts of pesticides have a negative impact on the functioning of the human body (review Grosicka-Maciąg, 2011). Chemical pesticides pose a real threat to the environment, contributing to the reduction of biodiversity in ecosystems. Studies conducted in Poland and other European

countries have shown that insecticides and fungicides are particularly dangerous, as they reduce the biodiversity of plants, insects and some birds. Therefore, in order to restore and protect biodiversity, it is necessary to almost immediately reduce the chemicalisation of agriculture. One way to reduce the use of plant protection products is to use films with biocide properties.

3.1. Antibacterial and antifungal activities

Films with antibacterial and anti-fungal properties are used to limit the spread of pathogens in crops. In our research, we have produced new materials containing thiabendazole in the range of 0.2–1.0%. We have shown that polylactide films with thiabendazole have antibacterial properties. The strongest antibacterial effect is characteristic for films containing thiabendazole in the highest concentrations of 0.6, 0.8, 1.0% (A3–A5, Fig. 1).





S. aureus

Figure 1. The presence or absence of zones of inhibition [mm] strain of *E. coli* and *S. aureus* in the presence of samples A1, A2, A3, A4, A5

Table 5. Antibacterial activity against *E. coli*.

Quantitative Assessment of Activity *E. coli* Concentration of starting inoculum $2.54x10^5$					
Sample Description	No. Bacteria Recovered	Log Value	R	% Reduction	Antimicrobial efficacy
A Control Sample	4.60×10^5	5.7	---	---	----
A1	4.87×10^4	4.7	1.0	89.4	poor
A2	4.16×10^4	4.6	1.1	91.0	satisfactory
A3	4.87×10^1	11.7	4.0	>99.9	very good
A4	$2{,}98x10^1$	1.5	4.2	>99.9	very good
A5	2.56×10^1	1.4	4.3	>99.9	very good

Note: (-) not determined.

Table 6. Antibacterial activity against *S. aureus*.

Quantitative Assessment of Activity *S. aureus* Concentration of atarting inoculum $2.54x10^5$					
Sample Description	No. Bacteria Recovered	Log Value	R	% Reduction	Antimicrobial efficacy
A Control Sample	1.34×10^7	7.1	---	---	---
A1	$2.08 x10^3$	3.3	3.8	>99.9	very good
A2	1.32×10^3	3.1	4.0	>99.9	very good
A3	1.10×10^1	3.0	4.1	>99.9	very good
A4	$2.98 x10^1$	1.5	5.6	>99.9	very good
A5	$<2.00 x10^1$	<1.3	>5.8	>99.9	very good

Note: (-) not determined.

Table 7. Size of the growth inhibition zones [mm] of *E. coli* and *S. aureus*.

Sample ID	Size of bacterial growth inhibition zones [mm]	Bacterial growth on nutrient medium for working sample	Rating
E. coli			
A	0	average	insufficient effect
A1	0	lack	good effect
A2	0	lack	good effect
A3	0	lack	good effect
A4	0	lack	good effect
A5	0	lack	good effect
S. aureus			
A	0	average	insufficient effect
A1	0	lack	good effect
A2	0	lack	good effect
A3	0.5	lack	good effect
A4	0.5	lack	good effect
A5	1	lack	good effect

For these films, the highest zones of growth inhibition were observed for the *S. aureus* strain (Fig. 1). It is known that during the first stage of colonization of polymeric materials by bacteria, a biofilm is created (Richert & Dąbrowska, 2020). The microscopic observations with SEM did not reveal the presence of a biofilm on A3–A5 foils (Fig. 4). Which confirms the antibacterial properties of these films.

Material susceptibility to antifungal is verified with the use of normalized research methods. Each of these techniques consists in submitting the polymeric material to the microorganisms. The growth of the microorganism on the film sample is tested on liquid and a solid surface depending on the experimental technique. Very often, such analyses use medium that does not contain a carbon source (Jayasekara et al., 2005; Richert et al., 2017; Richert et al., 2018).

In the research carried out for the purposes of this study, poor medium was also used to show fungal growth at the expense of the investigated film (Figs 2–3).

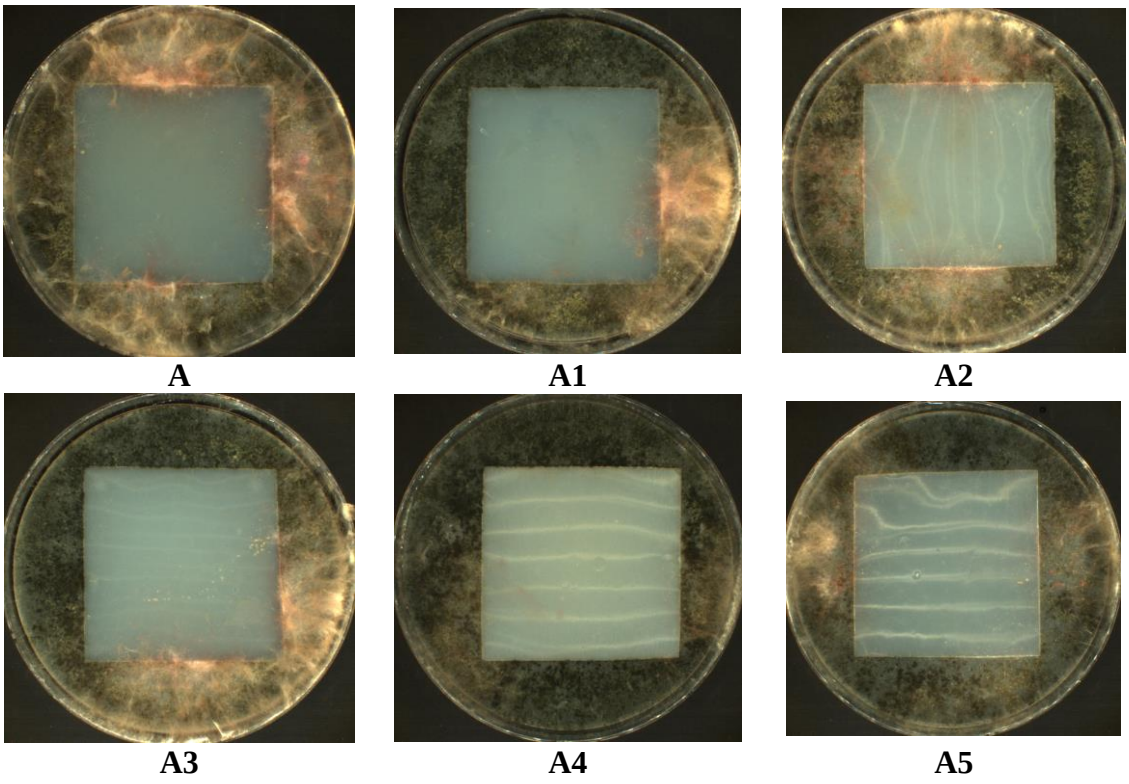


Figure 2. Visual rating of fungal growth observed (batch I), after 28 days

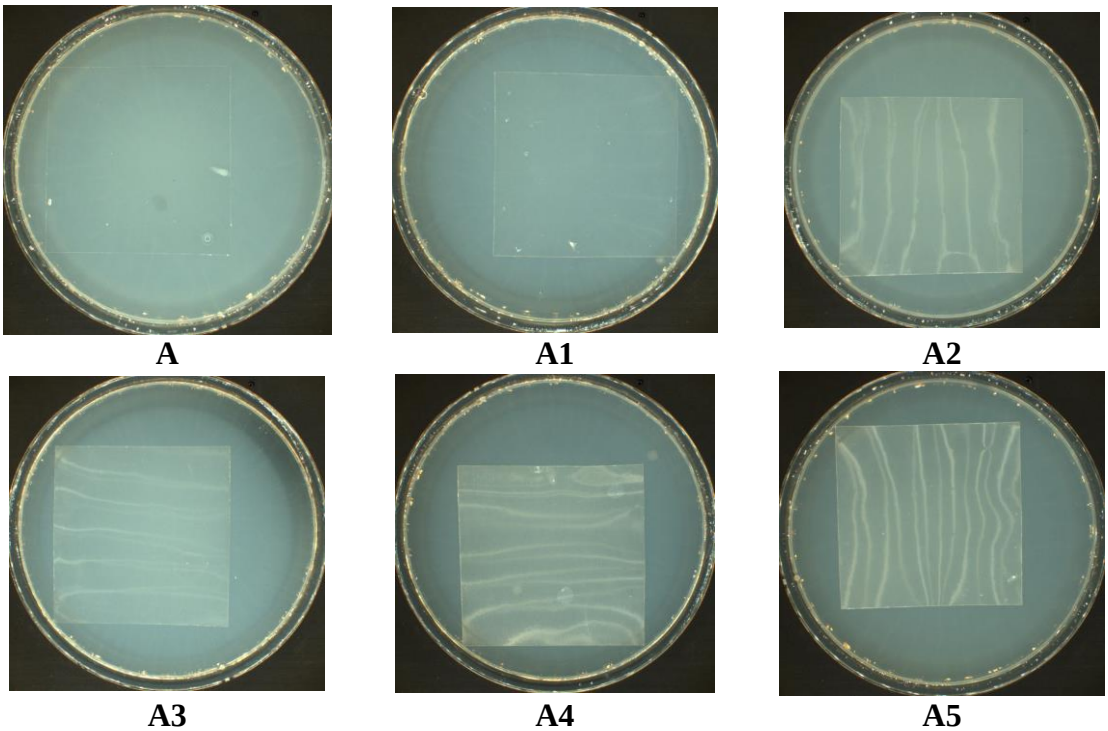


Figure 3. Results of the visual assessment of the batch (S) samples

The development of fungi on polymeric materials can be determined using different techniques, the growth of microorganisms itself depends mostly on experimental

conditions. It should be noted, however, that unification of experimental conditions for different strains can be problematic. According to Nishida and Tokiwa (1993), determination of microorganism quantity is an effective and proper method of assessing the suitability of a strain in polymeric material biodegradation. SEM analysis revealed the presence of mycelial fragments on thiabendazole-free (A) and thiabendazole-free (A1–A3) films. The increase of thiabendazole concentrations in films to the values of 0.8 and 1.0% (A4 and A5 films) resulted in their anti-fungal properties. However, no mycelial fragments were observed on A4 and A5 foils (Fig. 4).

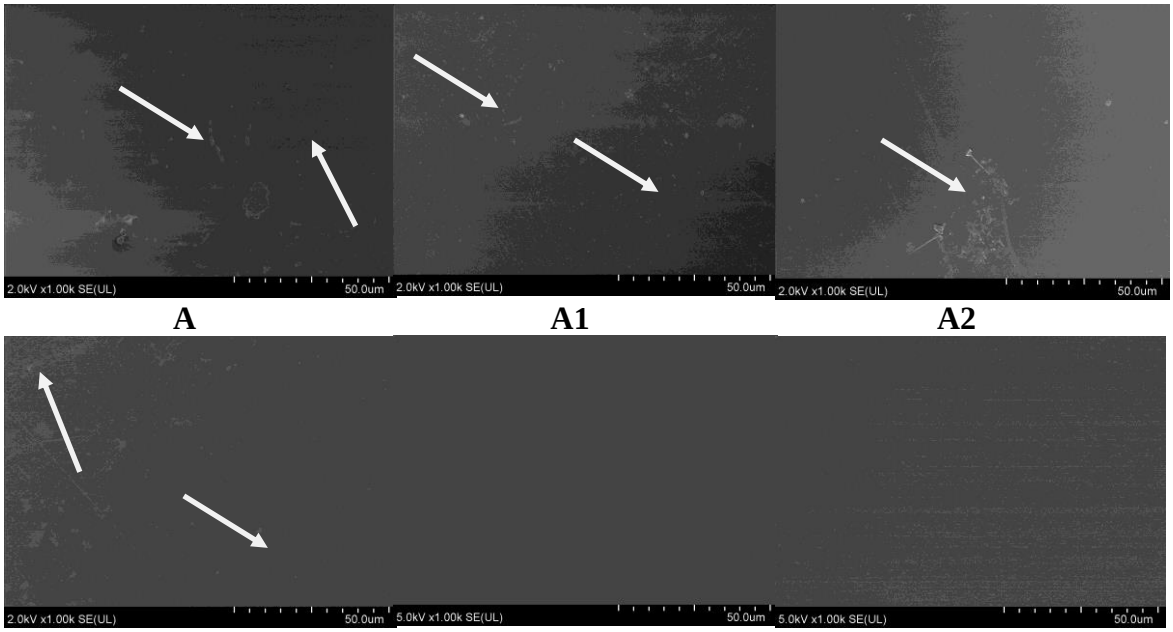


Figure 4. Images of the surface of the film A, A1, A2, A3, A4 and A5 after 28 days of incubation with fungi (magnified 1 000 times) (hyphae of fungi are marked with arrows)

Araceli et al. (2011) demonstrated that, of several fungi studied, *Aspergillus flavus* (ATCC 6051) caused a decrease in polyurethane mass of over 60% after one month of incubation.. Torres et al. (1996) while conducting research on PLA proved that only two out of fourteen strains showed the ability to use PLA lactic acid. The ability of *Trichoderma* sp. and strains of the genus *Bacillus* sp. To grow on PLA was analyzed by Dąbrowska et al. (2021, 2021a). Other authors have shown that both bacteria (*Serratia* sp. and *Arthrobacter* sp.) and fungi (*Laccaria laccatta* and *Clitocybe* sp.) are able to grow and degrade unmodified PLA in compost and cultivated soil (Janczak et al., 2018,

2020) The research by Richert and Dąbrowska (2021) showed that microorganisms of the soil environment degrade PLA faster than microorganisms of aquatic environment.

3.2. Assessment of plant growth

According to EN 13432, it is considered to be tested the substance is non-toxic if the index of sprouted seeds and the total fresh weight of plants do not differ by more than ± 10% from the control sample. The studies showed no negative effect of the analyzed films with thiabendazole (A0-A5) on the growth and development of seeds of monocotyledonous and dicotyledonous plants (Table 8 and 9, Figs 5 and 6).

Table 8. Changes in selected parameters in the *Raphanus sativus* phytotoxicity test.

Sample symbol	Number of plant sown	Number of germinated plants	Plant emergence, %	Yeld fresh weight g/pot	Dry matter yield g/pot	Soil pH
Soil - control	100	95	95	33.67	14.80	7.32
A	100	94	94	26.55	14.67	7.32
A1	100	93	93	30.04	14.97	7.23
A2	100	95	95	28.77	14.13	7.36
A3	100	91	91	30.86	14.54	7.33
A4	100	94	94	34.34	15.41	7.48
A5	100	92	92	32.60	15.60	7.56

Table 9. Changes in selected parameters in *Triticum aestivum* phytotoxicity test.

Sample symbol	Number of plant sown	Number of germinated plants	Plant emergence %	Yeld fresh weight g/pot	Dry matter yield g/pot	Soil pH
Soil - control	100	95	95	21.43	16.21	7.48
A	100	95	95	19.43	16.12	7.46
A1	100	91	91	22.27	15.58	7.34
A2	100	92	92	23.60	16.76	7.27
A3	100	91	91	24.30	16.49	7.40
A4	100	92	92	23.43	16.34	7.37
A5	100	92	92	24.53	16.48	7.47

A A1 A2 A3 A4 A5

Figure 5. Selected digital photographs of plants: *Raphanus sativus* after 28 days of growth

A **A1** **A2** **A3** **A4** **A5**

Figure 6. Selected digital photographs of plants: *Triticum aestivum* after 28 days of growth

In the experiment performed, no visible differences in the appearance of plants were observed, no inhibition of growth and no chloro- and necrotic changes of plants that grew in soil with composites and control material were found. The obtained results regarding poly (L-lactide) coincide with literature reports showing that PLA has no phytotoxic effect (Adamus et al., 2006). On the one hand, due to environmental protection (Richert & Olewnik-Kruszkowska, 2018) it seems appropriate to use biodegradable polymeric materials, and on the other it is also necessary to look for materials that are bacterial or fungicidal. It is important that such materials after complete use are completely safe for the environment and undergo natural biodegradation and are not phytotoxic (Richert et al., 2018; Richert & Olewnik-Kruszkowska, 2018; Tokiwa & Calabia, 2006).

3.3. Water vapor permeability

The results of studies on the effect of thiabendazole on water vapor permeability (Pv) are presented in Figure 7.

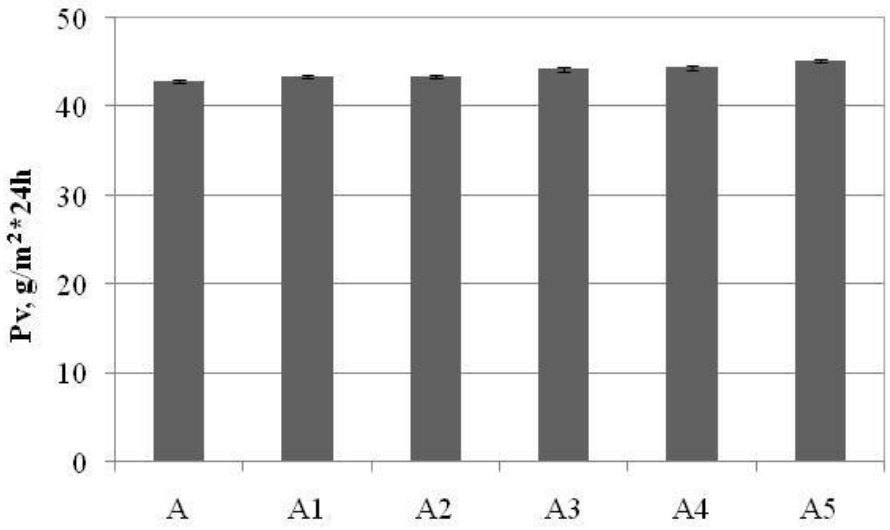


Figure 7. Water vapor permeability

The analysis showed that the content of thiabendazole increased water vapor permeability. For the A1-A5 films, the water vapor permeability increased in the range of 1.3 to 1.8%. For the A1-A5 film, the water vapor permeability also increased, reaching a level 5.3 higher (at a concentration of 1% by mass) than the value of the control film (PLA). In the case of films A1, A2, A3, A4 (0.2, 0.4, 0.6, 0.8), an analogous increase in water vapor permeability was observed along with an increase in thiabendazole concentration by: 1.3, 1.3, 3.5, 5.3%, respectively. This demonstrates the decreasing barrier of the analyzed films, and thus the achievement of the desired parameter in the case of films for agricultural or horticultural use. Our results regarding water vapor permeability coincide with the results obtained by other researchers. Jamshidian et al. (2012) conducted barrier tests of PLA samples with antioxidants in an amount of 1%. They showed that the water vapor permeability was lower for PLA than for other materials. Research has shown that the addition of 1% antioxidants did not cause a negative effect on other properties of PLA. Rhim et al. (2009) studied the water vapor permeability of composite PLA films with nanoclay, report that they contribute to improving barrier properties. The increase of water vapor permeability, and thus the reduction of barrier properties of the tested films is caused by the increased diffusion path of water vapor molecules between the polymer macromolecules, which is desirable in the case of films intended for agriculture and horticulture.

4. Conclusion

The tests have shown that polylactide can be modified by thiabendazole. An additive effectively reduced the survival of *E. coli* and *S. aureus* bacteria and demonstrated antibacterial properties in A1, A2, A3, A4, A5 material. The additive in the form of thiabendazole resulted in changes in water vapor permeability, while the barrier properties of the film decreased with the content of additives in the polylactide matrix. The most advantages of the proposed polylactide films modified with thiabendazole are expected for the purposes in agriculture. These include: (i) reduction of water evaporation (moisture loss) resulting in minimized irrigation frequency; (ii) limiting the development of bacterial and fungal pathogens on grown vegetables and fruits; (iii) weed control – reduction of pesticides amounts in the environment by limitation of excessive amounts of agrochemicals on the treated area; (iv) improvement of micro-conditions of plant's surrounding (well aerated surface of soil, roots have access to adequate oxygen thus elevating the microbial activity); (v) relatively fast disintegration/biodegradability – no wastes after the growing season; (iv) reduction of time and number of multiple agricultural application.

The new materials produced have the above properties and therefore can be used in agriculture and horticulture. Most importantly, they can contribute to the reduction of the use of plant protection products in order to protect human health and the environment.

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