Characterization of biodegradable poly-3-hydroxybutyrate films and pellets loaded with the fungicide tebuconazole

Tatiana Volova1 · Natalia Zhila1 · Olga Vinogradova1 · Anna Shumilova1 · Svetlana Prudnikova1 · Ekaterina Shishatskaya1

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Abstract Biodegradable polymer poly(3-hydroxybutyrate) (P3HB) has been used as a matrix to construct slow-release formulations of the fungicide tebuconazole (TEB). P3HB/TEB systems constructed as films and pellets have been studied using differential scanning calorimetry, X-ray structure analysis, and Fourier transform infrared spectroscopy. TEB release from the experimental formulations has been studied in aqueous and soil laboratory systems. In the soil with known composition of microbial community, polymer was degraded, and TEB release after 35 days reached 60 and 36 % from films and pellets, respectively. That was 1.23 and 1.8 times more than the amount released to the water after 60 days in a sterile aqueous system. Incubation of P3HB/TEB films and pellets in the soil stimulated development of P3HB-degrading microorganisms of the genera Pseudomonas, Stenotrophomonas, Variovorax, and Streptomyces. Experiments with phytopathogenic fungi F. moniliforme and F. solani showed that the experimental P3HB/TEB formulations had antifungal activity comparable with that of free TEB.

Keywords Poly(3-hydroxybutyrate) · Tebuconazole · Slow-release formulations · Controlled release · Antifungal activity

Introduction

Intensive farming involves increased use of various chemicals to protect crops from pests, weeds, and pathogens. However, no more than 10 % of the pesticides applied to crops reach their targets; the major part of these compounds accumulates in biological objects, contaminating the soil and aquatic environments, causing death of useful organisms, and unbalancing natural ecosystems (Hansen 2004).

There is growing concern about the possible dangers of pesticides to humans and the environment, which may include pollution of water bodies, soil, and other biological systems and the presence of residual levels of pesticides in food (Marc et al. 1996; Margni et al. 2002). To reduce the adverse effects of pesticides, modern-day formulations must meet the following requirements: (1) low acute toxicity to humans, useful animals, and other environmental objects; (2) the absence of adverse effects caused by long-term low-dose exposure, including mutagenic, carcinogenic, and teratogenic effects; and (3) short persistence time in the environment (rapid degradation, over one growing season at the most). The formulations must both effectively protect plants from harmful organisms and be cost-effective.

Pathogens such as root rot, powdery mildew, snow mold, leaf blotch, etc. do considerable harm to cultivated plants, causing crop losses. The strategy of plant protection from pathogens involves the following:

– Decreasing the infection stored in the environment (soil, plant waste, containers, storehouses, etc.) to innocuous levels
– Eradicating intermediate hosts (mainly weeds)
– Eradicating plant and seed pests and their carriers (insects, ticks, helminths, and rodents)
Creating favorable conditions for plant growth and seed harvesting and storage

The use of high-grade seed and planting material is a prerequisite for the high yields of agricultural crops. As seeds transmit more than 50% of all diseases, they need to be treated prior to sowing to eliminate the source of infection. Another approach is to apply pre-emergent chemicals that kill infectious agents to soil.

A promising direction of research is development of new-generation slow-release formulations with a targeted and controlled release of the active ingredient owing to the use of special coatings and/or matrices produced from biodegradable materials (Fernández-Pérez et al. 2007). The main condition for constructing this type of preparations is the availability of the material that must have such properties as safety for nature, chemical compatibility with agricultural chemicals, and controlled degradation to nontoxic products in the soil. Among materials that may be used to construct slow-release pesticide formulations are both synthetic and natural materials (Pong et al. 2006; Kumar et al. 2010). Degradable polymers that stand out from the rest are microbial polymers of hydroxy derivatives of alkanoic acids—polyhydroxyalkanoates (PHAs). These polymers are promising materials for agriculture as coatings for seeds, carriers for fertilizers and pesticides, degradable films, containers for greenhouse farming, etc. PHAs do not undergo rapid chemical hydrolysis in aqueous media but decompose via truly biological degradation, an enzymatic depolymerization process, and it takes months for them to be fully degraded (Sudesh et al. 2000; Jendrossek 2001). Such long degradation times of PHAs and their ability to be processed in different phase states make them suitable materials for constructing slow-release formulations that can be applied to soil and used as pre-emergent herbicides.

Research aimed at constructing such PHA-based formulations is still in its initial stages. Our team has described experimental formulations of α-hexachlorocyclohexane and lindane embedded in degradable poly-3-hydroxybutyrate/3-hydroxyvalerate (P3HB/3HV) matrices, showing that chemicals are gradually released into soil, as the polymer is hydrolysed (Vo inova et al. 2009). Prudnikova et al. (2013) reported results of constructing slow-release formulations of the herbicide Zellek Super and showed that the herbicide release rate can be controlled by varying the P3HB/3HV/herbicide ratio in the formulation. The Zellek formulations tested on the creeping bentgrass (Agrostis stolonifera L.) effectively suppressed the growth of that plant. Some authors reported encapsulation of the herbicides atrazine and ametrine in microspheres prepared from P3HB/3HV and encapsulation of the pesticide malathion in microspheres prepared from P3HB blended with polycaprolactone (Suave et al. 2010; Grillo et al. 2011; Lobo et al. 2011). A search of the literature revealed few studies that reported the use of PHAs to construct slow-release fungicide formulations. Savenkova et al. (2002) described P3HB films loaded with fungicides. Development of slow-release fungicide formulations is a relatively new branch of research, and data about the potential and effectiveness of such formulations are limited. There are published data on the use of fungicides encapsulated in polystyrene/maleic anhydride (Asrar et al. 2004), calcium carbonate (Qian et al. 2011), chitosan (Brunel et al. 2013), ethyl cellulose (Yang et al. 2014), and urea–formaldehyde (Zhang et al. 2015).

The purpose of this study was to construct and investigate slow-release formulations of the fungicide tebuconazole (TEB) embedded in films and pellets made from P3HB—a degradable polymer.

Materials and methods

Polymer characterization

P3HB was used as a degradable polymeric matrix for embedding the fungicide (Fig. 1). The polymer was synthesized by using bacterium Cupriavidus eutrophus B10646, at the Institute of Biophysics SB RAS. Cells were batch-cultured in a 7.5-L BioFlo/CelliGen115 (New Brunswic, USA) fermentor under strictly aseptic conditions, following the previously developed technology (Volova et al. 2013, 2014). Cells were grown in Schlegel’s mineral medium: Na2HPO4·H2O—9.1; KH2PO4—1.5; MgSO4·H2O—0.2; Fe3C6H5O7·7H2O—0.025; and CH4N2O—1.0 (g/L); the medium was supplemented with glucose in amounts corresponding to cell concentration in the medium. Due to the use of urea, no pH adjustment was needed. The pH level of the culture medium was stabilized at 7.0±0.1. A solution of iron citrate (5 g/L) was added to reach a concentration of 5 ml/L. Hoagland’s trace element solution was used: 3 ml of standard solution per 1 L of the medium. The standard solution contains H3BO3—0.288; CoCl2·6H2O—0.030; CuSO4·5H2O—0.08; MnCl2·4H2O—0.008; ZnSO4·7H2O—0.176; NaMoO4·2H2O—0.050; and NiCl2—0.008 (g/L). The air was pumped through the culture medium using a system of microbiological filters, with an air pump. When cell concentration reached 2.0–2.5 g/L, nitrogen (60 mg/g biomass) and glucose solutions were fed to the culture medium in two separate flows. Substrate feed rates were
regulated with a peristaltic pump. Nitrogen concentration in the culture medium was maintained at trace levels, and glucose concentration did not exceed 10 g/L. Nitrogen supply was stopped after 20–25 h, and the second phase was started; it lasted for 20–55 h, until P3HB reached 83±2 %. Polymer was extracted from cells with chloroform, and the extracts were precipitated using hexane. The extracted polymers were re-dissolved and precipitated again three to four times to prepare homogeneous specimens. P3HB had the following physicochemical parameters: weight average molecular weight (Mw) 920 kDa; polydispersity (Ð) 2.52; degree of crystallinity 74 %; and melting point and thermal decomposition temperature 179.1 and 284.3 °C, respectively.

**Fungicide**

Raxil Ultra (Bayer CropScience, Russia) is a systemic fungicide based on the active ingredient TEB, which has a broad spectrum of activity. It provides effective protection against various diseases in cereals and helianthus. This fungicide disinfects seeds and partially decontaminates soil and plant residues around the seeds. Its chemical formula is C16H22ClN3O, and its structural formula is shown in Fig. 1. The IUPAC name is (RS)-1-(4-Chlorophenyl)-4,4-dimethyl-3-(1H, 1,2,4-triazol-1-ethanol; molar mass (g/mol) 307.82; solubility in water 36 mg/L at 20 °C; and melting point is 105 °C. The time of degradation in soil is 177 days.

**Preparation and examination of P3HB/TEB polymeric mixtures**

Polymer/TEB mixtures were prepared by using purified P3HB and chemically pure TEB (Russian Federal Standard GSO 7669–99, purity 99.1 %). A polymer sample was dissolved in chloroform, and a solution of TEB in chloroform was added to the polymer solution. We used 2 % (w/v) polymer solutions in chloroform. The viscosity of the solution at 25 °C was 20.02 cP. The viscosity of the solutions was measured by using a HAAKE Höppler Falling Ball Viscometer C (Thermo Scientific, Germany). The P3HB/TEB solution was mixed on an MR Hei-Standart magnetic stirrer (Heidolph, Germany) for 3–4 h (until completely dissolved) and heated to 35–40 °C under a reflux condenser. The powder system was prepared as follows: the polymer was ground in a ZM 200 Ultra Centrifugal Mill (Retsch, Germany). The fractional composition of the polymeric powder was determined by using an AS 200 control analytical sieve shaker (Retsch, Germany): the fraction of the particles under 0.50 mm comprised 65 %, and the fraction of the particles between 0.80 and 1.00 mm constituted 45 %. The powdered polymer and TEB were mixed mechanically. Samples of the two powders of different fraction compositions (0.10 to 1.0 mm) were weighed on the analytical balance, homogenized with a laboratory stirrer for 2 min, and mixed with a TEB sample.

**Preparation of slow-release TEB formulations**

The polymeric systems (solutions and powders) were used to construct TEB-loaded films and pellets. The P3HB matrix was loaded with 25 % (w/w) TEB. Films loaded with the fungicide were prepared as follows: the P3HB/TEB solution was cast in Teflon-coated metal molds, and then solvent evaporation occurred. The films were left to stay at room temperature in a laminar flow cabinet for 24 h, and then they were placed into a vacuum drying cabinet (Labconco, USA) for 3–4 days, until complete solvent evaporation took place. Films were cut into disks of 10 and 20 cm diameter and 5×5 mm², which were weighed on the analytical balance of accuracy class 1 Discovery (Ohaus, Switzerland). The film thickness (0.080±0.005 mm) was measured with a digital micrometer (LEGIORER EDM-25-0.001, Germany). The pellets were prepared from the mixture of P3HB and TEB powders by cold-pressing, using a Carver AutoPellet 3887 press (Carver, USA) under pressing force of 8000. Pellets were 5 and 13 mm in diameter and were loaded with 25 % (w/w) active ingredient.

**Physicochemical analysis of P3HB/TEB formulations**

Thermal analysis was performed with a differential scanning calorimeter (DSC-1, Mettler Toledo, Switzerland). Films or powders (4.0±0.2 mg) were placed in aluminum crucibles and heated at 5 °C per minute. The melting point (Tm) and thermal decomposition temperature (Td) were determined from exothermic peaks on thermograms, using the STARe software. Infrared spectra of the polymer and TEB were taken with a Fourier transform infrared spectrometer (Lumex, Russia). X-ray structure analysis was performed using an X-ray spectrometer (D8 Advance, Bruker Corporation, Bremen, Germany) (graphite monochromator on a reflected beam) in a scan-step mode, with a 0.04 °C step and exposure time of 2 s, to measure intensity at point. The instrument was operating at 40 kV×40 μA.

Molecular weight and molecular weight distribution of the copolymer were examined with a gel permeation chromatograph (Agilent Technologies 1260 Infinity, USA) with a refractive index detector, using an Agilent PLgel Mixed-C column. Chloroform was the eluent, at a flow rate of 1.0 ml/min at 40 °C. Typical sample volumes were 50 μl at a polymer concentration of 2 mg/ml. Narrow polydispersity polystyrene standards (Agilent, USA) were used to generate a universal calibration curve, from which molecular weights (weight average, Mn, and number average, Mn) and polydispersity (Ð=...
Mₘ₀/Mₙ) were determined. The measurement accuracy was 2%.

TEB analysis

TEB is a volatile and thermostable compound; it was detected with gas chromatography. Plotting of calibration curves was based on Russian Federal Standard GSO 7669–99 (purity 99.1%). Measurements were done on a chromatograph mass spectrometer (7890/5975C, Agilent Technologies, USA). The chromatography conditions were as follows: an HP-5MS capillary column, 30 m long and 0.25 mm in diameter; carrier gas–helium rate 1.2 ml/min; sample introduction temperature 280 °C; initial temperature of chromatography 150 °C; temperature rise to 310 °C at 10 °C per min; 5 min isothermal conditions; transfer line temperature 230 °C, electron impact mode at 70 eV, and fragment scan from 50 to 550 m/z with a 0.5-s cycle time. The ion chromatogram and mass spectra of the components are shown in Fig. 2. Calibration curves were prepared by using two modes (“split” and “splitless”) and samples with TEB concentrations ranging between 1.0 ng/µl and 1.4 µg/µl. One microliter of each TEB sample was introduced into the chromatograph. At least three parallel measurements were performed, and the average area of the chromatographic peak was determined for each concentration. We plotted calibration curves of the relationship between the area of the chromatographic peak (calculated automatically, in relative units) and TEB concentration in the sample, in micrograms per microliter. The detection limit of the MS detector for TEB was 0.1 ng/µl; the standard error of the method was 1.0 %.

Release kinetics

Release kinetics of TEB from the polymeric matrices was studied in vitro in the aqueous and soil laboratory systems. In the first case, the films and pellets were sterilized and placed into 500-ml sterile conical flasks filled with sterile distilled water (100 ml). The number of films or pellets in a flask was determined in such a way that the samples in each flask contained equal total amounts of the active ingredient (50 mg). The flasks were incubated at 25 ºC in an Innova 44 New Brunswick temperature-controlled incubator shaker at 150 rpm. Samples for analysis were collected periodically, under aseptic conditions, and an aliquot of water was added to the flask to maintain a constant volume of liquid in it. TEB was extracted with chloroform three times to determine its concentration. The chloroform extracts were passed through sodium sulfate. Chloroform was removed in a rotary vacuum evaporator. The amount of TEB released (RT) was determined as percentage of the TEB encapsulated in the polymer matrix, using the following formula:

$$RT = \left( \frac{r}{EA} \right) \times 100\%,$$

where EA is the encapsulated amount, in milligrams, and r is the amount released, in milligrams.

In the soil system, vegetable garden soil (200 g) was placed in 250-mm³ containers (three replicates for each experimental point), and samples of formulations, which had been weighed in the same way as in the experiment with the aqueous system, were introduced into the soil. We used agrogenically transformed soil (the Krasnoyarsk Territory, the village of Subbotino). The containers were incubated in a temperature-controlled cabinet at a constant temperature of 21±0.1 °C and soil moisture content of 50 %. To monitor degradation of the polymeric matrix, the samples were regularly removed from the soil, thoroughly rinsed in water, and dried to constant weight (samples were weighed on the analytical balance of accuracy class 1 Discovery (Ohaus, Switzerland)). The samples were methanolized, and their polymer content was determined on the gas chromatograph equipped with a GCD plus mass spectrometric detector (Hewlett-Packard, USA). TEB was extracted from the soil with chloroform and purified, and then its concentration in the soil was determined by gas chromatography.

A microbiological study

The initial soil microbial communities and the microbial communities after 35 days of incubation of TEB-loaded films and pellets in soil were examined by plating soil samples on solid medium. Quantitative enumeration of microorganisms was done using the methods of soil microbiology by plating serial dilutions on nutrient medium. The number of ammonifying copiotrophic bacteria (CFU/g soil) was determined on fish-peptone agar (FPA); the number of mineral nitrogen-assimilating prototrophs was determined on starch and ammonia agar (SAA); nitrogen-fixing bacteria were counted on Ashby’s medium; and oligotrophs were counted on soil extract agar (SA) (Netrusov et al. 2005). Mineralization coefficient was determined as a ratio between microorganisms.
assimilating mineral nitrogen and ammonifying bacteria. Oligotrophy coefficient was determined as a ratio of oligotrophic to ammonifying bacteria. Concentrations of microorganisms (CFU/g soil) were calculated for the control soil and the soil layer on the surface of the specimens. All platings were performed in triplicate from 10^7 dilutions of soil suspension. The plates were incubated for 3–7 days at 30 °C. The number of microorganisms was determined taking into account the dilutions. Dominant microorganisms were isolated and identified by conventional methods, based on their cultural and morphological properties and using standard biochemical tests mentioned in identification keys (Holt et al. 1997; Boone et al. 2005).

Antifungal activity analysis

Fungi of the genus *Fusarium* (*F. moniliforme* and *F. solani*) were extracted from the field soil and grown on the malt extract agar (MEA, Sigma-Aldrich, USA) in Petri dishes at a temperature of 25 °C for 5–7 days. Then, 5-mm diameter slabs of agar were aseptically drilled from the culture regions with actively growing colonies. A slab with the fungal culture and a film or pellet with encapsulated fungicide were placed at opposite sides of the Petri dish containing sterile MEA. The mass of the film or pellet in a Petri dish was 100 mg, and the TEB/P3HB ratio was 1:3. The dishes were incubated in the temperature-controlled cabinet at 25 °C for 72 h; then, we measured the radius of the fungal mycelium and determined the degree of fungus growth inhibition relative to the control. As a negative control, we measured the radius of the fungal mycelium in the dish without the fungicide. As a positive control, we used 2 ml of the commercial TEB formulation (Raxil Ultra) placed in 10-mm diameter wells; this amount was equivalent to the amounts of TEB in films and pellets. All procedures were done in triplicate.

Statistical analysis

Statistical analysis of results was performed using the standard software package of Microsoft Excel, STATISTICA 8. Arithmetic means and standard deviations were determined using Student’s t test. Results are given as X±m.

Results

Characterization of P3HB/TEB formulations

P3HB/TEB solutions and powders were used to produce 0.080±0.005-mm thick 200-mg films and 200-mg pellets, which are shown in Fig. 3. Physicochemical properties of initial components and P3HB/TEB listed in Table 1 and shown in Figs. 4 and 5 suggest that loading of the polymer matrices with the fungicide influenced the degree of crystallinity (C_x) and temperature properties of the polymer systems.

DSC was used as one of the most informative methods for determining the thermal properties of polymers and polymer blends because the melting behaviors of components of the blend can be used to determine the degree of miscibility and interactions of the components. Thermograms were taken within a wide temperature range, including the polymer melting point and thermal decomposition temperature (Fig. 4).

Analysis of the number and shapes of endothermic peaks on the thermograms showed the formation of a stable mixture of the polymer and TEB, which was not separated under heating, as the thermogram had only one peak of melting and one peak of decomposition. The thermogram of the mixture had neither a melting peak nor a thermal decomposition peak of TEB, which is 104.7 and 296–304 °C, respectively. Embedding of TEB in P3HB decreased its melting temperature from 180 to 164 °C, i.e., by 16 °C. The T_m decrease suggests an increase in the viscosity of the melts and thus inhibition of polymer crystallization. In consequence, small crystals may form in the polymer, and these crystals may begin to melt at a lower temperature than the initial polymer, thus decreasing the melting temperature of the mixture and making the structure of the polymer more amorphous. This assumption was also supported by a decrease in the enthalpy of melting (Table 1): the peaks were somewhat smeared (Fig. 4), which is typical of melting of amorphous regions. X-ray structure analysis showed that the loading of P3HB with TEB increased the degree of crystallinity of the polymer from the initial 74 to 80 %.

FTIR analyses show that the most informative range of the wavenumbers was that between 1450 and 1700/cm. Pure P3HB has no absorption peaks within this range. The only absorption peaks (bands) observed in the P3HB/TEB system were those associated with the specific structural groups of TEB (Fig. 5). No groups that would form due to chemical interaction between P3HB and the fungicide were detected. Peaks of the existing groups became higher, indicating that the polymer/TEB system was a mechanical mixture of P3HB and TEB.

Thus, results of DSC, X-ray, and FTIR suggest that there were no chemical bonds between TEB and the polymer and that the system was a mechanical mixture of components. The decrease in the temperature and enthalpy of melting suggests that the active ingredients of the chemicals behave as fillers of the polymer matrix.

Release kinetics of TEB from P3HB/TEB films and pellets

TEB release from polymer films and pellets was studied in aqueous and soil laboratory systems. Figure 6 shows profiles of fungicide release to water from the films and pellets under
aseptic conditions, with no P3HB-degrading microorganisms present in the medium.

The water-soluble TEB was released at a higher rate from the films than from the pellets. After 60 days of incubation of the samples in the water, 46 % of the fungicide was released from the films while only 20 % leaked from the pellets.

In the soil, the TEB release rate was higher (Fig. 7). As the polymer incubated in the soil was degraded by microorganisms and this process influenced the fungicide release kinetics, we needed to study the structure of soil microbial community in the initial soil and monitor it during the experiment. The soil that we used in our experiments was rich in humus (17.4 %) and contained high concentrations of nitrate nitrogen N-NO₃ at 122.0 mg/kg, available phosphorus P₂O₅ 151.2 mg/100 g, and potassium K₂O 80 mg/100 g of soil. The pH level was close to neutral (6.6). The total number of CFU was 96.5 million in 1 g of soil. The percentage of copiotrophic bacteria was very high (over 60 %). Together with the high concentrations of biogenic elements (N, P, K), this is indicative of intense transformations of organic matter in the soil. Low mineralization and oligotrophy coefficients (0.07 and 0.46, respectively) confirm this conclusion. The presence of available nitrogen in the soil was responsible for the low numbers of nitrogen-fixing bacteria (3.5 million CFU in 1 g of soil). As shown in Fig. 8a, initial soil microbial community was dominated by four genera of bacteria—Bacillus, Micrococcus, Corynebacterium, and Pseudomonas—which comprised 43, 16, 12, and 8 %, respectively. Minor genera were Arthrobacter, Cellulomonas, Curtobacterium, Mycobacterium, and Streptomyces (between 2 and 7 %). Thus, soil conditions were favorable for intense transformation of organic matter, including the polymer, which is a substrate for many soil microorganisms (Jendrossek and Handrick 2002).

In the soil system, TEB was released from the polymer matrix at a higher rate than in the water (Fig. 7). For a considerably shorter period (35 days), about 60 % of the loaded fungicide was released to soil from the films and 36 % from the pellets, or 1.23 and 1.80 times, respectively, and more TEB was released to the soil over 35 days than to the water over 60 days.

TEB release occurred simultaneously with destruction of the polymer matrix. For the first 14 days of incubation, the mass of the films and pellets had decreased by 13 and 30 %, respectively. Then, P3HB degradation rate increased, and after 35 days, P3HB films were almost 60 % degraded, while pellet degradation reached only 40 %. The changes in the state of the samples are shown in Fig. 7. As films and pellets were degraded and their mass decreased, their surface became uneven; we observed formation of small pores and cracks (Fig. 3). The number and size of defects grew during the experiment. The number or perforations increased and, finally, the films broke up. The pellets retained their mass and shape for longer periods of time, i.e., were less destructible.

After the P3HB/TEB samples had been incubated in soil for 35 days, the composition of the soil microbial community changed as follows. The total number of CFU in 1 g of soil did not decrease dramatically, reaching 88.7 million. However, the proportions of the trophic groups of microorganisms and their composition changed relative to those of the initial microbial community. The proportion of copiotrophic bacteria

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<th>Table 1 Physicochemical properties of P3HB, TEB, and P3HB/TEB</th>
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ᵃ andᵇ show the presence of two peaks in molecular weight measurements
decreased, while the proportions of prototrophic and nitrogen-fixing bacteria increased. These results are indicative of high rates of organic matter mineralization and polymer biodegradation; degradation products were used by soil microflora as supplementary substrate. Rapid degradation of organics and assimilation of this substrate by bacteria in the soil containing high levels of nitrogen-containing organic matter caused stabilization of the numbers of organotrophic bacteria and an increase in the proportions of prototrophic and nitrogen-fixing bacteria. The composition of the major microbial species changed, too (Fig. 8b). The counts of spore-forming rods dropped dramatically, by 20 %, and the counts of Gram-positive cocci decreased by 12 %. The number of *Pseudomonas* and *Corynebacterium* increased by 11 and 12 %, respectively. The total counts of Gram-negative rods and actinobacteria increased; among them were representatives of *Pseudomonas, Stenotrophomonas, Variovorax,* and *Streptomyces,* which, as we have found before, degrade PHAs very effectively (Volova et al. 2007, 2010; Boyandin et al. 2012, 2013).

**Discussion**

In this study, we investigated formulations of the fungicide TEB embedded in the polymer matrix—films and pellets made from the natural degradable P3HB.

Research aimed at construction and use of new-generation slow-release targeted formulations of pesticides embedded in carriers made from degradable polymers, which are intended to protect cultivated plants from pests, weeds, and pathogens, follows the growing environmental and social concern over uncontrolled distribution and accumulation of chemical pesticides in the biosphere. Construction and testing of slow-release targeted formulations of fungicides is a relatively new line of research. Therefore, it is important to find and test new materials for microencapsulation of these chemicals.

Analysis of the available literature revealed a few studies on this subject. Qian et al. (2011) reported the use of nano-sized calcium carbonate as a carrier for validamycin and showed their durable effect against *Rhizoctonia solani.* Brunel et al. (2013) suggested using copper-loaded nanogels to inhibit fungal growth. The authors noticed a strong synergistic effect between chitosan and copper, whose release from the gel was triggered by a plant pathogen. The use of this formulation inhibited *Fusarium graminearum* growth.

Few studies have investigated the use of PHAs for constructing slow-release fungicide formulations. Savenkova et al. (2002) described film systems made from P3HB and loaded with fungicides Sumilex (with dicyclidine as the active ingredient) and Ronilan (with vinclozolin as the active ingredient). The films were incubated in the vegetable garden soil infected by pathogenic fungus *Botrytis cinerea.* The authors reported that after 4 weeks, the films were almost destroyed and the number of pathogenic fungi in the soil was considerably reduced. In the present study, we investigated degradation of the polymeric matrix and release kinetics of TEB loaded into P3HB films and pellets in a model soil microecosystem; we also studied changes in the soil microbial community. The polymeric matrix of the films and pellets incubated in soil at a temperature of 20 °C and moisture...
Fig. 5 Infrared-spectra of TEB (a), P3HB (b), and P3HB/TEB (c); fragments of spectra with higher wave number and absorption resolution.
content of 50 % for 35 days degraded up to the degree of 60 and 40 %, respectively. In the study of the composition of soil microbial community, we identified ecological trophic groups and major microorganisms and described changes occurring in the structure of the microbial component of the soil during decomposition of polymeric TEB formulations. While the total number of microorganisms remained rather stable, the structure of the community changed, as the percentages of prototrophic and nitrogen-fixing bacteria increased. The percent of Gram-negative bacteria of the genera *Pseudomonas*, *Stenotrophomonas*, and *Variovorax* increased, too, and they are the major PHA degraders, as we have shown previously (Boyandin et al. 2012, 2013). A similar favorable effect of incubating P3HB as a carrier of chemicals in soil on the number of microorganisms, including P3HB degraders, was described by other authors (Volova et al. 2008; Savenkova et al. 2002). That is, degradation of the polymer induces growth of soil microflora, which is supplied with additional carbon substrate. Our team studied PHA degradation in the Siberian and tropical soils as related to the weather conditions and the structures of microbial communities. We have found that the PHAs favor the development of microflora. Based on the cultural, morphological, and physiological characteristics and results of analysis of nucleotide sequences of the 16S and 28S rRNA genes, we identified the major PHA-degrading microorganisms. Species representing *Bacillus*, *Paecilomyces*, and *Penicillium* effectively degraded PHAs in all study regions; other microorganisms were specific for their natural ecosystem. The major PHA degraders in Siberian soils were bacteria representing *Variovorax*, *Stenotrophomonas*, *Acinetobacter*, *Pseudomonas*, *Bacillus*, and *Xanthomonas* and micromycetes *Penicillium*, *Paecilomyces*, *Acremonium*, *Verticillium*, and *Zygosporium*. In tropical soils, the major bacteria were *Burkholderia*, *Bacillus*, *Cupriavidus*, *Streptomyces*, *Nocardiosis*, and *Mycobacterium* and micromycetes *Gongronella*, *Penicillium*, *Acremonium*, *Paecilomyces*, and *Trichoderma* (Prudnikova and Volova 2012; Volova et al. 2007, 2010; Boyandin et al. 2012, 2013). In this study, while investigating degradation of TEB-loaded P3HB films and pellets, we observed enhanced growth of such PHA degrades as *Pseudomonas*, *Stenotrophomonas*, *Variovorax*, and *Streptomyces*.

TEB is an effective modern fungicide containing triazole components, which have a wide range of fungicidal activity and regulate plant growth (Hedden et al. 1989; Child et al. 1993). A number of researchers have reported construction of new formulations of this fungicide. Asrar et al. (2004) described microparticles prepared from poly(methyl methacrylate) and poly(styrene-co-maleic anhydride) and loaded with TEB (Asrar et al. 2004). This formulation effectively controlled wheat rust *Puccinia recondita*. Khalikov et al. (2013) mixed TEB and water-soluble polymers, using a mechano-physical technique, to enhance the effectiveness and reduce the toxicity of TEB. To prepare a nanodispersive powder formulation and improve solubility of the fungicide in water, TEB was mixed with water-soluble polymers (arabinogalactan (Ag), pectin (P), cyclodextrin (CD), polyvinylpyrrolidone (PVP), and
hydroxyethyl starch (HES)) in a planetary-type mill. Formulations with Ag and HES showed higher fungicidal activity against root rot agents (*Biopolaris* and *Fusarium*) than the commercial formulation Raxil (Bayer CropScience); water solubility did not influence the effectiveness of the formulations. Free TEB may adversely affect seeds and the development of the root system of plants, causing biomass loss. By contrast, as reported by Yang et al. (2014), encapsulated TEB not only suppresses the infection but also may have a favorable effect on photosynthesis pigment content and productivity of maize. Zhang et al. (2015) described a monodisperse microencapsulation system for prolonged and effective control of drug administration. Cyanobacteria cells served as a natural environmentally friendly wall material to encapsulate the fungicide TEB, and then urea–formaldehyde (UF) resins were automatically coated on it via electrostatic interactions. By this means, monodisperse TEB–PCC@UF microcapsules were achieved, which could not only effectively control the drug release rate but also depress the initial “burst effect” to some degree. A bioactivity experiment showed that TEB–PCC@UF microcapsules authentically prolonged the antifungal effects and were very efficacious in controlling wheat powdery mildew compared with the commercial formulation.

In this study, we investigated release kinetics of TEB embedded in the matrix prepared from the biodegradable P3HB in the aqueous and soil laboratory systems. As the polymer was degraded, TEB was gradually released, and after 35 days of incubation of P3HB/TEB systems in soil, the amount of TEB released from films and pellets reached 60 and 36 %, respectively. That was 1.23 and 1.80 times greater that the amounts released to water after a longer incubation period (60 days); i.e., in the soil, where polymeric matrix was degraded, the fungicide was released at a higher rate. Furthermore, the gradual and relatively slow TEB release and the influence of the shape of the formulation on this process suggest the possibility of controlling fungicide release and its amounts in soil. Experiments with test organisms, *F. moniliforme* and *F. solani*, showed that the antifungal activity of TEB encapsulated in P3HB pellets is comparable with the antifungal activity of free TEB. Thus, experimental formulations of TEB embedded in the slowly degrading P3HB matrix hold promise for constructing slow-release formulations of this fungicide.

**Conclusions**

Experimental slow-release formulations of the fungicide TEB were constructed by using P3HB as a degradable polymeric matrix. The P3HB/TEB films and pellets were prepared from polymer solutions and powder and examined by DSC, X-ray

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**Fig. 8** Major bacteria in the soil before (*A*) and after incubation of P3HB/TEB (*B*)

**Fig. 9** Sensitivity of *Fusarium* species to different forms of TEB; *a* negative control, *b* positive control, *c* TEB in pellets, *d* TEB in films

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*Fusarium moniliforme*  *Fusarium solani*
structure analysis, and Fourier transform infrared spectroscopy, which showed that the P3HB/TEB system was a mechanical mixture of the components. TEB release was studied in the aqueous and soil systems. The soil microbial community was characterized, and polymer matrix degradation was monitored in experiments with TEB formulations; the TEB release kinetics and polymer degradation rate were found to be influenced by the geometry of the P3HB/TEB system. Experiments with the cultures of fungi *F. moniliforme* and *F. solani* showed that the polymer-embedded TEB had antifungal activity comparable with that of free TEB. The formulations of TEB embedded in the slowly degrading P3HB matrix constructed in this study hold promise for the development of slow-release fungicide formulations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References


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