Research Article

Received: 1 February 2016 Revised: 4 July 2016 Accepted article published: 22 July 2016 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.4367

Efficacy of tebuconazole embedded in biodegradable poly-3-hydroxybutyrate to inhibit the development of *Fusarium moniliforme* in soil microecosystems

Tatiana G Volova,[a*] Svetlana V Prudnikova,[a,b] Natalia O Zhila,[a] Olga N Vinogradova,[a] Anna A Shumilova,[a] Elena D Nikolaeva,[a] Evgeniy G Kiselev[a] and Ekaterina I Shishatskaya[a]

Abstract

BACKGROUND: An important line of research is the development of a new generation of formulations with targeted and controlled release of the pesticide, using matrices made from biodegradable materials. In this study, slow-release formulations of the fungicide tebuconazole (TEB) have been prepared by embedding it into the matrix of poly-3-hydroxybutyrate (P3HB) in the form of films, microgranules and pellets.

RESULTS: The average rates of P3HB degradation were determined by the geometry of the formulation, reaching, for 63 days, 0.095–0.116, 0.081–0.083 and 0.030–0.055 mg day^{-1} for films, microgranules and pellets respectively. The fungicidal activity of P3HB/TEB against the plant pathogen *Fusarium moniliforme* was compared with that of the commercial formulation Raxil Ultra. A pronounced fungicidal effect of the experimental P3HB/TEB formulations was observed in 2–4 weeks after application, and it was retained for 8 weeks, without affecting significantly the development of soil aboriginal microflora.

CONCLUSION: TEB release can be regulated by the process employed to fabricate the formulation and the fungicide loading, and the TEB accumulates in the soil gradually, as the polymer is degraded. The experimental forms of TEB embedded in the slowly degraded P3HB can be used as a basis for developing slow-release fungicide formulations.

Keywords: tebuconazole; poly-3-hydroxybutyrate; degradation; embedding; tebuconazole release; fungicidal effect

1 INTRODUCTION

Science and industry are developing and manufacturing increasing amounts of new pesticides to fulfil the needs of agriculture, as the use of chemicals is one of the most common ways to enhance crop production. New types of pesticide are more effective against pests than the previously used ones. Thus, high levels of research effort are focused on designing novel formulations and studying their behaviour in the environment. The main goals of this line of research are to invent less toxic and more targeted pesticides and to reduce their application rates.

Pathogens such as root rot, powdery mildew, snow mould, leaf blotch, etc., do considerable harm to cultivated plants, causing crop losses. Among the great diversity of fungicides, triazole chemicals occupy a special place, as they have a wide range of fungicidal activity and regulate plant growth.[1–3] These include fungicides containing tebuconazole (TEB) as the active ingredient, which are now commonly used. Tebuconazole is an effective multifunctional systemic fungicide used to protect a number of agricultural crops (wheat, barley, rape, corn, rice, vineyards, etc.) against powdery mildew, rust, rots, leaf blotches and other spot diseases. TEB rapidly penetrates into the plants through their vegetative organs and roots, inhibits ergosterol synthesis, preventing the formation of cell membranes, and disrupts metabolic processes, causing the death of pathogens.[4] The most common commercial TEB formulations are suspensions or emulsions, which are used to spray the vegetative organs of plants. Standard formulations are present at higher concentrations than required for activity at the time of application in order to guarantee the duration of effect at later time intervals. As TEB is potentially phytotoxic, this method of application of the fungicide inhibits plant growth, causing economic losses and posing a threat to the health of people and the environment.[5] The unfavourable side effect of TEB occurs via the following mechanism: triazoles shift the balance of phytohormones in plant tissues and inhibit biosynthesis of gibberellins,

* Correspondence to: Tatiana G Volova, Institute of Biophysics, Siberian Section, Russian Academy of Sciences, 50/50 Akademgorodok, Krasnoyarsk, Russian Federation. E-mail: volova45@mail.ru

a Institute of Biophysics, Siberian Section, Russian Academy of Sciences, Krasnoyarsk, Russia

b Siberian Federal University, Krasnoyarsk, Russia

causing a temporary increase in the content of abscisic acid in plants.[2,6] Another way of using TEB chemicals is to treat seeds prior to planting, but this may reduce seed germination ability, suppress the growth of seedlings and inhibit root growth.[7] The TEB used as seed dressing is quickly depleted, and the vegetative organs of plants have to be sprayed with the fungicide. Thus, new TEB formulations need to be developed to increase the efficacy of TEB and minimise its harmful effects on the environment.

The latest research direction is the development and agricultural use of environmentally safe new-generation pesticides with targeted and controlled release of active ingredients embedded in biodegradable matrices or covered with biodegradable coatings. These matrices and coatings are degraded in soil and other biological media by soil microflora to form products that are harmless to living and non-living nature, and the pesticides are gradually released into the environment. The use of such formulations can reduce the amounts of the chemicals added to the soil and enables their sustained and controlled delivery over a growing season, preventing the immediate release of the active ingredients that occurs in the case of using the most common formulations of TEB, i.e. emulsifiable concentrates and wettable powders.[5]

The crucial part of constructing such preparations is the availability of appropriate materials with the following properties: degradability; ecological compatibility with the environment and global biosphere cycles; safety for living and non-living nature; long-term (weeks and months) presence in the natural environment and controlled degradation, followed by the formation of non-toxic products; chemical compatibility with pesticides and fertilisers; processability by available methods, compatible with pesticide and fertiliser production technologies. Among materials that can be used to construct slow-release pesticide formulations are both synthetic and natural materials.[8,9]

Research aimed at designing slow-release tebuconazole formulations was started quite recently. Asrar *et al.*[10] described microparticles prepared from poly(methyl methacrylate) and poly(styrene-*co*-maleic anhydride) and loaded with TEB. Yang *et al.*[7] described encapsulation of TEB in ethyl cellulose microcapsules to be used to suppress the pathogen causing loose smut. Qian *et al.*[11] reported encapsulation of TEB in silica nanospheres. The study by Khalikov *et al.*[12] described the preparation of nanoparticulate powders with enhanced water solubility by mixing TEB with water-soluble polymers in a planetary-type mill. The authors used arabinogalactan (AG), pectin (P), cyclodextrin (CD), polyvinylpyrrolidone (PVP) and hydroxyethyl starch (HES). TEB/AG and TEB/HES samples showed higher fungicidal activity against root rot pathogens (*Biopolaris* and *Fusarium*) than the commercial formulation Raxil.

Natural degradable polymers – polyhydroxyalkanoates (PHAs) – are promising materials for embedding agrochemicals. These polymers do not undergo rapid chemical hydrolysis in aqueous media, but slowly (over a period of months) decompose via truly biological degradation, an enzymatic depolymerisation process.[13,14] The long degradation times of PHAs, which are eventually degraded to such harmless products as CO_2 and H_2O, 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 pesticides. Prudnikova *et al.*[15] reported results of constructing slow-release formulations of the herbicide Zellek (haloxyfop-*P*-methyl) embedded in PHA and showed that the herbicide release rate can be controlled by varying the polymer/herbicide ratio in the formulation. The Zellek formulations tested on creeping bentgrass (*Agrostis stolonifera* L.) effectively suppressed the growth of that plant. A recent study by our team described various techniques for constructing slow-release formulations of TEB, which was embedded in the degradable polymer matrix of poly-3-hydroxybutyric acid (P3HB) prepared from polymer/TEB physical mixtures in the form of solutions and powders.[16]

The purpose of the present study was to investigate the release profile of tebuconazole embedded in films, microgranules and pellets of degradable P3HB incubated in soil microecosystems and the fungicidal activity of the formulations against the plant pathogen of the genus *Fusarium*.

2 MATERIALS AND METHODS

2.1 Fungicide

The chemicals used in this study were the systemic fungicide Raxil Ultra (Bayer Crop Science, Russia), with tebuconazole (TEB) as the active ingredient, and chemically pure TEB (Russian Federal Standard GSO7669-99, purity 99.1%). TEB is a multifunctional systemic fungicide, which is effective against a very wide range of fungal diseases of cereal crops. The chemical formula of TEB is $C_{16}H_{22}ClN_3O$; molar mass 307.82 g mol^{-1}; solubility in water 36 mg L^{-1} at 20 °C; melting point 104.7 °C; the substance is not hydrolysed at pH values between 4 and 9 and is stable upon exposure to light and elevated temperature; degradation time in soil 177 days.

2.2 P3HB characterisation

P3HB was used as a matrix for embedding TEB. The polymer was synthesised by using bacterium *Cupriavidus eutrophus* B10646 at the Institute of Biophysics, Siberian Section, Russian Academy of Sciences. Cells were aseptically cultured in a 7.5 L BioFlo/CelliGen115 (New Brunswick Scientific, Edison, NJ) fermentor, following previously developed technology.[17,18] Polymer was extracted from cells with chloroform, and the extracts were precipitated using hexane. The extracted polymers were redissolved and precipitated again 3-4 times to prepare homogeneous specimens. P3HB had the following physicochemical parameters: weight-average molecular weight (M_w) 920 kDa; polydispersity (D) 2.52; degree of crystallinity 74%; melting point and thermal decomposition temperature 179.1 and 284.3 °C respectively.

2.3 Preparation of fungicide formulations

Films, pellets and granules with tebuconazole loadings of 10 and 50% were prepared as experimental formulations. Films loaded with the fungicide were prepared as follows. A solution of TEB in chloroform was added to the 2% polymer solution in chloroform. The P3HB/TEB solution was mixed on an MR Hei-Standart magnetic stirrer (Heidolph, Schwabach, Germany) for 2–3 h (until completely dissolved) and heated to 35–40 °C under reflux for 3–4 h. Then, the P3HB/TEB solution was cast in Teflon-coated metal moulds, where solvent evaporation occurred. The films were left at room temperature in a laminar flow cabinet for 24 h, and they were then placed into a vacuum drying cabinet (Labconco, Kansas City, MO) for 3–4 days until complete solvent evaporation took place. Films were cut into 5 × 5 mm squares, which were weighed on a Discovery analytical balance of accuracy class 1 (Ohaus, Greifensee, Switzerland). The film thickness was measured with a LEGIONER EDM-25-0.001 digital micrometer (Germany). TEB-loaded pellets were prepared as follows. The polymer was ground in a ZM 200

ultracentrifugal mill (Retsch, Haan, Germany). The fractional composition of the polymeric powder was determined by using an AS 200 control analytical sieve shaker (Retsch). The <0.50 mm fraction of particles comprised 55%, and the 0.80–1.00 mm fraction of particles constituted 45%. Samples of the P3HB and TEB powders were weighed on the analytical balance, mixed and homogenised by stirring. The 3 mm diameter pellets were prepared from the P3HB/TEB powder by cold pressing, using a laboratory bench-top hand-operated screw press (Carl Zeiss, Jena, Germany) under a pressing force of 6000 F. Granules were prepared as follows. The necessary amounts of TEB were added to the 10% P3HB solution, which was thoroughly mixed. A peristaltic pump was used to drop the P3HB/TEB solution into the sedimentation tank which contained hexane (by the microdrop technique); the needle size was 20 G, and the thickness of the layer of the precipitating agent (hexane) was 200 mm. As the polymer is insoluble in hexane, when the drops passed through the layer of the precipitating agent, granule formation occurred.

2.4 Characterisation of soil microecosystems

The effect of the experimental tebuconazole formulations on plant-pathogenic fungi was studied in laboratory soil microecosystems. Soil microecosystems were prepared as follows. The agrogenically transformed soil (the village of Minino in the Krasnoyarsk Krai, Russia) was placed into 250 cm^3 plastic containers (200 g soil per container). The soil used in the experiment is a typical agricultural soil occurring in Siberian agroecosystems,[19,20] similar to the soils of Northern Europe – the Netherlands[21] – or eastern England.[22] The agrogenically transformed field soil was cryogenic micellar agrochernozem with a high humus content in the 0–20 cm layer (7.9–9.6%). The soil was weakly alkaline (pH 7.1–7.8), with high total exchangeable bases (40.0–45.2 mequiv 100 g^{-1}). The soil contained 6 mg kg^{-1} of nitrate nitrogen N-NO_3, 6 mg kg^{-1} of P_2O_5 and 22 mg 100 g^{-1} of K_2O (according to Machigin). The soil had high mineralisation and oligotrophy coefficients (1.52 and 11.74 respectively), indicating soil maturity and low contents of available nitrogen forms. The number of copiotrophic bacteria was 16.3 ± 5.1 million CFUs g^{-1} – 1.5 and 11.7 times lower than the number of prototrophic and oligotrophic bacteria, respectively, while the number of nitrogen-fixing bacteria was very high (26.1 ± 4.7 million CFUs g^{-1}).

Tebuconazole formulations (films, granules, pellets – 6 mg per container) were buried in the soil. The positive control was TEB in the form of commercial formulation Raxil Ultra, and the negative control was soil without TEB. The analysis of the chemical composition of the soil included measuring the pH of the aqueous extract (following Russian Federal Standard 26423-85) and concentrations of nitrate nitrogen (by the method developed at the Central Research Institute for Agrochemical Support of Agriculture, CRIASA, following Russian Federal Standard 26488-85), mobile phosphorus and exchangeable potassium (by the method developed by Machigin and modified at CRIASA, following Russian Federal Standard 26204-91).

The structure of the soil microbial community was analysed by conventional methods of soil microbiology.[23] The total number of organotrophic bacteria was determined on nutrient agar medium (NA, HiMedia); microscopic fungi were counted on malt extract agar (MEA) (Sigma-Aldrich, St Louis, MO). The ecological trophic groups of microorganisms were identified by plating them onto diagnostic media. Ammonifying (copiotrophic) bacteria were identified on NA; microorganisms (including actinomycetes) capable of utilising mineral nitrogen (prototrophs) were identified on starch ammonium agar (SAA). Microorganisms involved in mineralisation of humus substances (oligotrophs) were identified on soil extract agar (SEA).[23] Platings were performed in triplicate from 10^3–10^7 dilutions of soil suspension. The plates were incubated for 3–7 days at a temperature of 30 °C for counting bacteria and for 7–10 days at 25 °C for counting fungi. The mineralisation coefficient was determined as the ratio between microorganisms on SAA and on NA. The oligotrophy coefficient was determined as the ratio of microorganisms on SEA and on NA. To prepare pure cultures of microorganisms, 8–10 morphotypes of each isolate were transferred to the plates with the corresponding media. The morphology of bacterial cells was examined by gram staining.

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.[24,25] Soil microscopic fungi were identified by their micro- and macromorphological features (the structure and colour of colonies and the structure of mycelium and spore-forming organs), which are objective parameters for identifying these microorganisms.[26,27]

In addition to conventional examination, we also used molecular genetic methods. DNA was extracted by using an Aqua-Pure Genomic DNA Isolation Reagent kit (Bio-Rad, Hercules, CA), following the manufacturer's guidelines. The 16S rRNA gene of bacteria was amplified using universal primers 27F (5′-AGAGT TTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), corresponding to positions 8–27 and 1510–1492 of the *Escherichia coli* gene respectively. For eukaryotes, the 28S rRNA gene was amplified using yeast primers D1/D2 R (5′-TTGGTCCGTGT TTCAAGACG-3′) and D1/D2 U (5′-GCATATCAATAAGCGGAGGA-3′). A polymerase chain reaction (PCR) was run in a total volume of 50 μL, which contained 50–100 ng of DNA, a 1× reaction buffer, 0.3 μM of each primer, 0.2 μM of each dNTP, 2 μM of $MgSO_4$ and 1 U of high-fidelity Platinum Taq polymerase (Invitrogen, Carlsbad, CA), using a Mastercycler Gradient DNA amplifier (Eppendorf, Hamburg, Germany). The reaction involved initial denaturation at 95 °C for 3 min, followed by 25 cycles with 30 s at 95 °C, 40 s at 55 °C and 1 min 30 s at 68 °C. Final extension was performed for 10 min at 68 °C. The size, amount and purity of PCR products were tested by electrophoresis in a 1.5% agarose gel, using a 0.5% TAE buffer. Visualisation was achieved by staining the gels with ethidium bromide, followed by documentation in a Doc Print transilluminator (Vilber Lourmat, Marne-la-Vallée, France).

High-quality sequences were obtained by cloning unpurified PCR products into vector pCR4-TOPO (Invitrogen), used to transform TOP10 *E. coli* cells. The resultant clones were tested by restriction analysis for the presence of the insert of the proper size in the vector. Plasmid DNA was extracted with a PureLink Quick Plasmid Miniprep kit (Invitrogen), following the manufacturer's guidelines. Sequencing was performed in two directions in an ALFexpress II automatic DNA analyser (Amersham Pharmacia Biotech Ltd, Piscataway, NJ), using universal primers T3 and T7 and a Thermo Sequenase Cy5 Dye Terminator kit. The nucleotide sequences were deposited in GenBank (accession numbers KT321679-KT321682, KT321700-KT321701, KU052942, KU052947 and JX524023); they were compared with the sequences in EMBL/DDBJ/GenBank using the BLAST program for searching highly homologous sequences of the NCBI web resource (http://www.ncbi.nlm.nih.gov/BLAST).

2.5 Infecting the soil with a plant pathogen

Fungi of the genus *Fusarium* (*F. moniliforme*) were used in experiments. Inoculum was prepared by growing fungi in culture tubes

on MEA (Sigma-Aldrich) for 14 days. Then, spore suspension was prepared in sterile tap water, 2×10^7 spores mL^{-1}. The number of spores was counted in a Goryaev chamber. A quantity of 10 mL of *F. moniliforme* spore suspension was added to each container with soil. The initial concentration of *F. moniliforme* spores was 1×10^6 spores g^{-1} soil.

P3HB/TEB films, granules and pellets were weighed on an analytical balance of accuracy class 1, placed in close-meshed gauze bags (three specimens of the same formulation per bag) and buried in the soil at the same time as the spore suspension was added to it. Two concentrations of Raxil Ultra, which were comparable with the percentages of the active ingredient (TEB) in the experimental formulations, were added to the soil in the positive control. No fungicide was added to the soil infected with the *F. moniliforme* spores in the negative control. All manipulations were performed in triplicate.

The fungicidal action of P3HB/TEB formulations was compared with that of the commercial fungicide based on tebuconazole, Raxil Ultra, by counting the *F. moniliforme* colonies plated on MEA and by detecting purple-pink pigmentation – an indicator enabling identification of this plant pathogen and estimation of its physiological activity.[28]

2.6 TEB release kinetics and P3HB matrix degradation dynamics

The containers with soil were incubated in an A1000 environmental chamber (Conviron, Winnipeg, Canada) at a constant temperature of 25 ± 0.1 °C and a soil moisture content of 50%. The experiment lasted 63 days. The soil moisture content and pH were maintained at predetermined levels during the experiment.

The weight loss was measured gravimetrically: P3HB/TEB formulations were periodically taken out of the bags and cleaned to remove soil; the specimens were photographed to record the degradation process. The surface microstructure was examined by scanning electron microscopy (SEM). The specimens were placed on the microscope stage and gold sputtered by using an Emitech K575X sputter coater (10 mA, 2×40 s). SEM images were obtained with an S-5500 electron microscope (Hitachi, Tokyo, Japan). Surface properties were studied on a DSA-25E drop shape analyser (Krüss GmbH, Hamburg, Germany) for measuring contact angles of water and diiodomethane drops by the Owens, Wendt, Rabel and Kaelble method: the surface free energy (SFE) and its dispersive and polar components were measured, and the data were processed by DSA-4 software.

2.7 TEB release

During the experiment, the specimens were periodically removed from the soil to monitor changes in their weight (gravimetrically) and to determine the concentration of the active ingredient in the soil and its residual content in the formulations. Tebuconazole was extracted from soil and water with chloroform. To determine the residual TEB content in the polymer, the specimens were dissolved in chloroform, and then the polymer was precipitated with hexane. The polymer was separated from the solvents and weighed to determine the polymer content of the formulation. The solvents were removed in a rotary vacuum evaporator. After removal of chloroform, 100–500 μL of acetone was added to the polymer. The quantity of the active ingredient was measured by gas chromatography. Measurements were made with a chromatograph mass spectrometer (7890/5975C; Agilent Technologies, Santa Clara, CA), using a capillary column, under varied temperature conditions. The chromatography conditions were as follows: a DB-35MS capillary column, 30 m long and 0.25 mm in diameter; carrier gas helium, rate 1.2 mL min^{-1}; sample introduction temperature 220 °C; initial temperature of chromatography 180 °C; temperature rise to 310 °C at 10 °C min^{-1}; 5 min isothermal conditions. The TEB concentration was determined using the calibration curve; the curves were plotted as described elsewhere.[16]

The amount of TEB released was determined as the percentage of the TEB encapsulated in the polymer matrix, using the following formula:

$$RA = 100\% - (r/EA * 100\%)$$

where RA is the encapsulated amount of the active ingredient (mg), and r is the amount retained in the matrix (mg).

Based on the experimental curves of the release kinetics, we used the Korsmeyer–Peppas model to describe controlled fungicide release. In contrast to the Higuchi model or the first-order model, this equation very accurately describes the release process, taking into account such mechanisms as Fickian diffusion, convection, polymer matrix relaxation and edge effects:[29]

$$M_t/M_\infty = kt^n$$

where M_t and M_∞ are the amount of the chemical released over time and the initial amount of the chemical in the matrix respectively, k is a kinetic constant, which contains structural and geometric data on the polymer system, and n is the exponential parameter characterising the mechanism of release of the chemical from the polymer matrix.

At $n = 0.5$, the chemical is released via diffusion, in accordance with the Fickian diffusion mechanism. At $n = 1$, the release mechanism is described as case II transport, determined by relaxation processes and transitions in the carrier rather than by diffusion laws. This type of release occurs when the diffusion layer is dissolved and the matrix is partly destroyed and degraded. Values of n between 0.5 and 1 indicate superposition, or anomalous release.[30]

2.8 Indicators of the fungicidal activity of the formulations

Counting of the total microscopic fungi, including *F. moniliforme*, was performed by plating soil suspension onto petri dishes with MEA, which was supplemented with chloramphenicol (100 μg L^{-1} of the medium) to suppress cell growth. All platings were performed in triplicate from 10^5 dilutions of soil suspension. The dishes were incubated in a chamber thermostatically set to specific conditions. Microscopic analysis of the colonies was done using an AxioStar microscope (Carl Zeiss). Microscopic fungi were identified by their cultural and morphological properties, with identification guides:[26,27]

3 RESULTS

3.1 Characterisation of tebuconazole experimental formulations

Figure 1 shows photographs of the experimental slow-release formulations of tebuconazole (TEB) embedded in the polymer matrix of degradable poly-3-hydroxybutyrate (P3HB). The films prepared by solvent evaporation from the 2% polymer solution were 25 ± 0.3 μm thick; 2–3 mm diameter granules were prepared from the 10% P3HB solution by the microdrop technique; pellets prepared from polymer powder and TEB were 3 mm in diameter and 1 mm thick. The formulations were loaded with TEB at 10 or 50% of the polymer matrix weight. The surface properties of the

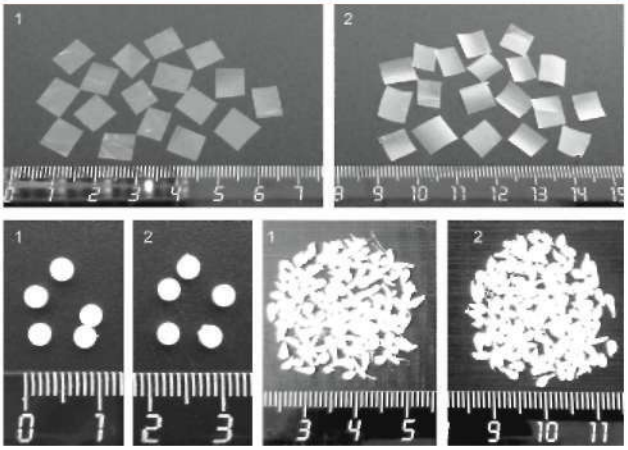

Figure 1. Photographs of P3HB/TEB films, pellets and granules with TEB loadings of 10 and 50% of the polymer weight: 1 and 2 respectively.

TEB-loaded pellets were not significantly different from the surface properties of the pellets without TEB (Table 1). Some differences were observed between surfaces of P3HB and P3HB/TEB films: the water contact angle of the P3HB films was 68.4°, while the water contact angles of P3HB/TEB films with 10% and 50% TEB loading were 75.9 and 72.1° respectively, i.e. the surfaces of the TEB-loaded films were somewhat more hydrophobic. Their surface energy and its dispersive and polar components were also different. Analysis of the microstructure showed that the surfaces of all formulations with the higher TEB loading were more uneven, with 1–2 μm pores, bumps and erosions (Fig. 2). These changes were more pronounced on films.

3.2 Characterisation of laboratory soil microecosystems

Based on morphological, physiological and biochemical studies and molecular genetic examination of the 16S and 28S rRNA gene fragments, we determined the major microorganisms in the soil samples (Fig. 3). The microbial community was dominated by actinobacteria such as *Streptomyces* (24%), *Arthrobacter* species (18%) and *Corynebacterium* species (12%); *Pseudoxanthomonas* were the major gram-negative bacilli (12%) (Fig. 3A). Microscopic fungi were dominated by *Penicillium* species (58–65%); fungi of the genera *Fusarium*, *Trichoderma* and *Aspergillus* constituted 8–11% of the population of microscopic fungi in soil samples (Fig. 3B). *Fusarium* species isolated from the initial soil samples were represented by *F. solani* and *F. lateritium*. No *F. moniliforme* was detected in the initial microbial community.

3.3 TEB release kinetics and dynamics of degradation of P3HB/TEB formulations in soil

Results of studying P3HB/TEB degradation dynamics and TEB release kinetics are shown in Figs 4 and 5. Dynamics of degradation of P3HB/TEB formulations was influenced by their geometry and the percentage of TEB loaded into them (Fig. 4).

The highest degradation rate was recorded for P3HB/TEB films: after 7 days of incubation in soil, their weight loss reached 40% of their initial weight, irrespective of the TEB loading. Then, the degradation process was influenced by the percentage of loaded TEB. The weight of the specimens with 50% TEB loading had dropped to 2–5% of their initial weight by day 28, while the specimens with 10% TEB loading showed a comparable weight loss at day 51. The average degradation rate of the films was 0.106 mg day^{-1}. The second fastest degradation rates were recorded for microgranules. P3HB/TEB microgranules with different TEB percentages showed similar average degradation rates: 0.081–0.083 mg day^{-1}. At the end of the experiment, their residual weight was no higher than 15% of the initial weight. The pressed pellets were degraded at the slowest rate. Formulations with 10 and 50% TEB loadings showed average degradation rates of 0.030 and 0.055 mg day^{-1} respectively, and their residual weights were about 70 and 40% of the initial weights.

There were lag phases in the degradation of all polymer formulations: 2–3 days for films, 4–6 days for microgranules and over 2 weeks for pellets. The reason for the occurrence of the lag phase is that it takes some time for microorganisms to get attached to the surface of the polymer samples and become adapted to the synthesis of the depolymerising enzyme systems corresponding to the substrate (polymer). We monitored changes in the structure and physicochemical properties of the polymer specimens during degradation and observed changes in the surface structure of all specimens in the early degradation phase. In the later phases, films broke into fragments, while pellets underwent surface erosion, with the polymer weight dropping and crystallinity rising. The increase in the degree of crystallinity was caused by the quicker degradation of the amorphous region of the polymer, which is a more readily available growth substrate to the microorganisms than the crystalline region.

The geometry of the form influenced tebuconazole release kinetics (Fig. 5). The amount of TEB released from the most rapidly degraded specimens (films) reached its maximum in the soil after 43 days of incubation: 15 and 3 μg g^{-1} soil in the samples with formulations loaded at 10 and 50% TEB. That was comparable with the concentration of TEB added to the soil in free form. The degrading P3HB/TEB microgranules released similar amounts of TEB, increasing its concentration in the soil to about 2.4 and 13.5 μg g^{-1} soil. TEB concentrations in the soil with P3HB/TEB pellets (degraded at the slowest rate) were lower: about 1.1 and 8.3 μg g^{-1} soil after 51 days of incubation of formulations with 10 and 50% TEB loadings. Thus, TEB release can be regulated by loading different percentages of the fungicide (10 and 50% of the polymer weight) into P3HB films, microgranules and pellets. Embedding TEB into polymer matrices enabled slow release of the fungicide (for more than 60 days). The percentage of released TEB varied between 40–50 and 90–100% of the amount loaded into the specimens.

Constant k and exponent n, characterising the kinetics of tebuconazole release from the experimental P3HB/TEB formulations, which were obtained by using the Korsmeyer–Peppas model, are given in Table 2. The time when tebuconazole is released with the highest rate is characterised by parameter t^{50} – the time needed for the fungicide content of the specimen to reach $M_t/M_\infty \leq 0.5$.

Tebuconazole release from the films and granules can be described as the zero-order anomalous release. The exponent had the following values: for films loaded at 10%, $n = 0.81$, and for films loaded at 50%, $n - 0.66$; for granules loaded at 10%, $n = 0.85$, and for granules loaded at 50%, $n = 0.49$. In both cases, as the loading was increased, the exponent decreased and constant k, which contains the diffusion coefficient and the geometric and structural characteristics of the specimens, increased. Changes in constant k are indicative of the heterogenic structure of the specimens of the same geometry but with different loadings. SEM data lead to the same conclusion. Pellets have more stable structural and geometric parameters, as suggested by the values of k, and

Table 1. Surface properties of experimental P3HB/TEB films and pellets with different TEB loadings

Sample, TEB loading	Water contact angle (deg)	Surface energy (mN m^{-1})	Dispersive component (mN m^{-1})	Polar component (mN m^{-1})
Initial P3HB:				
film	68.4 ± 4.3	59.2 ± 1.9	43.8 ± 0.9	15.4 ± 0.9
pellet	77.0 ± 2.7	58.4 ± 3.2	45.5 ± 0.9	5.7 ± 0.9
		P3HB/TEB films		
TEB loading (%):				
10	75.9 ± 3.1	44.2 ± 0.3	38.5 ± 0.2	5.6 ± 0.2
50	72.1 ± 0.5	47.9 ± 0.6	41.4 ± 0.5	6.5 ± 0.1
		P3HB/TEB pellets		
TEB loading (%):				
10	77.0 ± 3.4	58.2 ± 2.9	44.8 ± 0.93	5.6 ± 0.97
50	75.0 ± 5.4	56.7 ± 3.7	43.4 ± 0.70	5.1 ± 0.89

the value of exponent n characterises the release mechanism as Fickian diffusion.

The release time of the greater percentage of the fungicide, kinetic constant and exponent of the specimens correlate with polymer degradation kinetics. For the films with 10 and 50% TEB loadings, t^{50} was 14 and 12 days respectively, and the polymer weight loss was 59 and 69% respectively. For the granules with 10 and 50% TEB loadings, t^{50} was 43 and 28 days respectively, and the polymer weight loss was 49 and 56% respectively. Pellets were more resistant to degradation, and TEB release from pellets loaded at 10% was 40% for 63 days. For 50% loaded pellets, t^{50} was 43 days. The polymer weight loss for the time when TEB release occurred at the highest rate was 32 and 29% for the 10% and 50% loaded pellets respectively.

Thus, tebuconazole release was influenced by the geometry of the specimens and their structure, which was related to the percentage of the loaded fungicide and the process employed to fabricate the formulation.

3.4 Fungicidal activity of P3HB/TEB formulations

Results characterising the fungicidal activity of the experimental P3HB/TEB formulations are shown in Figs 6 and 7. The parameters evaluated in this study were the total number of CFUs and the production of purple or pink pigment, which identifies *F. moniliforme* and indicates its physiological activity.[28] Microbiological analysis showed that, after 7 days, the number of *F. moniliforme* CFUs decreased in all treatments, but it remained the dominant species, and the number of *F. moniliforme* colonies on the dishes with the nutrient medium was higher than the number of colonies of saprotrophic microscopic fungi.

The application of the commercial formulation Raxil Ultra reduced the number of *F. moniliforme* CFUs by a factor of 400–500 compared with the initial number of CFUs of this plant pathogen – to 1800–3200 CFUs g^{-1} soil – and kept it at that level for the following 7 weeks, to the end of the experiment. The polymer-embedded TEB was less effective in the early phase of incubation, but the number of *Fusarium* CFUs decreased steadily throughout the experiment, as TEB was gradually released from the polymer matrix.

P3HB/TEB granules and films were more effective than pellets. In the soil with microgranules, the *F. moniliforme* population decreased by a factor of 70–90 compared with the initial population after 7 days of incubation. During the first 2 weeks, P3HB/TEB films were less effective than granules, but after 14 days the effects of the two types of formulation did not differ significantly; after 4–6 weeks the effectiveness of the P3HB/TEB granules and films became comparable with that of the commercial formulation Raxil Ultra (Fig. 6). P3HB/TEB pellets had a weaker fungicidal effect than films or granules, as the release of the fungicide from the pressed pellets was slower during the first month of incubation in soil. After 8 weeks, the fungicidal effect of the TEB embedded in polymer matrices was not significantly different from the effect of Raxil Ultra. The higher TEB loading and the increase in the TEB concentration in the control with Raxil Ultra did not significantly influence its fungicidal effect.

TEB exhibited a fungistatic effect not only towards plant pathogens but also towards all saprotrophic fungi. The microbiological examination of the structure of microbial communities during the experiments showed that the use of Raxil Ultra inhibited the growth of such aboriginal fungi as *Penicillium*, *Alternaria* and *Aspergillus*. In 3 days after the commercial formulation was added to the soil, no growth of colonies was observed on petri dishes. After 7 days of incubation, the fungi resumed growth, but the total number of microscopic fungi in the positive control was lower than in the treatments (with P3HB/TEB formulations) (Fig. 7). A possible explanation for this is that the concentration of TEB added to the soil as the active ingredient of the commercial formulation was initially much higher than the concentration of TEB released from the experimental formulations. TEB release from the P3HB/TEB formulations occurred as the polymer was degraded, and the amount of TEB in the soil increased gradually. The initially low TEB concentrations could not inhibit the growth of aboriginal fungi, mainly *Penicillium*, which dominated aboriginal saprophytic fungi.

The total counts of bacteria varied very slightly between treatments and did not differ significantly from the bacterial counts in the initial soil and controls. Hence, none of the TEB formulations inhibited the growth of saprotrophic bacteria in soil.

Thus, the fungicide tebuconazole embedded in the polymer matrix of P3HB was effective against plant pathogens of the genus *Fusarium*, and the effect was long lasting (for 8 weeks). After 2–4 weeks of incubation in soil, the effect of TEB embedded in polymer granules was comparable with the effect of the commercial TEB formulation Raxil Ultra. P3HB/TEB formulations did not exhibit a toxic effect towards saprotrophic microscopic fungi, while Raxil Ultra produced a strong fungistatic effect on all fungi in soil microbial communities.

4 DISCUSSION

The present study addresses the fungicidal effect of tebuconazole embedded in the polymer matrix of natural degradable polymer of

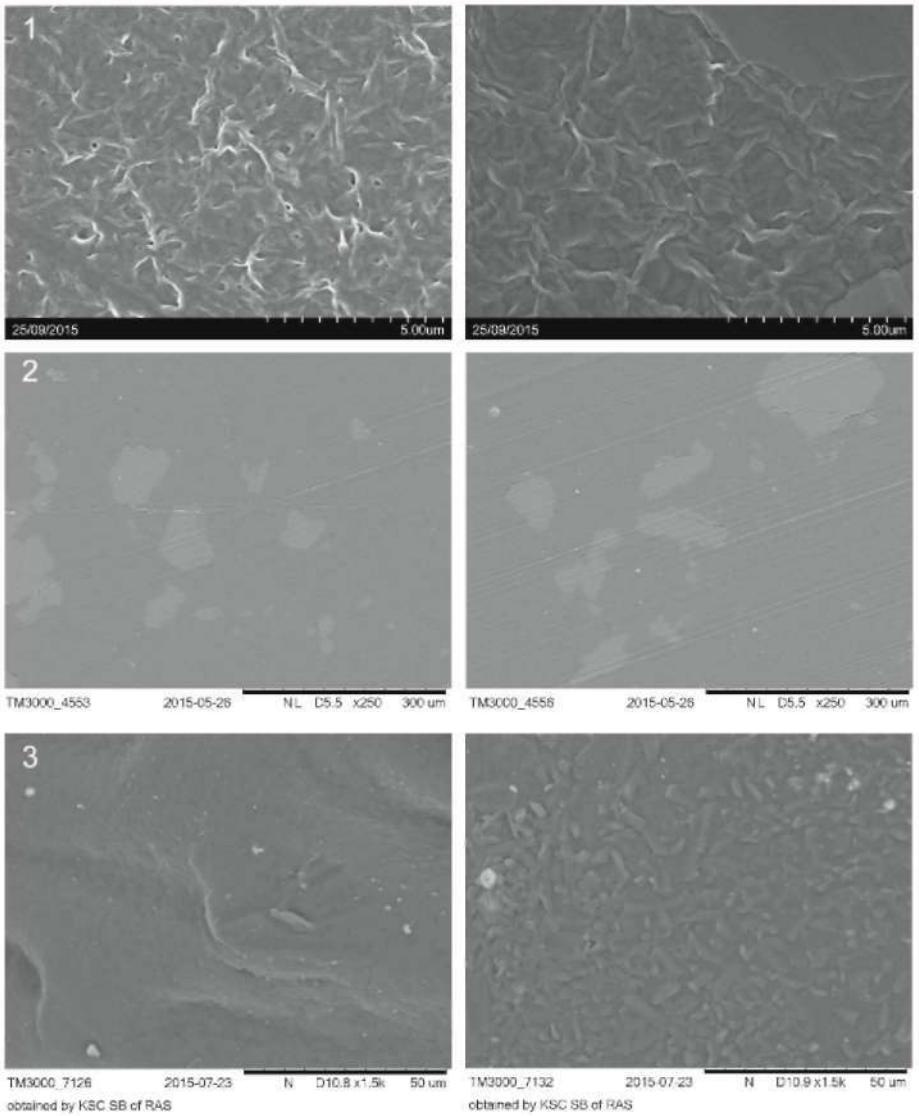

Figure 2. SEM images of the experimental P3HB/TEB formulations: 1 – films, 2 – pellets, 3 – granules, with TEB loadings of 10 and 50% of the polymer weight.

Table 2. Kinetic constants of tebuconazole release from the experimental P3HB/TEB formulations in laboratory soil microecosystems, obtained by using the $M_t/M_\infty = kt^n$ equation

Formulation type	TEB loading (%)	$k(h^{-n})$	n	R^2	t^{50} (days)
Films	10	0.005	0.81	0.99	14
	50	0.014	0.66	0.99	12
Granules	10	0.001	0.85	0.92	43
	50	0.032	0.49	0.93	28
Pellets	10	0.045	0.30	0.98	63
	50	0.044	0.36	0.99	43

3-hydroxybutyric acid (poly-3-hydroxybutyrate, P3HB) in the form of films, microgranules and pellets. These slow-release formulations were incubated in laboratory soil microecosystems infected with the spores of the plant pathogen *Fusarium* in long-term experiments.

Tebuconazole is a multifunctional systemic fungicide containing components of triazole. Triazole chemicals are commonly used to protect socially and economically important plants, including the major cereal crops and oil plants. Free TEB used to spray the vegetative organs of plants or treat seeds is very effective against pathogens but has a number of limitations. To increase the efficacy of the fungicide and minimise its environmental impact, special studies need to be conducted to develop and use new formulations of TEB characterised by targeted delivery, slow release and low percentages of the active ingredient.

TEB-related studies were reported in several papers, which showed that tebuconazole could be embedded in microparticles, micelles and microgranules and used as a component of powder systems, based on different materials such as poly(methyl methacrylate) and poly(styrene-*co*-maleic anhydride),[10] ethyl cellulose[7] and silica nanospheres.[11] Khalikov *et al.*[12] prepared and studied TEB mixtures with water-soluble polymers (arabinogalactan, pectin, cyclodextrin, polyvinylpyrrolidone and hydroxyethyl starch). Those studies described TEB encapsulation

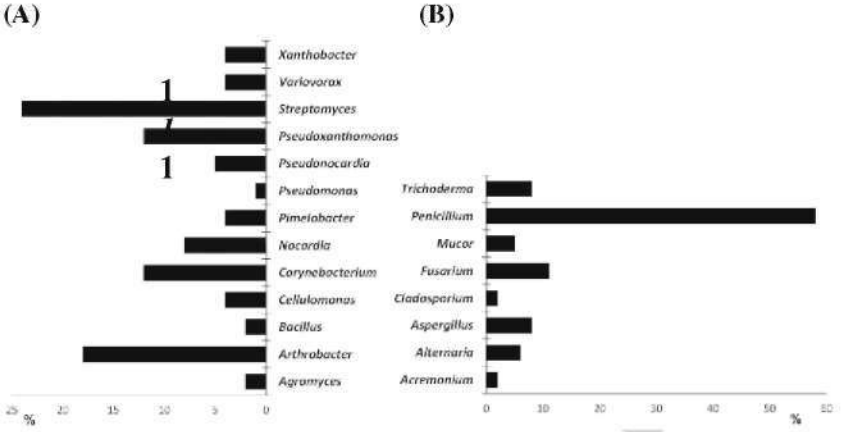

Figure 3. Dominant bacteria (A) and fungi (B) in the initial soil samples.

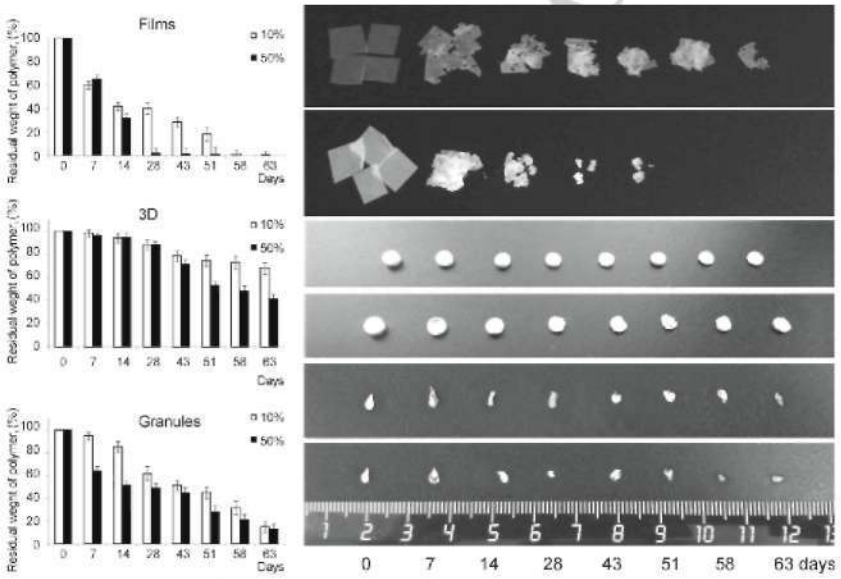

Figure 4. Degradation dynamics and external appearance of P3HB/TEB formulations incubated in soil.

efficiency and its release duration and mechanism in laboratory systems such as sterile water and soil. Some studies compared the efficacy of TEB formulations and the free fungicide against plant pathogens such as wheat rust *Puccinia recondite*,[10] root rot pathogens *Biopolaris* and *Fusarium*,[12] maize head smut[7] and wheat powdery mildew.[5] None of these studies either investigated TEB release kinetics as related to the degradation of the matrix or examined the structure of soil microbial communities and their role in the behaviour and fate of slow-release TEB formulations.

In the present study, we describe the construction and testing of TEB formulations based on the natural degradable polymers polyhydroxyalkanoates (PHAs). The literature data on the use of this class of polymers as matrices for slow-release fungicide formulations are scant. Savenkova *et al.*[31] 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 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 a study conducted by our team, biodegradable polymer P3HB was used as a matrix to construct slow-release formulations of the fungicide tebuconazole. P3HB/TEB systems constructed as films and pellets were studied using differential scanning calorimetry, X-ray structure analysis and Fourier transform infrared spectroscopy. TEB release from the experimental formulations was studied in aqueous and soil laboratory systems. In soil with known composition of the microbial community, the 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 water after 60 days in a sterile aqueous system. Incubation of P3HB/TEB films and pellets in the soil stimulated the 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.[16]

The present work was aimed at investigating three sustained-release TEB formulations – P3HB/TEB films, microgranules and pellets – with different loadings of the active ingredient (10 and 50% of the weight of the polymer matrix). All formulations were

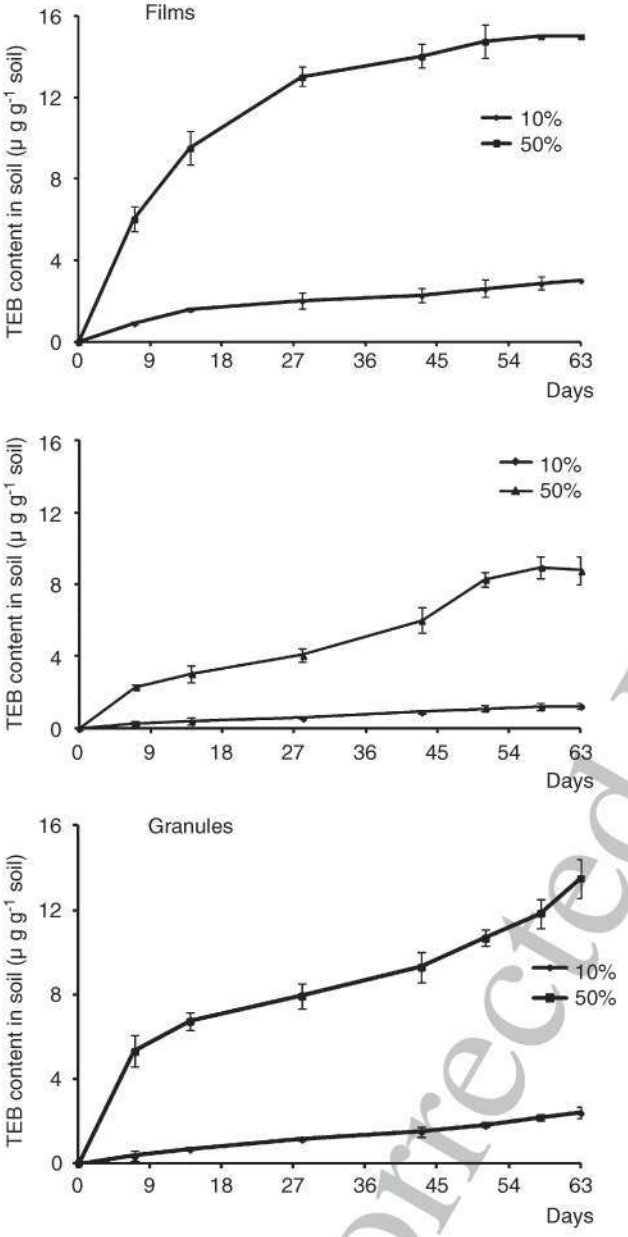

Figure 5. Cumulative release of TEB into soil from different P3HB/TEB forms (films, granules, pellets) with 10 and 50% TEB.

incubated in laboratory soil microecosystems that had been previously examined to characterise the structure of their microbial communities. The major representatives of the bacterial component and the fungal component had been isolated and identified; the soil of the microecosystems had been infected with spores of the plant pathogen *F. moniliforme* (the agent of plant root rot). TEB release was studied as dependent on the geometry of the specimens and TEB loading, taking into account the dynamics of polymer matrix degradation.

The kinetic constants and exponents showed that TEB release from films and granules occurred through the anomalous release mechanism, while TEB release from pellets was a diffusion process. That was consistent with the data reported by Siepmann and Peppas[30] for specimens with different geometries. In all specimens, there was a relationship between kinetic parameters and the degree of degradation of the carriers. A possible explanation for the anomalous release from films and granules may be a shorter degradation time compared with the pellet degradation time. Zero-order release was reported by Korsmeyer *et al.*[32] for polymers whose degradation occurred by dissolution of the diffusion layer.

A shift of the kinetics towards linearity during degradation of the polymer matrix was reported by Catellani *et al.*[33] An increase in exponent n with decrease in kinetic constant k was observed by a number of authors.[34–36]

The present study showed that TEB release from experimental formulations based on degradable natural polymer P3HB could be regulated by varying the formulation fabrication process and TEB loading. It is important that all formulations enabled slow release of the fungicide, which lasted 60 days or longer.

All TEB formulations inhibited *F. moniliforme* growth, but during the early phase of the experiment (the first 2–3 weeks) the effectiveness of P3HB/TEB formulations was somewhat lower than the effectiveness of the free TEB (commercial formulation Raxil Ultra at a similar concentration used in the control). The fungicidal effect of TEB embedded in microgranules was the strongest, followed by films and, lastly, pellets, which had the slowest degradation rate. In later phases of the experiment, as P3HB was degraded and TEB concentration in the soil increased, the fungicidal activity of the embedded TEB became comparable with the effect of the free fungicide. The problem of delay in the effect of the fungicide embedded into slowly degraded P3HB can be managed by using P3HB blends with more rapidly degraded fillers than the polymer matrix.

As reported in the few available TEB-related studies, TEB affects soil microflora. Muñoz-Leoz *et al.*[37] showed that application of different TEB concentrations (between 5 and 500 mg kg^{-1} dry soil) inhibited soil basal respiration, substrate-induced respiration, activity of a number of enzymes and nitrification rate. The functional profiles of microbial communities detected by the activity of several enzymes (urease, arylsulfatase, β-glucosidase, alkaline phosphatase and dehydrogenase) showed changes in the profiles of the cultured heterotrophic bacteria. By contrast, in a study by Cycon *et al.*,[38] application of 7 and 13.5 mg TEB kg^{-1} sandy soil stimulated substrate-induced respiration of soil and heterotrophic microflora; concentrations of nitrates and ammonium ions and the total counts of cultured bacteria, of denitrifying and nitrogen-fixing bacteria and of fungi remained unchanged or were stimulated by the application of the pesticide to the soil. Higher TEB concentrations (270 mg kg^{-1} soil) reduced microbial respiration in the first 24 h after application and stimulated it in later stages (at days 14 and 28) compared with the control.

Microbiological study of the structure of the microbial communities in soil microecosystems with the experimental P3HB/TEB formulations buried in them showed that TEB did not affect the total number of bacteria and did not inhibit the growth of saprophytic fungi. However, the proportions of the trophic groups and the composition of the microbial community had changed. The percentage of copiotrophic bacteria decreased, while the percentages of prototrophic and nitrogen-fixing bacteria increased. Among the major microorganisms, the number of spore-forming bacilli dropped by 20%, and the number of gram-positive cocci by 12%, while the number of *Pseudomonas* and *Corynebacterium* species increased by 11–12%. We observed an increase in the total number of gram-negative bacilli and actinobacteria, including representatives of *Pseudomonas*, *Stenotrophomonas*, *Variovorax* and *Streptomyces*, which are effective PHA degraders.[39–42] A similar

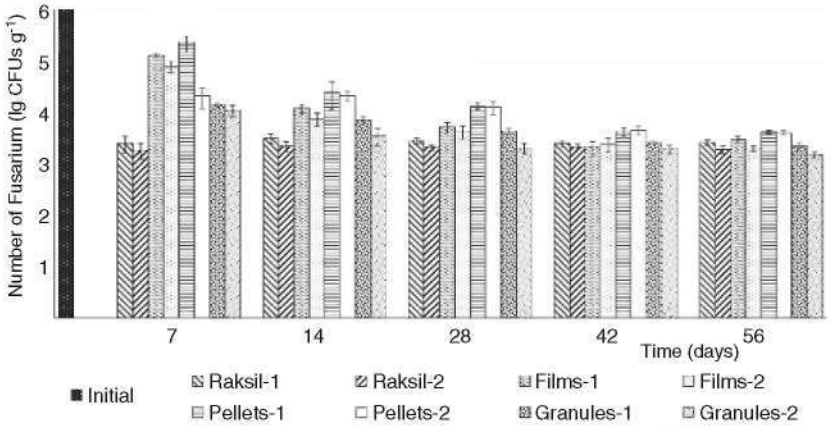

Figure 6. The fungicidal effect of various forms of TEB on the plant pathogen *F. moniliforme*; the tebuconazole concentration in the formulation was 600 mg (1) or 3000 mg (2).

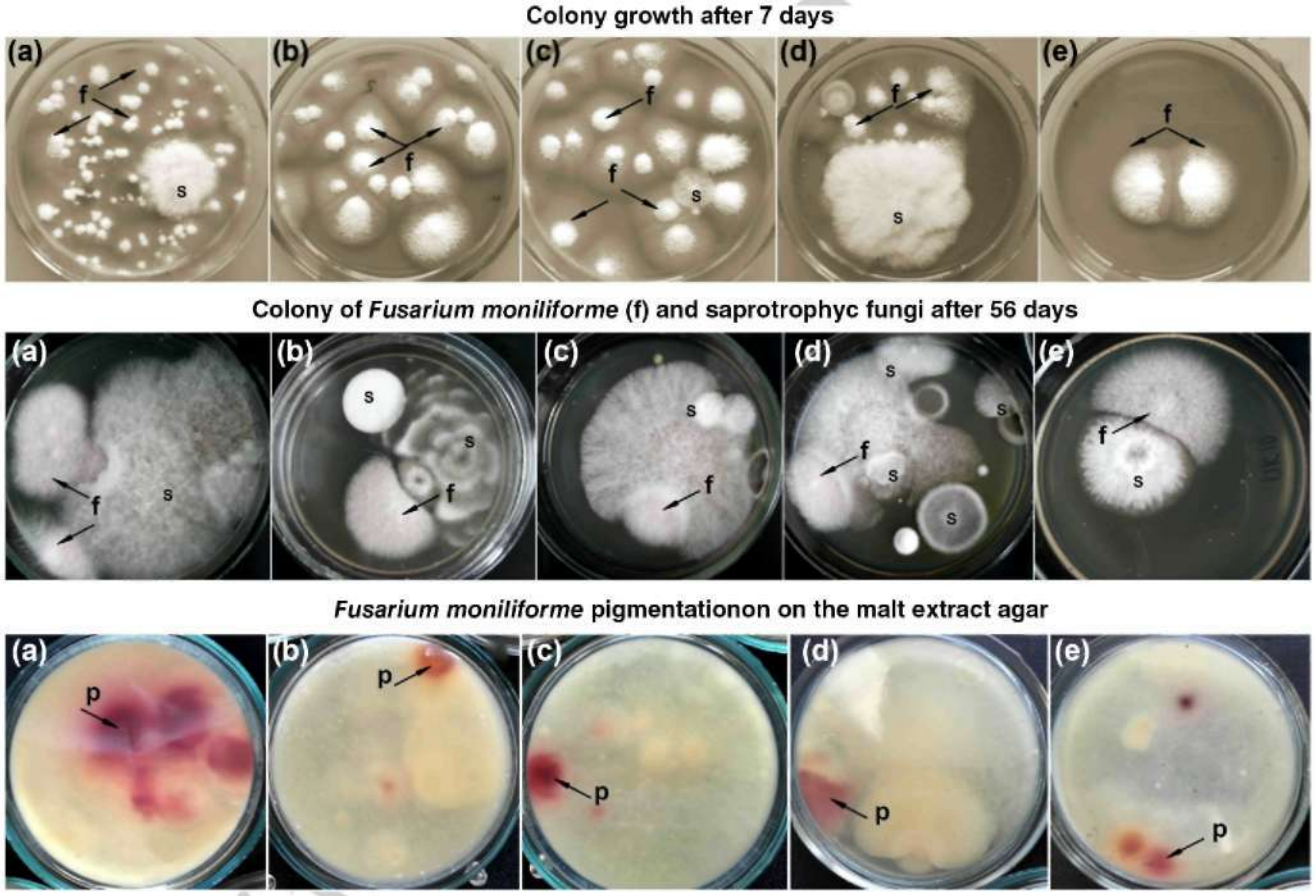

Figure 7. The growth of *F. moniliforme* colonies: a – negative control; b – P3HB/TEB films; c – P3HB/TEB pellets; d – P3HB/TEB granules; e – Raxil Ultra. Arrows indicate colonies of *Fusarium* (f) and purple-pink pigment formation (p); s – colonies of saprotrophic fungi.

effect of TEB embedded in P3HB films and pellets was described in our previous study.[16]

Thus, fungicide TEB embedded in the polymer matrix of P3HB in the form of films, microgranules and pellets was effective against plant pathogens of the genus *Fusarium*, and that was a long-lasting effect (for 8 weeks). After 2–4 weeks of incubation in soil, P3HB/TEB formulations were as effective as the commercial formulation Raxil Ultra. The experimental forms of TEB embedded in the slowly degraded P3HB can be used as a basis for developing slow-release fungicide formulations.

ACKNOWLEDGEMENT

This study was supported by the Russian Science Foundation (grant number 14-26-00039). The authors declare no competing financial interest.

REFERENCES

1 Child RD, Evans DE, Allen J and Arnold GM, Growth response in oilseed rape (*Brassica napus* L.) to combined applications of the triazole chemicals triapenthenol and tebuconazole and interactions with gibberelli. *Plant Growth Regul* **13**:203–212 (1993).

2 Fletcher RA, Gilley A, Sankhla N and Davis TD, Triazoles as plant growth regulators and stress protectants, in *Horticultural Reviews*, ed. by Janick J. John Wiley & Sons, Inc., Oxford, UK, pp. 55–138 (1999).

3 Hedden P, Croker SJ, Rademacher W and Jung J, Effect of the triazole plant growth retardant BAS 111W on gibberellin levels in oilseed rape, *Brassica napus*. *Physiol Plant* **75**:445–451 (1989).

4 Tomlin CDS, *The e-Pesticide Manual. 2002–2003: a World Compendium, Version 2.2*, 12th edition. The British Crop Protection Council, Croydon, UK (2003).

5 Zhang B, Zhang T, Wang Q and Ren T, Microorganism-based monodisperse microcapsules: encapsulation of the fungicide tebuconazole and its controlled release properties. *RSC Adv* **5**:25 164–25 170 (2015).

6 Grossmann K, Plant growth retardants as tools in physiological research. *Physiol Plant* **78**:640–648 (1990).

7 Yang D, Wang N, Yan X, Shi J, Zhang M, Wang Z *et al.*, Microencapsulation of seed-coating tebuconazle and its effects on physiology and biochemistry of maize seedlings. *Colloids Surf B: Biointerfaces* **114**:241–246 (2014).

8 Puoci F, Iemma F, Spizzirri UG, Cirillo G, Curcio M and Picci N, Polymer in agriculture: a review. *Am J Agric Biol Sci* **3**:299–314 (2008).

9 Dubey S, Jhelum V and Patanjali PK, Controlled release agrochemicals formulations: a review. *J Sci Ind Res* **70**:105–112 (2011).

10 Asrar J, Ding Y, La Monica RE and Ness LC, Controlled release of tebuconazole from a polymer matrix microparticle: release kinetics and length of efficacy. *J Agric Food Chem* **52**:4814–4820 (2004).

11 Qian K, Shi T, He S, Luo L, Liu X and Cao Y, Release kinetics of tebuconazole from porous hollow silica nanospheres prepared by miniemulsion method. *Micropor Mesopor Mater* **169**:1–6 (2013).

12 Khalikov SS, Dushkin AV, Dvaleti RD and Evsenko VI, The creation of innovative fungicides based on tebuconazole and mechanochemical processes (in Russian). *Fundam Issled* **10**:2695–2700 (2013).

13 Sudesh K, Abe H and Doi Y, Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog Polym Sci* **25**:1503–1555 (2000).

14 Jendrossek D, Microbial degradation of polyesters. *Adv Biochem Eng Biotechnol* **71**:293–325 (2001).

15 Prudnikova SV, Boyandin AN, Kalacheva GS and Sinskey AJ, Degradable polyhydroxyalkanoates as herbicide carriers. *J Polym Environ* **21**:675–682 (2013).

16 Volova T, Zhila N, Vinogradova O, Shumilova A, Prudnikova S and Shishatskaya E, Characterization of biodegradable poly-3-hydroxybutyrate films and pellets loaded with the fungicide tebuconazole. *Environ Sci Pollut Res* DOI: 10.1007/s11356-015-5739-1 (2015).

17 Volova T, Kiselev E, Shishatskaya E, Zhila N, Boyandin A, Syrvacheva D *et al.*, Cell growth and accumulation of polyhydroxyalkanoates from CO_2 and H_2 of a hydrogen-oxidizing bacterium, *Cupriavidus eutrophus* B-10646. *Biores Technol* **146**:215–222 (2013).

18 Volova T, Kiselev E, Vinogradova O, Nikolaeva E, Chistyakov A, Sukovatiy A *et al.*, A glucose-utilizing strain, *Cupriavidus euthrophus* B-10646: growth kinetics, characterization and synthesis of multicomponent PHAs. *PLoS ONE* **9**:1–15 (2014).

19 Artamonova VS, Microbiological Characteristics of Anthropogenically Transformed Soils of Western Siberia *(in Russian)*. Siberian Section, Russian Academy of Sciences, Novosibirsk (2002).

20 Senchakova TYu, Micromycetes of chernozems as a bioindication object in anthropogenically transformed ecosystems (in Russian). *Nauka Sovrem* **6**:55–59 (2010).

21 Smit E, Leeflang P, Gommans S, van den Broek J, van Mil S and Wernars K, Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Appl Environ Microbiol* **67**:2284–2291 (2001).

22 Girvan MS, Bullimore J, Pretty JN, Osborn AM and Ball AS, Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. *Appl Environ Microbiol* **69**:1800–1809 (2003).

23 N[illegible]ov AI, Egorov MA, Zakharchuk LM and Kolotilova NN, *Workshop on Microbiology: a Textbook for Students of Higher Educational Institutions*. Akademiya, Moscow (2005).

24 Brenner DJ, Krieg NR, Staley JT and Garrity GM, *Bergey's Manual of Systematic Bacteriology. Vol. 2. The Proteobacteria*, 2nd edition. Springer-Verlag, New York, NY (2005).

25 Dworkin M, Falkow S, Rosenberg E, Schleifer K-H and Stackebrandt E, *The Prokaryotes. Vol. 5. Proteobacteria: Alpha and Beta Subclasses*. Springer-Verlag, New York, NY (2006).

26 Sutton D, Fothergill A and Rinaldi M, Key Pathogenic and Opportunistic Fungi *(in Russian)*. Mir, Moscow (2001).

27 Watanabe T, *Pictorial Atlas of Soil Fungi: Morphologies of Fungi and Key Species*, 2nd edition. CRC Press, Boca Raton, FL (2002).

28 Pradeep FS, Begam MS, Palaniswamy M and Pradeep BV, Influence of culture media on growth and pigment production by *Fusarium moniliforme* KUMBF1201 isolated from paddy field soil. *World Appl Sci J* **22**:70–77 (2013).

29 Narasimhan B and Peppas NA, The role of modeling in the development of future controlled release devices, in *Controlled Drug Delivery: Challenges and Strategies*, ed. by Park K. American Chemical Society, Washington, DC, pp. 529–557 (1997).

30 Siepmann J and Peppas NA, Modelling of drug release from delivery systems based on hydroxypropyl nethylcellulose (HPMC). *Adv Drug Deliv Rev* **48**:139–157 (2001).

31 Savenkova L, Gercberga Z, Muter O, Nikolaeva V, Dzene A and Tupureina V, PHB-based films as matrices for pesticides. *Process Biochem* **37**:719–722 (2002).

32 Korsmeyer RW, Gurny R, Doelker E, Buri P and Peppas NA, Mechanism of solute release from porous hydrophilic polymers. *Int J Pharm* **15**:25–35 (1983).

33 Catellani P, Colombo P, Vitali T and Caramella C, Modified release tablet formulation. *Prog Biomed Eng* **5**:169–174 (1988).

34 Akbuga J, Use of chitosonium malate as a matrix in sustained-release tablets. *Int J Pharm* **89**:19–24 (1993).

35 Quadir MA, Rahman MS, Karim MZ, Akter S, Awkat MT and Reza MS, Evaluation of hydrophobic materials as matrices for controlled-release drug delivery. *Pak J Pharm Sci* **16**:7–28 (2003).

36 Sato H, Miyagawa Y, Okabe T, Miyajima M and Sunada H, Dissolution mechanism of diclofenac sodium from wax matrix granules. *J Pharm Sci* **86**:929–934 (1997).

37 Muñoz-Leoz B, Ruiz-Romera E, Antigüedad I and Garbisu C, Tebuconazole application decreases soil microbial biomass and activity. *Soil Biol Biochem* **43**:2176–2183 (2011).

38 Cycon M, Piotrowska-Seget Z, Kaczynska A and Kozdroj J, Microbiological characteristics of a sandy loam soil exposed to tebuconazole and λ-cyhalothrin under laboratory conditions. *Ecotoxicology* **15**:639–646 (2006).

39 Prudnikova S and Volova T, The Ecological Role of Polyhydroxyalkanoates: Laws of Biodegradation in the Environment and Interaction with Microorganisms *(in Russian)*. Krasnoyarsk writer, Krasnoyarsk (2012).

40 Voinova ON, Kalacheva GS, Grodnitskaya ID and Volova TG, Microbial polymers as a degradable carrier for pesticide delivery. *Appl Biochem Microbiol* **45**:384–388 (2009).

41 Volova T, Gladyshev M, Trusova M and Zhila N, Degradation of polyhydroxyalkanoates in eutrophic reservoir. *Polym Degrad Stab* **92**:580–586 (2007).

42 Volova TG, Boyandin AN, Vasiliev AD, Karpov VA, Prudnikova SV, Mishukova OV *et al.*, Biodegradation of polyhydroxyalkanoates (PHAs) in tropical coastal waters and identification of PHA-degrading bacteria. *Polym Degrad Stab* **95**:2350–2359 (2010).