Fungicidal activity of slow-release P(3HB)/TEB formulations in wheat plant communities infected by *Fusarium moniliforme*

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ABSTRACT

Fungicidal activity of experimental tebuconazole (TEB) formulations was investigated in laboratory soil ecosystems in wheat plant communities infected by plant pathogen *Fusarium moniliforme*. Tebuconazole was embedded in the matrix of degradable polymer of microbial origin, poly-3-hydroxybutyrate, shaped as films and microgranules. These formulations were buried in the soil with wheat plants, and their efficacy was compared with that of commercial formulation Raxil and with the effect of pre-sowing treatment of seeds. In the experiment with the initially infected seeds and a relatively low level of natural soil infection caused by *Fusarium* fungi (3.1×10^3 CFU·g^{-1}), the effects of the experimental P(3HB)/TEB formulations and Raxil were comparable. However, when the level of soil infection was increased by adding *F. moniliforme* spores (1×10^6 spores g^{-1} soil), P(3HB)/TEB granules and films reduced the total counts of fungi and the abundance of *F. moniliforme* more effectively than Raxil. Seed treatment or soil treatment with Raxil solution showed an increase in the percentage of rot-damaged roots in the later stages of the experiment. In the early stage (between Days 10 and 20), the percentage of rot-damaged roots in the soil with TEB embedded in the slowly degraded P(3HB) matrix was similar to that in the soil with Raxil. However, the efficacy of P(3HB)/TEB formulations lasted longer, and in later stages (between Days 20 and 30), the percentage of rot-damaged roots in that group did not grow. In experiments with different TEB formulations and, hence, different fungicidal activities, the increase in plant biomass was 15-17 to 40-60% higher than in the groups where TEB was applied by using conventional techniques.

KEYWORDS: *tebuconazole, poly-3-hydroxybutyrate, fungicidal effect, Fusarium moniliforme, wheat plant communities, root rot*

1. Introduction

Weeds, insects, and diseases cause enormous damage to world agriculture, leading to crop losses of up to 40% of the total yield. Phytopathogenic fungi are responsible for about 10% of the crop loss. Modern efficient agriculture is impossible without fungicides. Such important sources of plant protein as maize, wheat, and rice, whose yields constitute over 55% of the total yield of cereal crops, are particularly susceptible to the adverse effects of pathogens. Annual production of grain as the major food source has exceeded 2250 million tons; cereal crops occupy more than 670 million ha (Zernovyye kultury, 2008).

Phytopathogenic fungi not only decrease the crop yield but also considerably impair the quality of the produce. To protect crops against fungal infections, farmers employ cultural control methods as well as seed treatment and soil application of chemicals inhibiting the growth of plant pathogens. One of the modern approaches to plant protection is the use of composite formulations, or tank-mixes. The tank-mixes usually contain both biological and chemical pesticides and, thus, exert strong protective and beneficial effects. Moreover, they do not significantly inhibit plant growth and are less toxic to plants. Consequently, fewer treatments are needed, and control measures become less expensive.

Among the great diversity of modern fungicides, triazole chemicals occupy a special place. One of them is tebuconazole (TEB). TEB is an effective multifunctional systemic fungicide used to protect a number of agricultural crops (maize, wheat, rice, barley, rape, vineyards, etc.) against rots, powdery mildew, rust, and leaf blotches. TEB rapidly penetrates into the plants through both their vegetative organs and roots. It inhibits ergosterol synthesis, preventing the formation of cell membranes, and disrupts metabolic processes, causing the death of pathogens. The most common way of using TEB is to treat seeds prior to planting, but this may decrease seed germination ability, suppress the growth of seedlings, and inhibit root growth (Yang et al., 2014). Moreover, TEB used as seed dressing is rather quickly depleted, and the vegetative organs of plants have to be sprayed with the fungicide. However, when TEB is used as suspensions or emulsions to spray the vegetative organs of plants, the active ingredient is released too quickly to be sufficiently effective, and the fungicide has to be applied again, in greater quantities. As TEB is potentially phytotoxic, this method of application of the fungicide inhibits plant growth, causing economic losses and posing threat to the health of people and the environment (Zhang et al., 2015). Thus, new fungicide formulations need to be developed to increase the efficacy of TEB and minimize its harmful effects on the environment.

The efficacy of pesticides is determined not only by the type of the active ingredient and its activity but also by the type of formulation (Tropin, 2007), which must preserve the useful properties of the active ingredient, prolong its effectiveness, and minimize its adverse effects on the useful biota and the entire environment.

The newest trend in research is 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, which are degraded in soil and other biological media by soil microflora to form products that are harmless to living and nonliving nature and which are gradually released into the environment. Research aimed at designing slow-release tebuconazole formulations was started quite recently. A number of authors described TEB formulations shaped as microparticles and microcapsules based on various materials: poly(methyl methacrylate) and poly(styrene-co-maleic anhydride) (Asrar et al., 2004); ethyl cellulose (Yang et al., 2014); silica nanospheres (Qian et al., 2013).

Natural degradable polymers synthesized by microorganisms – the so-called polyhydroxyalkanoates (PHAs) – have been studied as potential carriers of pesticides for a relatively short time. The long degradation times of PHAs, which are eventually degraded by natural microflora to such harmless products as CO_2 and H_2O, and their ability to be processed into polymer products from solutions, emulsions, powders, and melts make them suitable materials for constructing slow-release formulations that can be applied to soil and used as pre-emergent pesticides. Recent studies showed that poly-3-hydroxybutyrate [P(3HB)] – the best studied representative of PHAs – was a suitable degradable material for constructing slow-release formulations of herbicides Zellek Super (Prudnikova et al., 2013) and metribuzin (Volova et al., 2016). A study was carried out in which TEB was embedded in films, microgranules, and microparticles of P(3HB), and its fungicidal activity against *Fusarium moniliforme* was comparable to that of commercial formulation Raxil (Volova et al., 2015). TEB release kinetics, degradation of P(3HB) matrix, and inhibition of *Fusarium* growth were investigated in laboratory soil microecosystems (unpublished data).

The purpose of the present study was to investigate the efficacy of slow-release P(3HB)/TEB formulations in wheat plant communities infected by *Fusarium moniliforme*.

2. Materials and methods

2.1. Poly(3-hydroxybutyrate) characterization

Poly(3-hydroxybutyrate) [P(3HB)] was used as a matrix for embedding tebuconazole (TEB). The polymer was synthesized by using bacterium *Cupriavidus eutrophus* B10646, at the Institute of Biophysics SB RAS. Cells were aseptically cultured in a 7.5-L BioFlo/CelliGen115 (New Brunswick, U.S.) fermentor, following the previously developed technology (Volova et al., 2013; 2014). Polymer was extracted from cells with chloroform, and the extracts were precipitated using hexane. The extracted polymers were re-dissolved and precipitated again 3-4 times to prepare homogeneous specimens. P(3HB) had the following physicochemical parameters: weight average molecular weight (M_w) 920 kDa; polydispersity (Đ) 2.52; degree of crystallinity 74%; melting point and thermal decomposition temperature 179.1 and 284.3 °C, respectively.

2.2. 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 (g mol^{-1}): 307.82. Solubility in water: 36 mg L^{-1} at 20°C. Melting point is 104.7°C. The substance is not hydrolyzed at pH of between 4 and 9; it is stable upon exposure to light and elevated temperature. The time of degradation in soil is 177 days.

2.3. Wheat

Experiments were performed in communities of soft spring wheat cv. Altaiskaya 70.

2.4. Plant pathogen

Fungi of the genus *Fusarium* Link (*F. moniliforme* J. Sheld) were used in experiments. Inoculum was prepared by growing fungi in culture tubes on malt extract agar (MEA, Sigma, U.S.) for 14 days. Then, spore suspension was prepared in sterile tap water, 5.2×10^7 spores ml^{-1}. The number of spores was counted in the Goryaev chamber. Ten ml *F. moniliforme* spore suspension was added to each container with soil.

2.5. Preparation of fungicide formulations

Films, and granules, with tebuconazole loadings of 25% 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 P(3HB)/TEB solution was mixed on an MR Hei-Standard magnetic stirrer (Heidolph, Germany) for 2-3 h (until completely dissolved) and heated to 35-40°C under reflux condenser for 3-4 h. Then, the P(3HB)/TEB solution was cast in Teflon-coated metal molds, where 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, U.S.) for 3-4 days, until complete solvent evaporation took place. Films were cut into 5×5 mm squares, which were weighed on the analytical balance of accuracy class 1 Discovery (Ohaus, Switzerland). The film thickness was measured with an EDM-25-0.001 digital micrometer (Legioner, Germany).

TEB granules were prepared as follows. The necessary amounts of TEB were added to the 10% P(3HB) solution, which was thoroughly mixed. A peristaltic pump was used to drop the P(3HB)/TEB solution into the sedimentation tank that 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 was insoluble in hexane, when the drops passed through the layer of the precipitating agent, granule formation occurred. The granules had a diameter of 3 mm.

2.6. Characterization of soil microecosystems with plants

The effect of the experimental TEB formulations on plant pathogenic fungi was studied in laboratory soil microecosystems. Soil microecosystems were prepared as follows. The agrogenically-transformed soil (from the village of Minino, the Krasnoyarsk Territory, Siberia, Russia) was placed into 500-cm^3 plastic containers (500 g soil per container). Wheat seeds were sown into the soil, 100.45 g seeds per 1 m^2. The plants were grown in the Conviron A1000 growth chamber (Canada) for 30 d under stable conditions: illumination 100-300 μmoles/m^2/s under the 12L:12D photoperiod, temperature of 18-25°C, and humidity of 65%.

Two experiments were carried out, each lasting 30 days. In Experiment 1, wheat seeds were infected with plant pathogens. There were five experimental groups. In Group 1 (negative control), seeds sown into the soil had not been treated with the fungicide, and no TEB was added to the soil. In Group 2 (positive control), at the time of planting, the soil was treated with commercial formulation Raxil at a

concentration comparable with the TEB concentrations in the treatments: 3 μg TEB/g soil. In two treatment groups, the seeds had not been treated with the fungicide prior to sowing, but P(3HB)/TEB films (Group 3) and granules (Group 4) were buried in the soil. In Group 5, the seeds were soaked in the Raxil solution for 10 min prior to sowing; no TEB was added to the soil.

In Experiment 2, the efficacy of the P(3HB)/TEB formulations was tested under harsher conditions. In addition to using the infected wheat seeds, we also added spores of the plant pathogen *F. moniliforme* into the soil. There were four experimental groups. In Group 1, the wheat seeds had not been treated with the fungicide before sowing; no TEB was added to the soil. In the treatment groups, the seeds sown into the soil had not been treated with the fungicide, but P(3HB)/TEB films (Group 2) and granules (Group 3) were buried in the soil. In Group 4 (control), at the time of planting, the soil was treated with commercial formulation Raxil at a concentration comparable with the TEB concentrations in the treatments: 3 μg TEB/g soil.

2.7. Chemical and microbial compositions of the soil

The analysis of the chemical composition of the soil included measuring 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 analyzed by conventional methods of soil microbiology (Netrusov et al., 2005). 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). The ecological-trophic groups of microorganisms were identified by plating soil samples onto diagnostic media. Ammonifying (copiotrophic) bacteria were identified on NA; microorganisms (including actinomycetes) capable of utilizing mineral nitrogen (prototrophs) were identified on starch ammonium agar (SAA). Microorganisms involved in mineralization of humus substances (oligotrophs) were identified on soil extract agar (SEA) (Netrusov et al., 2005).

Soil microscopic fungi were identified by their micro- and macro-morphological features (the structure and color of colonies and the structure of mycelium and spore-forming organs), which are objective parameters for identifying these microorganisms (Sutton et al., 2001; Watanabe, 2002). All manipulations were performed in triplicate.

2.8. Analysis of TEB concentrations

During the experiment, to monitor changes in TEB concentrations in the soil, tebuconazole was extracted from soil and water with chloroform. To determine 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 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, U.S.), 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 per min; 5 min isothermal conditions. TEB concentration was determined using the calibration curve; the curves were constructed as described elsewhere (Volova et al., 2015).

2.9. Evaluation of P(3HB)/TEB fungicidal activity

The fungicidal activity of the experimental P(3HB)/TEB formulations was compared with the activity of commercial formulation Raxil by evaluating changes in the following parameters: the counts of *F. moniliforme* in soil; the percentage of plant roots affected by the rot disease; the amount of aboveground biomass of the plants (determined by weighing the biomass preliminarily dried to constant weight). These parameters were measured at Days 10, 20, and 30 of the experiment.

Counting of the total microscopic fungi, including *F. moniliforme*, was performed by plating soil suspension onto Petri dishes with malt extract agar, which was supplemented with chloramphenicol (100 μg L^{-1} of the medium) to suppress cell growth. All platings were performed in triplicate from 10^2-10^5 dilutions of soil suspension. The dishes were incubated at a temperature of 25°C for 7-10 days. 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 (Sutton et al., 2001; Watanabe, 2002).

The degree to which the roots were damaged by fusarium infection was evaluated as follows: the plant roots were carefully removed from the soil, rinsed first in running water and then three times in sterile tap water, and placed onto paper filters wetted to the maximum water holding capacity in Petri dishes. The Petri dishes were incubated in the thermostat at 25 °C. The degrees to which the roots were infected by the fungi *Fusarium*, *Alternaria*, and *Bipolaris* were determined at Day 5-10, by microscopic examination of mycelium and spore formation of the fungi on wheat roots.

The initial degree of infection of wheat seeds by plant pathogens (internal infection) was determined by germinating the seeds in Petri dishes on sterile nutrient medium MEA (Russian Federal Standard 12044-93). The state of the plants and their growth were evaluated by photographing the plant communities and the roots.

2.10. 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.

3. Results

Laboratory soil ecosystems with wheat plants contained agrogenically-transformed field soil (from the village of Minino, the Krasnoyarsk Territory): cryogenic-micellar agro-chernozem with 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 nitrate nitrogen $N\text{-}NO_3$ – 6 mg kg^{-1}, and P_2O_5 –6 and K_2O – 22 mg 100 g^{-1} soil (according to Machigin). The soil had high mineralization and oligotrophy coefficients (1.52 and 11.74, respectively), indicating soil maturity and low contents of available nitrogen forms.

The total number of organotrophic bacteria was 16.3±5.1 x 10^6 CFU g^{-1}. The phylum *Actinobacteria* dominated and included the following genera: *Streptomyces* (24%), *Arthrobacter* (18%), and *Corynebacterium* (12%); *Pseudoxanthomonas* were the major Gram-negative rods (12%) in the soil samples. Among microscopic fungi, the *Penicillium* species dominated (58-65%); fungi of the genera *Fusarium, Trichoderma*, and *Aspergillus* constituted 8-11% of the fungal population in soil samples. *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.

Equal amounts of wheat seeds were sown into the soil-filled containers: 100.45 g seeds per 1 m^2. In the control groups, Raxil was added to the soil simultaneously with seed sowing. In the treatment groups, P(3HB)/TEB formulations were buried in soil 10 days before the sowing. Our previous studies showed that it took some time for soil microorganisms to get attached to the surface of the polymer samples and get adapted to the polymer matrix of P(3HB) as the substrate. The release of the active ingredient such as TEB from the polymer matrix only occurred when the polymer was gradually degraded, and the release rate was determined by the kinetics of polymer biodegradation (Volova et al., 2015). By the time of sowing, in the group with the P(3HB)/TEB films, TEB concentration in the soil was somewhat lower than in the control group – 2.7 μg TEB/g soil. In the soil containing P(3HB)/TEB granules, TEB concentration was lower – 1.1 μg TEB/g soil.

3.1. The effect of tebuconazole on plant pathogens and saprotrophic microflora of wheat as dependent on the fungicide delivery method

Phytosanitary analysis of wheat seeds germinated on the nutrient medium showed the presence of infections caused by the fungi of the genera *Fusarium* Link, *Alternaria* Nees, and *Bipolaris* Shoem. (Fig. 1, 2). Wheat seeds infected by plant pathogens constituted 9.5±1.2%, 5.6±0.2% of which were infected by *Fusarium* species. Thus, natural infections of the seeds were caused not only by *Fusarium* species, which were detected in the initial soil, but also by the phytopathogenic microscopic fungi that developed when the seeds containing internal infection were germinated.

Analysis of microscopic fungi in soil samples showed that in the initial soil, their total counts reached $(28.3\pm9.4)\times10^3$ CFU·g^{-1}, while at the end of the experiments, this number had dropped by a factor of 2.3, to $(12.3\pm2.5)\times10^3$ CFU·g^{-1} (Fig. 3A). That was caused by changes in the structure of the microbial community that occurred due to the selective effects of wheat on the rhizosphere microflora: wheat roots exuded organic substances readily available to bacteria, leading to an increase in their abundance. The total bacterial counts increased considerably: from $(16.3\pm5.2)\times10^6$ CFU·g^{-1} in the initial soil to $(164.7\pm8.5)\times10^6$ CFU·g^{-1} in the soil samples analyzed after 30 days of the experiment. Thus, as wheat

plants were growing, the structure of the microbial community was changing. The decrease in the counts of microscopic fungi seemed to be caused by their competition with rhizosphere bacteria.

The degrees of infection caused by phytopathogenic fungi in the initial soil differed considerably between the two experiments, and that resulted in different efficacy of TEB. In Experiment 1, wheat was grown in the soil with natural infection mainly caused by *Fusarium* fungi, whose counts reached 3.1×10^3 CFU·g^{-1}. In Experiment 2, when the soil was additionally inoculated with *F. moniliforme* spores, the counts of plant pathogens in the initial soil were higher by three orders of magnitude – 1×10^6 spores g^{-1} soil.

The evaluation of the fungicidal effect of experimental TEB formulations in Experiment 1, with the soil containing relatively low concentrations of plant pathogens, did not reveal any significant differences between the effects of the experimental formulations and commercial formulation Raxil. Both P(3HB)/TEB and Raxil decreased not only the counts of such plant pathogens as *Fusarium*, *Alternaria*, and *Bipolaris*, but also the total counts of microscopic fungi – by a factor of 1.7-2.3 compared to the TEB-free soil (negative control) (Fig. 3A).

A different result was achieved in Experiment 2, with soil additionally inoculated with *F. moniliforme* spores. At the time of sowing, the total counts of saprotrophic fungi and the abundance of plant pathogens of the genus *Fusarium* (including *F. moniliforme* and minor species) reached 25.2×10^3 CFU g^{-1} and 1×10^6 CFU g^{-1}, respectively, but at Day 30, in the negative control, the total counts of the introduced *Fusarium* fungi dropped by three orders of magnitude – to 21.2×10^3 CFU g^{-1}, and the counts of indigenous saprotrophic microflora decreased by a factor of 5.8 – to 4.9×10^3 CFU g^{-1} (Fig. 3B). That was most probably caused by trophic and competitive interactions between the introduced species and the indigenous microflora (Simberloff, Stiling, 1996; Ricciardi et al., 2013). In the soil with Raxil, the counts of phytopathogenic and saprotrophic fungi were 8.4×10^3 and 9.2×10^3 CFU g^{-1}, respectively. Neither of the experimental P(3HB)/TEB formulations inhibited the growth of saprotrophic fungi, and both were 3.0-3.6 times more effective against *F. moniliforme* than Raxil. Thus, in the soil with a high level of *F. moniliforme* infection, the fungicidal activity of the experimental P(3HB)/TEB formulations was higher than that of the commercial Raxil.

3.2. The state of wheat roots and the degree of rot infection

The roots of the initially infected wheat plants grown in the soil infected by plant pathogens were damaged by rot. In Experiment 1, with naturally infected soil, fusarium infection was detected in all plant groups, including the groups with tebuconazole added to the soil, in the first 10 days (Fig. 4 A, B). The reason for that was the internal infection of the seeds, which developed in the early, seedling, stage. Then, in the negative control group, the infection of the roots caused by phytopathogenic microscopic fungi increased. Between Days 10 and 30, the percentage of roots damaged by rot increased from 17 to 30% (of the total root mass). The major contribution to the etiology of root rot was made by fusarium infection – 50-80% of all infections.

In the soil with commercial formulation Raxil, the degree of root rot infection was significantly lower than in the negative control, but the infection of the roots increased, reaching 25% by the end of the experiment. The pre-sowing seed treatment with Raxil restrained the development of the overall root infection in the first 10 days, but then rot infection damaged more roots and persisted at a high level – 21-27% (Fig. 4A)

The experimental P(3HB)/TEB films were effective against all root rots, including fusarium infection, restraining their development. Between Days 10 and 20, the efficacy of this formulation was comparable to that of Raxil. Moreover, TEB embedded in the polymer matrix showed extended fungicidal effect, and between Days 20 and 30, root infection did not increase, in contrast to the groups with Raxil and pretreated seeds (Fig. 4A, B). P(3HB)/TEB granules did not show any fungicidal effect in the first 10 days. TEB release from the granules occurred at a slower rate than from the films, and TEB concentration in the soil was too low: 1.1 μg/g soil versus 2.7 μg/g soil in the soil with P(3HB)/TEB films. At Day 30, however, inhibition of root rot development in this group was comparable to the effect of commercial formulation Raxil.

In Experiment 2, with *F. moniliforme* spores added to the soil, the percent of the damaged roots was considerably higher (Fig. 5 B). In the group with no TEB added to the soil (negative control), after 30 days, the percentage of infected roots reached 61.5%, the roots damaged by fusarium infection caused by *F. moniliforme* constituting 53.8%. TEB in the form of commercial Raxil (positive control) was effective against root rots in the early stage. Between Days 10 and 20, the total percentage of infected roots was 1.8-1.9 times lower and the percentage of fusarium infection-damaged ones 3.3-2.2 times lower than in

the negative control group. Later, however, the fungicidal effect of Raxil became weaker, and the percentage of infected roots increased. Similarly to Experiment 1 (with the naturally infected soil), the fungicidal effects of the two experimental P(3HB)/TEB formulations was comparable to that of commercial formulation Raxil in the first 20 days, but it lasted longer and restrained the development of root rots, including fusarium infection, during the final stage (Days 20-30). At Day 30, the total percentage of the infected roots was 1.6 times lower and the percentage of fusarium infection 1.4 time lower than in the group with Raxil (Fig. 5A, B).

Figure 6 shows photographs of wheat roots at Day 30 of the experiment in the groups with different TEB delivery methods, illustrating the beneficial effect of the experimental P(3HB)/TEB formulations.

3.3. Characterization of wheat plant communities grown on the soil with different TEB formulations

Results of evaluating the productivity of wheat communities growing on soils infected by *F. moniliforme* to various degrees, with different percentages of roots damaged by root rot, are shown in Figure 7. In Experiment 1, with milder damage to roots, measurements of aboveground biomass in the early stage (10 days) in the negative and positive control groups and in the group with pretreated seeds gave comparable values. In the treatment groups, the biomass was somewhat (15-20%) lower (Fig. 7 A). At Day 20, however, all groups showed comparable values. In the later stage (at Day 30), the biomass of the plants grown without TEB amounted to 180 g/m^2, which was 40% lower than in the group with Raxil and pretreated seeds and 60% lower than in the treatment groups. The difference in biomass between the groups with pretreated seeds and Raxil, on the one hand, and the groups with the experimental P(3HB)/TEB formulations, on the other, reached about 15-17%.

The effectiveness of the experimental P(3HB)/TEB formulations was more noticeable in Experiment 2, with higher degrees of soil infection and root damage caused by rot (Fig. 7 B). At Day 30, in the group with Raxil, the aboveground biomass reached 190 g/m^2, while in the treatment groups, it was 26% higher – 233-240 g/m^2.

4. Discussion

In this work, we investigated the fungicidal activity of experimental formulations of tebuconazole embedded in the matrix of natural degradable polymer poly-3-hydroxybutyrate in laboratory wheat plant communities infected by *Fusarium moniliforme* – a fusarium infection causal agent.

Fusarium infection, causing development of root and foot rot of cereals, results in yield decrease and impairment of grain quality. Yield losses may reach between 5 and 30%. Causal agents of fusarium infection are ubiquitous *Fusarium* fungi, which damage wheat, rye, barley, grass, and, to a lesser extent, oat and many other crops (over 200 species). Fusarium root rot may affect the ears and grain, contaminating the grain with mycotoxins and making it unsuitable and even unsafe food for humans and animals. *Fusarium* fungi are producers of very potent mycotoxins, the most common of which are fusarium toxins such as deoxynivalenol (vomitoxin), zearalenone, and T-2 mycotoxin (Kravchenko, Tutelyan, 2005; Binder et al., 2007).

The spread of fusarium infection is mainly associated with the extensive development of agroecosystems and an increase in the area occupied by cereals. This upsets the ecological balance in the soil – plant system. The composition of the soil microbial community changes: the percentage of harmful microflora increases with the growing abundance of microscopic fungi, which produce toxins hazardous to the plants, animals, and even humans.

Modern means of protection of cereal crops against root rots should be based on the ecosystem approach to reduce the adverse effects of human-made pollutants and to produce health foods. The most common way to control fusarium root rot is to treat seeds with fungicides prior to sowing, but this treatment cannot protect plants throughout their growing season; moreover, it may decrease the germinating capacity of seeds and inhibit the development of the roots (Yang et al., 2014). Therefore, it is important to develop and use ecofriendly targeted slow-release formulations capable of inhibiting the development of plant pathogens without posing significant risks to useful biota and the entire environment.

Construction of formulations in which agrochemicals are embedded in the degradable matrix seems to be a propitious approach. Gradual degradation of the matrix in soil should enable gradual and targeted release of the active ingredient. This is the way to reduce the amounts of the chemicals added to the soil and decrease their uncontrolled distribution and accumulation in agroecosystems. It is very important to find proper material to be used as a matrix for embedding agrochemicals.

Natural degradable polymers – polyhydroxyalkanoates (PHAs) – are promising materials for constructing new-generation formulations of agrochemicals. PHAs are synthesized by prokaryotes as energy and carbon storage; they are degraded both intracellularly and extracellularly to safe products (CO_2 and H_2O) by soil and water microflora (Sudesh et al., 2000; Jendrossek, 2001). Although this is a relatively new application of PHAs, they have been successfully used to embed herbicides (Prudnikova et al., 2013; Volova et al., 2016a), fungicides (Savenkova et al., 2002; Volova et al., 2015), and nitrogen fertilizers (Volova et al., 2016b).

In this work, we investigated fungicidal activity of tebuconazole (TEB) in wheat plant communities infected by *Fusarium*. TEB was embedded in the degradable matrix of poly-3-hydroxybutyrate [P(3HB)] in the form of films and microgranules. Tebuconazole is an effective systemic fungicide that penetrates not only into the vegetative organs of the plants but also into their roots, and, thus, it can be used as both post- and pre-emergence fungicide (Zhang et al., 2015). Experiments in laboratory soil ecosystems with higher plants were conducted after preliminary studies, in which we had developed the process of embedding TEB into the polymer matrix and investigated the properties of the formulations, kinetics of degradation of the polymer matrix, and kinetics of tebuconazole release into water and soil (Volova et al., 2015). Those studies showed that the fungicidal effect of P(3HB)/TEB films, microparticles, microgranules, and pellets buried in the soil containing plant pathogens was comparable with that of commercial formulation Raxil Ultra, but the fungicidal activity of the experimental formulations was related to the kinetics of degradation of the polymer matrix and TEB release into the soil (unpublished data).

Tebuconazole-based formulations are commonly used to protect cultivated plants, including socially and economically important cereal crops (wheat, maize, etc.). In addition to its fungicidal effect, TEB also regulates plant growth. However, this fungicide is potentially toxic to plants if it is used in large quantities to spray leaves or treat seeds. The adverse effects of the fungicide are expressed as low germinating capacity of seeds, inhibition of the growth of seedlings, and, especially, inhibition of root elongation (Yang et al., 2014). The unfavorable regulatory effect of TEB occurs via the following mechanism: triazoles, TEB, in particular, shift the balance of phytohormones in plant tissues and inhibit biosynthesis of gibberellins, causing a temporary increase in the content of abscisic acid in plants (Grossman, 1990; Fletcher et al., 2000).

Several studies compared the efficacy of slow-release TEB formulations with that of free TEB against such plant pathogens as wheat rust *Puccinia recondita* (Asrar et al., 2004), root rot pathogens *Biopolaris* and *Fusarium* (Khalikov et al., 2013), maize head smut (Yang et al., 2014), and wheat powdery mildew (Zhang et al., 2015). TEB encapsulated in microcapsules of ethyl celluloses did not affected adversely the germinating capacity of maize seeds, in contrast to Raxil. Phytohormonal analysis showed that the microencapsulated and continuously released tebuconazole had a beneficial effect on the balance of phytohormones during maize seed germination. Encapsulated TEB provided better protection against maize head smut than the conventionally used Raxil (Yang et al., 2014). Zhang et al. (2015) reported a study in which cells of cyanobacterium *Synechocystis* sp. *strain PCC* 6803 used to encapsulate tebuconazole were coated with the rubber-like urea/formaldehyde material. That formulation remained 80% effective against powdery mildew for 12 days, as TEB was slowly and steadily released from microgranules, while the efficacy of Raxil was 52.25%. In another study (Asrar et al., 2004), TEB was encapsulated into microcapsules prepared from poly(methyl methacrylate) (PMMA) and poly(styrene-co-maleic anhydride) (PSMA) by using different techniques and used to spray plants. The bio-effectiveness of microparticles against wheat rust *Puccinia recondita* in the wheat varied depending on the form and production technique employed; however, all forms of microparticles provided better protection against wheat rust than commercial foliar-applied tebuconazole, Raxil.

In this study, we evaluated the efficacy of the experimental P(3HB)/TEB formulations against plant pathogen *Fusarium moniliforme* in laboratory wheat plant communities and compared it with the results achieved by pre-sowing seed treatment and soil treatment with a Raxil solution. In the experiment with the initially infected seeds and a relatively low level of natural soil infection caused by *Fusarium* fungi (3.1×10^3 CFU·g^{-1}), the effects of the experimental P(3HB)/TEB formulations and Raxil were comparable. However, when the level of soil infection was increased by adding *F. moniliforme* spores (1×10^6 spores g^{-1} soil), P(3HB)/TEB granules and films reduced the total counts of fungi and the abundance of *F. moniliforme* more effectively than Raxil.

As fusarium infection causes root rot in the plants of any age, we examined the state and degree of infection of the wheat roots during the experiments with different modes of TEB delivery. The commonly used seed treatment or soil treatment with Raxil solution showed a significant decrease in the

percentage of rot-damaged roots, which, though, increased in later stages of the experiment. In the early stage (between Days 10 and 20), the percentage of rot-damaged roots in the soil with TEB embedded in the slowly degraded P(3HB) matrix was similar to that in the soil with Raxil. However, the efficacy of P(3HB)/TEB formulations lasted longer, and in later stages (between Days 20 and 30), the percentage of rot-damaged roots in that group did not grow, in contrast to the group with the soil treated with Raxil and in the group with the pre-treated seeds.

Differences in the fungicidal activity of TEB could be seen not only in the dissimilar levels of soil infection caused by the plant pathogen and percentages of rot-damaged roots but also in different plant growth, evaluated by the increase in aboveground biomass. In experiments with different TEB formulations and, hence, different fungicidal activities, the increase in plant biomass was 15-17 to 40-60% higher than in the groups where TEB was applied by using conventional techniques.

5. Conclusion

The fungicidal activity of the experimental slow-release formulations of TEB embedded in the matrix of degradable poly-3-hydroxybutyrate against fusarium infection of wheat was comparable to that of TEB in commercial formulation Raxil in early stages. In the later stages, P(3HB)/TEB formulations more effectively suppressed the development of *Fusarium* in soil and inhibited the growth of plant root rot.

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References

Asrar J, Ding Y, La Monica RE, Ness LC. Controlled release of tebuconazole from a polymer matrix microparticle: release kinetics and length of efficacy. J Agric Food Chem 2004;52:4814–4820. doi**:**10.1021/jf0306385.

Binder EM, Tan LM, Chin LM, Handl J, Richard J. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Anim Feed Sci Technol 2007;137:265-282. doi:10.1016/j.anifeedsci.2007.06.005.

Fletcher RA, Gilley A, Davis TD, Sankhla N. Triazole as plant growth regulators and stress protectants. Hortic Rev 2000;24:55-138.

Fletcher RA, Gilley A, Sankhla N, Davis TD. Triazoles as plant growth regulators and stress protectants. In: Janick J, ed. Horticultural Reviews. Oxford: John Wiley and Sons Inc; 1999. p. 55-138.

GOST 12044-93. Semena selkokhozyaistvennykh kultur. Metody opredeleniya zarazhennosti boleznyami. (Russian Federal Standard 12044-93. Seeds of farm crops. Methods of determining infections.) Publication date: 01.01.2004. Latest revision date: 19.07.2010 (in Russian)

Grossmann K. Plant growth retardants as tools in physiological research. Physiol Plant 1990;78:640-648. doi: 10.1111/j.1399-3054.1990.tb05254.x.

Jendrossek D. Microbial degradation of polyesters. Adv Biochem Eng Biotechnol 2001;71:293–325.

Khalikov S, Dushkin A, Dvaleti R, Evsenko V. The creation of innovatice fungicides based on tebuconazole and mechanochemical processes. Fundamental'nie Issledovaniya 2013;10:2695–2700 (in Russian).

Kravchenko LV, Tutelyan VA. Review. Biosafety. Mycotoxins – natural contaminants of wheat. Voprosy pitaniya (Dietary issues) 2005;3:3-13 (in Russian).

Netrusov AI, Egorov MA, Zakharchuk LM and Kolotilova NN. Workshop on microbiology: a textbook for students of higher educational institutions. Moscow: Academy Publ; 2005 (in Russian).

Prudnikova SV, Boyandin AN, Kalacheva GS, Sinskey AJ. Degradable polyhydroxyalkanoates as herbicide carriers. J Polym Environ 2013;21:675–682. doi:10.1007/s10924-012-0561-z.

Qian K, Shi T, He S, Luo L, Liu X, Cao Y. Release kinetics of tebuconazole from porous hollow silica nanospheres prepared by miniemulsion method. Micropor Mesopor Mater 2013;169:1-6. doi:10.1016/j.micromeso.2012.10.017.

Ricciardi A, Hoopes MF, Marchetti MP, Lockwood JL. Progress toward understanding the ecological impacts of nonnative species. Ecological Monographs 2013;83:263-282. doi:10.1890/13-0183.1.

Savenkova L, Gercberga Z, Muter O, Nikolaeva V, Dzene A, Tupureina V. PHB-based films as matrices for pesticides. Process Biochem 2002;37:719-722. doi:10.1016/S0032-9592(01)00263-1.

Simberloff D, Stiling P. How risky is biological control? Ecology 1996;77:1965-1974. doi: 10.2307/2265693.

Sudesh K, Abe H, Doi Y, Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 2000;25:1503–1555. doi:10.1016/S0079-6700(00)00035-6.

Sutton D, Fothergill A, Rinaldi M. Key pathogenic and opportunistic fungi. Moscow:Mir; 2001 (In Russian).

Tropin VP. Advanced formulations of pesticides and their application techniques. Zashchita i karantin rastenii (Plant protection and quarantine) 2007;7:32–33. (in Russian).

Volova T, Kiselev E, Shishatskaya E, Zhila N, Boyandin A, Syrvacheva D, Vinogradova O, Kalacheva G, Vasiliev A, Peterson I. Cell growth and accumulation of polyhydroxyalkanoates from CO_2 and H_2 of a hydrogen-oxidizing bacterium, *Cupriavidus eutrophus* B-10646. Biores Technol 2013;146:215–222. doi:10.1016/j.biortech.2013.07.070.

Volova T, Kiselev E, Vinogradova O, Nikolaeva E, Chistyakov A, Sukovatiy A, Shishatskaya E. A glucose-utilizing strain, *Cupriavidus euthrophus* B-10646: growth kinetics, characterization and synthesis of multicomponent PHAs. PLoS One 2014;9:1–15. doi:10.1371/journal.pone.0087551.

Volova TG, Zhila NO, Vinogradova ON, Shumilova AA, Prudnikova SV, Shishatskaya EI. Characterization of biodegradable poly-3-hydroxybutyrate films and pellets loaded with the fungicide tebuconazole. Environ Sci Pollut Res 2015. doi:10.1007/s11356-015-5739-1.

Volova TG, Zhila NO, Vinogradova ON, Nikolaeva ED, Kiselev EG, Shumilova AA, Shershneva AM, Shishatskaya EI. J Env Sci Health, Part B 2016a;51:113-125. doi:10.1080/03601234.2015.1092833.

Volova TG, Prudnikova SV, Boyandin AN. Biodegradable poly-3-hydroxybutyrate as a fertilizer carrier. J Sci Food Agric 2016b. doi:10.1002/jsfa.7621.

Watanabe T. Pictorial atlas of soil fungi: morphologies of fungi and key species. 2nd ed. Boca Raton: CRC Press LLC; 2002.

Yang D, Wang Na, Yan X, Shi J, Zhang M, Wang Z, Yuan H. Microencapsulation of seed-coating tebuconazole and its effect on physiology and biochemistry of maize seedlings. Coll Surf B Biointerfaces 2014;114:241–246. doi:10.1016/j.colsurfb.2013.10.014.

Zernovyye kultury: vyrashchivaniye, uborka i ispolzovaniye (Cereal crops: cultivation, harvesting, and use). Shpaar D, editor. Moscow: Publishing House DLV AGRODELO; 2008 (in Russian).

Zhang B, Zhang T, Wang Q, Ren T. Microorganism-based monodisperse microcapsules: encapsulation of the fungicide tebuconazole and its controlled release properties. RSC Advances 2015;32:25164–25170. doi:10.1039/C5RA01629K, Paper.

Figures

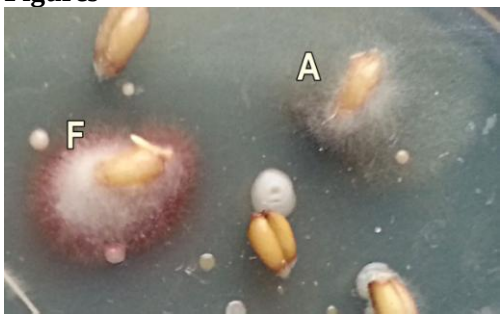

Fig. 1 Infected wheat seeds: A – *Alternaria sp.*, F – *Fusarium sp.*

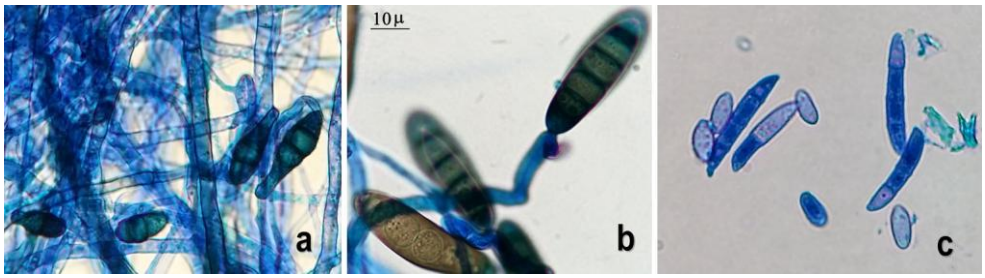

Fig. 2 Conidia of phytopathogenic fungi: a – *Alternaria sp.*, b – *Bipolaris sp.*, c – *Fusarium sp.*

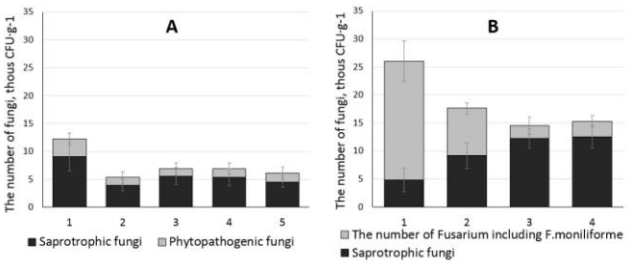

Fig. 3 The fungicidal effect of different forms of TEB in soil after 30 days; A – naturally infected soil, B – soil inoculated with spores of *Fusarium moniliforme*; *1* – negative control, *2* – positive control, *3* – P(3HB)/TEB film, 4 – P(3HB)/TEB granules, *5* – pre-sowing seed treatment by Raxil Ultra.

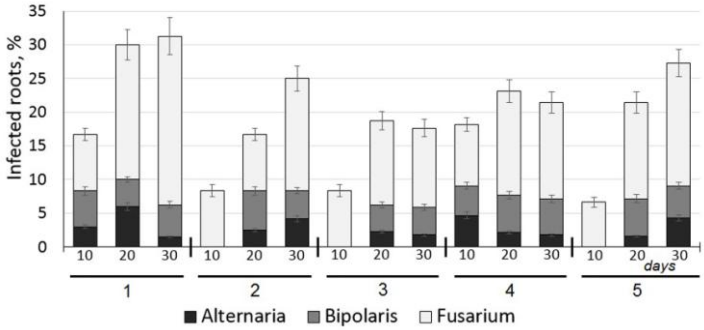

Fig. 4 The effect of TEB delivery mode on the percentage of wheat roots damaged by rot: *1* – negative control, *2* – positive control (Raxil applied to soil), *3* – P(3HB)/TEB films, *4* – P(3HB)/TEB granules, *5* – pre-sowing treatment of seeds with Raxil .

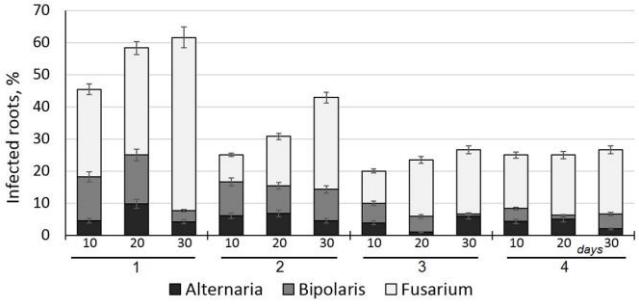

Fig. 5 The effect of TEB delivery mode on the percentage of wheat roots damaged by rot in the experiment with *Fusarium moniliforme* spores added to the soil: *1* – negative control, *2* – positive control (Raxil applied to soil), *3* – P(3HB)/TEB films, *4* – P(3HB)/TEB granules.

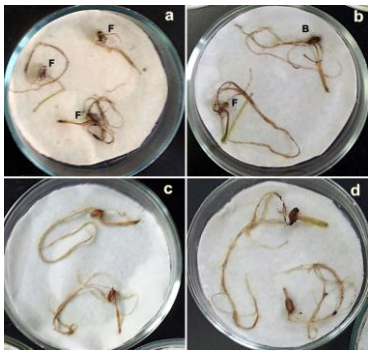

Fig. 6 Wheat root rot under different modes of TEB delivery: a – negative control, b – Raxil Ultra, c – P(3HB)/TEB films, d – P(3HB)/TEB granules; F – *Fusarium* infection, B – *Bipolaris* infection.

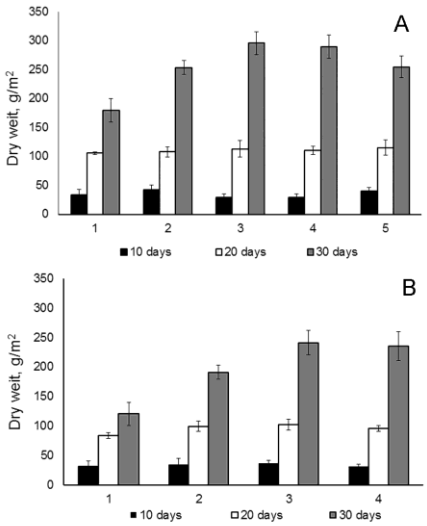

Fig. 7 The effect of TEB delivery mode on the increase in wheat aboveground biomass on the naturally infected soil (A) and on the soil to which *Fusarium* was added (B): *1* – negative control, *2* – positive control (Raxil applied to soil), *3* – P(3HB)/TEB films, *4* – P(3HB)/TEB granules, *5* – pre-sowing treatment of seeds with Raxil