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

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9
10 **ABSTRACT**

11 Fungicidal activity of experimental tebuconazole (TEB) formulations was investigated in laboratory soil
12 ecosystems in wheat plant communities infected by *Fusarium moniliforme*. TEB was embedded in the matrix of
13 poly-3-hydroxybutyrate, shaped as films and microgranules. These formulations were buried in the soil with wheat
14 plants, and their efficacy was compared with that of commercial formulation Raxil and with the effect of pre-sowing
15 treatment of seeds. In the experiment with the initially infected seeds and a relatively low level of natural soil
16 infection caused by *Fusarium* fungi, the effects of the experimental P(3HB)/TEB formulations and Raxil were
17 comparable. However, when the level of soil infection was increased by adding *F. moniliforme* spores, P(3HB)/TEB
18 granules and films reduced the total counts of fungi and the abundance of *F. moniliforme* more effectively than
19 Raxil. Seed treatment or soil treatment with Raxil solution showed an increase in the percentage of rot-damaged
20 roots in the later stages of the experiment. In the early stage (between Days 10 and 20), the percentage of rot-
21 damaged roots in the soil with TEB embedded in the slowly degraded P(3HB) matrix was similar to that in the soil
22 with Raxil. However, the efficacy of P(3HB)/TEB formulations lasted longer, and in later stages (between Days 20
23 and 30), the percentage of rot-damaged roots in that group did not grow. In experiments with different TEB
24 formulations and, hence, different fungicidal activities, the increase in plant biomass was 15-17 to 40-60% higher
25 than in the groups where TEB was applied by using conventional techniques.
26

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

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

65 The newest trend in research is development and agricultural use of environmentally safe new-generation
66 pesticides with targeted and controlled release of active ingredients embedded in biodegradable matrices or covered
67 with biodegradable coatings, which are degraded in soil and other biological media by soil microflora to form
68 products that are harmless to living and nonliving nature and which are gradually released into the environment.
69 Research aimed at designing slow-release tebuconazole formulations was started quite recently. A number of
70 authors described TEB formulations shaped as microparticles and microcapsules based on various materials:
71 poly(methyl methacrylate) and poly(styrene-co-maleic anhydride) (Asrar et al 2004); ethyl cellulose (Yang et al
72 2014); silica nanospheres (Qian et al 2013).

73 Natural degradable polymers synthesized by microorganisms – the so-called polyhydroxyalkanoates
74 (PHAs) – have been studied as potential carriers of pesticides for a relatively short time. The long degradation times
75 of PHAs, which are eventually degraded by natural microflora to such harmless products as CO_2 and H_2O, and their
76 ability to be processed into polymer products from solutions, emulsions, powders, and melts make them suitable
77 materials for constructing slow-release formulations that can be applied to soil and used as pre-emergent pesticides.
78 Recent studies showed that poly-3-hydroxybutyrate [P(3HB)] – the best studied representative of PHAs – was a
79 suitable degradable material for constructing slow-release formulations of herbicides Zellek Super (Prudnikova et al
80 2013) and metribuzin (Boyandin et al 2016; Volova et al 2016a,b). A study was carried out in which TEB was
81 embedded in films, microgranules, and microparticles of P(3HB), and its fungicidal activity against *Fusarium*
82 *moniliforme* was comparable to that of commercial formulation Raxil (Volova et al 2016c). TEB release kinetics,
83 degradation of P(3HB) matrix, and inhibition of *Fusarium* growth were investigated in laboratory soil
84 microecosystems (Volova et al 2016d).

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

87

88 **Materials and methods**

89

90 **Poly(3-hydroxybutyrate) characterization**

91 Poly(3-hydroxybutyrate) [P(3HB)] was used as a matrix for embedding tebuconazole (TEB). The polymer
92 was synthesized by using bacterium *Cupriavidus eutrophus* B10646, at the Institute of Biophysics SB RAS. Cells
93 were aseptically cultured in a 7.5-L BioFlo/CelliGen115 (New Brunswick, U.S.) fermentor, following the previously
94 developed technology (Volova et al 2013; 2014). Polymer was extracted from cells with chloroform, and the extracts
95 were precipitated using hexane. The extracted polymers were re-dissolved and precipitated again 3-4 times to
96 prepare homogeneous specimens. P(3HB) had the following physicochemical parameters: weight average molecular
97 weight (M_w) 920 kDa; polydispersity (Đ) 2.52; degree of crystallinity 74%; melting point and thermal
98 decomposition temperature 179.1 and 284.3 °C, respectively.
99
100 **Fungicide**
101
102 The chemicals used in this study were the systemic fungicide Raxil Ultra (Bayer Crop Science, Russia),
103 with tebuconazole (TEB) as the active ingredient, and chemically pure TEB (Russian Federal Standard GSO7669-
104 99, purity 99.1%). TEB is a multifunctional systemic fungicide, which is effective against a very wide range of
105 fungal diseases of cereal crops.
106
107 **Wheat**
108
109 Experiments were performed in communities of soft spring wheat cv. Altaiskaya 70.
110
111 **Plant pathogen**
112
113 Fungi of the genus *Fusarium* Link (*F. moniliforme* J. Sheld) were used in experiments. Inoculum was
114 prepared by growing fungi in culture tubes on malt extract agar (MEA, Sigma, U.S.) for 14 days. Then, spore
115 suspension was prepared in sterile tap water, 5.2×10^{7} spores ml^{-1}. The number of spores was counted in the Goryaev
116 chamber. Ten ml *F. moniliforme* spore suspension was added to each container with soil.
117
118 **Preparation of fungicide formulations**
119
120 Films, and granules, with tebuconazole loadings of 25% were prepared as experimental formulations.

121 Films loaded with the fungicide were prepared as follows. A solution of TEB in chloroform was added to
122 the 2% polymer solution in chloroform. The P(3HB)/TEB solution was mixed on an MR Hei-Standard magnetic
123 stirrer (Heidolph, Germany) for 2-3 h (until completely dissolved) and heated to 35-40°C under reflux condenser for
124 3-4 h. Then, the P(3HB)/TEB solution was cast in Teflon-coated metal molds, where solvent evaporation occurred.
125 The films were left to stay at room temperature in a laminar flow cabinet for 24 h, and then they were placed into a
126 vacuum drying cabinet (Labconco, U.S.) for 3-4 days, until complete solvent evaporation took place. Films were cut
127 into 5×5 mm squares, which were weighed on the analytical balance of accuracy class 1 Discovery (Ohaus,
128 Switzerland). The film thickness was measured with an EDM-25-0.001 digital micrometer (Legioner, Germany).

129 TEB granules were prepared as follows. The necessary amounts of TEB were added to the 10% P(3HB)
130 solution, which was thoroughly mixed. A peristaltic pump was used to drop the P(3HB)/TEB solution into the
131 sedimentation tank that contained hexane (by the microdrop technique); the needle size was 20 G, and the thickness
132 of the layer of the precipitating agent (hexane) was 200 mm. As the polymer was insoluble in hexane, when the
133 drops passed through the layer of the precipitating agent, granule formation occurred. The granules had a diameter
134 of 3 mm.

135

136 **Characterization of soil microecosystems with plants**

137

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

144 Two experiments were carried out, each lasting 30 days. In Experiment 1, wheat seeds were infected with
145 plant pathogens. There were five experimental groups. In Group 1 (negative control), seeds sown into the soil had
146 not been treated with the fungicide, and no TEB was added to the soil. In Group 2 (positive control), at the time of
147 planting, the soil was treated with commercial formulation Raxil at a concentration comparable with the TEB
148 concentrations in the treatments: 3 μg TEB/g soil. In two treatment groups, the seeds had not been treated with the
149 fungicide prior to sowing, but P(3HB)/TEB films (Group 3) and granules (Group 4) were buried in the soil. In
150 Group 5, the seeds were soaked in the Raxil solution for 10 min prior to sowing; no TEB was added to the soil.

151 In Experiment 2, the efficacy of the P(3HB)/TEB formulations was tested under harsher conditions. In 152 addition to using the infected wheat seeds, we also added spores of the plant pathogen *F. moniliforme* into the soil. 153 There were four experimental groups. In Group 1, the wheat seeds had not been treated with the fungicide before 154 sowing; no TEB was added to the soil. In the treatment groups, the seeds sown into the soil had not been treated 155 with the fungicide, but P(3HB)/TEB films (Group 2) and granules (Group 3) were buried in the soil. In Group 4 156 (control), at the time of planting, the soil was treated with commercial formulation Raxil at a concentration 157 comparable with the TEB concentrations in the treatments: 3 μg TEB/g soil.

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159 **Chemical and microbial compositions of the soil**

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161 The analysis of the chemical composition of the soil included measuring pH of the aqueous extract 162 (following Russian Federal Standard 26423-85) and concentrations of nitrate nitrogen (by the method developed at 163 the Central Research Institute for Agrochemical Support of Agriculture, CRIASA, following Russian Federal 164 Standard 26488-85), mobile phosphorus and exchangeable potassium (by the method developed by Machigin and 165 modified at CRIASA, following Russian Federal Standard 26204-91).

166 The structure of the soil microbial community was analyzed by conventional methods of soil microbiology 167 (Netrusov et al 2005). The total number of organotrophic bacteria was determined on nutrient agar medium (NA, 168 HiMedia); microscopic fungi were counted on malt extract agar (MEA, Sigma-Aldrich). The ecological-trophic 169 groups of microorganisms were identified by plating soil samples onto diagnostic media. Ammonifying 170 (copiotrophic) bacteria were identified on NA; microorganisms (including actinomycetes) capable of utilizing 171 mineral nitrogen (prototrophs) were identified on starch ammonium agar (SAA). Microorganisms involved in 172 mineralization of humus substances (oligotrophs) were identified on soil extract agar (SEA) (Netrusov et al 2005).

173 Soil microscopic fungi were identified by their micro- and macro-morphological features (the structure and 174 color of colonies and the structure of mycelium and spore-forming organs), which are objective parameters for 175 identifying these microorganisms (Sutton et al 2001; Watanabe 2002). All manipulations were performed in 176 triplicate.

177

178 **Analysis of TEB concentrations**

179

180 During the experiment, to monitor changes in TEB concentrations in the soil, tebuconazole was extracted
181 from soil and water with chloroform. To determine residual TEB content in the polymer, the specimens were
182 dissolved in chloroform, and then the polymer was precipitated with hexane. The polymer was separated from the
183 solvents and weighed to determine polymer content of the formulation. The solvents were removed in a rotary
184 vacuum evaporator. After removal of chloroform, 100-500 μl of acetone was added to the polymer. The quantity of
185 the active ingredient was measured by gas chromatography. Measurements were made with a chromatograph mass
186 spectrometer (7890/5975C, Agilent Technologies, U.S.), using a capillary column, under varied temperature
187 conditions. The chromatography conditions were as follows: a DB-35MS capillary column, 30 m long and 0.25 mm
188 in diameter; carrier gas – helium, rate 1.2 ml min^{-1}; sample introduction temperature 220°C; initial temperature of
189 chromatography – 180°C; temperature rise to 310°C at 10°C per min; 5 min isothermal conditions. TEB
190 concentration was determined using the calibration curve; the curves were constructed as described elsewhere
191 (Volova et al 2016c).
192
193 **Evaluation of P(3HB)/TEB fungicidal activity**
194
195 The fungicidal activity of the experimental P(3HB)/TEB formulations was compared with the activity of
196 commercial formulation Raxil by evaluating changes in the following parameters: the counts of *F. moniliforme* in
197 soil; the percentage of plant roots affected by the rot disease; the amount of aboveground biomass of the plants
198 (determined by weighing the biomass preliminarily dried to constant weight). These parameters were measured at
199 Days 10, 20, and 30 of the experiment.
200 Counting of the total microscopic fungi, including *F. moniliforme*, was performed by plating soil
201 suspension onto Petri dishes with malt extract agar, which was supplemented with chloramphenicol (100 μg L^{-1} of
202 the medium) to suppress cell growth. All platings were performed in triplicate from 10^2-10^5 dilutions of soil
203 suspension. The dishes were incubated at a temperature of 25°C for 7-10 days. Microscopic analysis of the colonies
204 was done using an AxioStar microscope (Carl Zeiss). Microscopic fungi were identified by their cultural and
205 morphological properties, with identification guides (Sutton et al 2001; Watanabe 2002).
206 The degree to which the roots were damaged by fusarium infection was evaluated as follows: the plant
207 roots were carefully removed from the soil, rinsed first in running water and then three times in sterile tap water, and
208 placed onto paper filters wetted to the maximum water holding capacity in Petri dishes. The Petri dishes were
209 incubated in the thermostat at 25 °C. The degrees to which the roots were infected by the fungi *Fusarium,*

210 *Alternaria*, and *Bipolaris* were determined at Day 5-10, by microscopic examination of mycelium and spore
211 formation of the fungi on wheat roots.
212 The initial degree of infection of wheat seeds by plant pathogens (internal infection) was determined by
213 germinating the seeds in Petri dishes on sterile nutrient medium MEA (Russian Federal Standard 12044-93). The
214 state of the plants and their growth were evaluated by photographing the plant communities and the roots.
215
216 **Statistical analysis**
217
218 Statistical analysis of results was performed using the standard software package of Microsoft Excel,
219 STATISTICA 8. Arithmetic means and standard deviations were determined using Student's t test. Results are given
220 as X±m.
221
222 **Results**
223
224 Laboratory soil ecosystems with wheat plants contained agrogenically-transformed field soil (from the
225 village of Minino, the Krasnoyarsk Territory): cryogenic-micellar agro-chernozem with high humus content in the
226 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
227 mequiv 100 g^{-1}). The soil contained nitrate nitrogen N-NO_3 – 6 mg kg^{-1}, and P_2O_5 –6 and K_2O – 22 mg 100 g^{-1} soil
228 (according to Machigin). The soil had high mineralization and oligotrophy coefficients (1.52 and 11.74,
229 respectively), indicating soil maturity and low contents of available nitrogen forms.
230 The total number of organotrophic bacteria was 16.3±5.1 x 10^6 CFU g^{-1}. The phylum *Actinobacteria*
231 dominated and included the following genera: *Streptomyces* (24%), *Arthrobacter* (18%), and *Corynebacterium*
232 (12%); *Pseudoxanthomonas* were the major Gram-negative rods (12%) in the soil samples. Among microscopic
233 fungi, the *Penicillium* species dominated (58-65%); fungi of the genera *Fusarium, Trichoderma*, and *Aspergillus*
234 constituted 8-11% of the fungal population in soil samples. *Fusarium* species isolated from the initial soil samples
235 were represented by *F. solani* and *F. lateritium*. No *F. moniliforme* was detected in the initial microbial community.
236 Equal amounts of wheat seeds were sown into the soil-filled containers: 100.45 g seeds per 1 m^2. In the
237 control groups, Raxil was added to the soil simultaneously with seed sowing. In the treatment groups, P(3HB)/TEB
238 formulations were buried in soil 10 days before the sowing. Our previous studies showed that it took some time for
239 soil microorganisms to get attached to the surface of the polymer samples and get adapted to the polymer matrix of

240 P(3HB) as the substrate. The release of the active ingredient such as TEB from the polymer matrix only occurred
241 when the polymer was gradually degraded, and the release rate was determined by the kinetics of polymer
242 biodegradation (Volova et al 2016c). By the time of sowing, in the group with the P(3HB)/TEB films, TEB
243 concentration in the soil was somewhat lower than in the control group – 2.7 µg TEB/g soil. In the soil containing
244 P(3HB)/TEB granules, TEB concentration was lower – 1.1 µg TEB/g soil.
245

246 **The effect of tebuconazole on plant pathogens and saprotrophic microflora of wheat as dependent on**
247 **the fungicide delivery method**
248

249 Phytosanitary analysis of wheat seeds germinated on the nutrient medium showed the presence of
250 infections caused by the fungi of the genera *Fusarium* Link, *Alternaria* Nees, and *Bipolaris* Shoem. (Fig. 1, 2).
251 Wheat seeds infected by plant pathogens constituted 9.5±1.2%, 5.6±0.2% of which were infected by *Fusarium*
252 species. Thus, natural infections of the seeds were caused not only by *Fusarium* species, which were detected in the
253 initial soil, but also by the phytopathogenic microscopic fungi that developed when the seeds containing internal
254 infection were germinated.
255 Analysis of microscopic fungi in soil samples showed that in the initial soil, their total counts reached
256 $(28.3\pm9.4)\times10^3$ CFU·g^{-1}, while at the end of the experiments, this number had dropped by a factor of 2.3, to
257 $(12.3\pm2.5)\times10^3$ CFU·g^{-1} (Fig. 3A). That was caused by changes in the structure of the microbial community that
258 occurred due to the selective effects of wheat on the rhizosphere microflora: wheat roots exuded organic substances
259 readily available to bacteria, leading to an increase in their abundance. The total bacterial counts increased
260 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
261 analyzed after 30 days of the experiment. Thus, as wheat plants were growing, the structure of the microbial
262 community was changing. The decrease in the counts of microscopic fungi seemed to be caused by their competition
263 with rhizosphere bacteria.
264 The degrees of infection caused by phytopathogenic fungi in the initial soil differed considerably between
265 the two experiments, and that resulted in different efficacy of TEB. In Experiment 1, wheat was grown in the soil
266 with natural infection mainly caused by *Fusarium* fungi, whose counts reached 3.1×10^3 CFU·g^{-1}. In Experiment 2,
267 when the soil was additionally inoculated with *F. moniliforme* spores, the counts of plant pathogens in the initial soil
268 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 and 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.

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-

298 sowing seed treatment with Raxil restrained the development of the overall root infection in the first 10 days, but
299 then rot infection damaged more roots and persisted at a high level – 21-27% (Fig. 4A)

300 The experimental P(3HB)/TEB films were effective against all root rots, including fusarium infection,
301 restraining their development. Between Days 10 and 20, the efficacy of this formulation was comparable to that of
302 Raxil. Moreover, TEB embedded in the polymer matrix showed extended fungicidal effect, and between Days 20
303 and 30, root infection did not increase, in contrast to the groups with Raxil and pretreated seeds (Fig. 4A, B).
304 P(3HB)/TEB granules did not show any fungicidal effect in the first 10 days. TEB release from the granules
305 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
306 μg/g soil in the soil with P(3HB)/TEB films. At Day 30, however, inhibition of root rot development in this group
307 was comparable to the effect of commercial formulation Raxil.

308 In Experiment 2, with *F. moniliforme* spores added to the soil, the percent of the damaged roots was
309 considerably higher (Fig. 5 B). In the group with no TEB added to the soil (negative control), after 30 days, the
310 percentage of infected roots reached 61.5%, the roots damaged by fusarium infection caused by *F. moniliforme*
311 constituting 53.8%. TEB in the form of commercial Raxil (positive control) was effective against root rots in the
312 early stage. Between Days 10 and 20, the total percentage of infected roots was 1.8-1.9 times lower and the
313 percentage of fusarium infection-damaged ones 3.3-2.2 times lower than in the negative control group. Later,
314 however, the fungicidal effect of Raxil became weaker, and the percentage of infected roots increased. Similarly to
315 Experiment 1 (with the naturally infected soil), the fungicidal effects of the two experimental P(3HB)/TEB
316 formulations was comparable to that of commercial formulation Raxil in the first 20 days, but it lasted longer and
317 restrained the development of root rots, including fusarium infection, during the final stage (Days 20-30). At Day
318 30, the total percentage of the infected roots was 1.6 times lower and the percentage of fusarium infection 1.4 time
319 lower than in the group with Raxil (Fig. 5A, B).

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

322

323 **Characterization of wheat plant communities grown on the soil with different TEB formulations**

324

325 Results of evaluating the productivity of wheat communities growing on soils infected by *F. moniliforme* to
326 various degrees, with different percentages of roots damaged by root rot, are shown in Figure 7. In Experiment 1,
327 with milder damage to roots, measurements of aboveground biomass in the early stage (10 days) in the negative and

328 positive control groups and in the group with pretreated seeds gave comparable values. In the treatment groups, the
329 biomass was somewhat (15-20%) lower (Fig. 7 A). At Day 20, however, all groups showed comparable values. In
330 the later stage (at Day 30), the biomass of the plants grown without TEB amounted to 180 g/m^2, which was 40%
331 lower than in the group with Raxil and pretreated seeds and 60% lower than in the treatment groups. The difference
332 in biomass between the groups with pretreated seeds and Raxil, on the one hand, and the groups with the
333 experimental P(3HB)/TEB formulations, on the other, reached about 15-17%.

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

337

338 **Discussion**

339

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

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

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

354 Modern means of protection of cereal crops against root rots should be based on the ecosystem approach to
355 reduce the adverse effects of human-made pollutants and to produce health foods. The most common way to control
356 fusarium root rot is to treat seeds with fungicides prior to sowing, but this treatment cannot protect plants throughout
357 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; Boyandin et al 2016; Volova et al 2016a,b), fungicides (Savenkova et al 2002; Volova et al 2016c,d), and nitrogen fertilizers (Volova et al 2016e).

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 2016c). 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 (Volova et al 2016d).

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

388 growth of seedlings, and, especially, inhibition of root elongation (Yang et al 2014). The unfavorable regulatory
389 effect of TEB occurs via the following mechanism: triazoles, TEB, in particular, shift the balance of phytohormones
390 in plant tissues and inhibit biosynthesis of gibberellins, causing a temporary increase in the content of abscisic acid
391 in plants (Grossman 1990; Fletcher et al 2000).

392 Several studies compared the efficacy of slow-release TEB formulations with that of free TEB against such
393 plant pathogens as wheat rust *Puccinia recondita* (Asrar et al 2004), root rot pathogens *Biopolaris* and *Fusarium*
394 (Khalikov et al 2013), maize head smut (Yang et al 2014), and wheat powdery mildew (Zhang et al 2015). TEB
395 encapsulated in microcapsules of ethyl celluloses did not affected adversely the germinating capacity of maize
396 seeds, in contrast to Raxil. Phytohormonal analysis showed that the microencapsulated and continuously released
397 tebuconazole had a beneficial effect on the balance of phytohormones during maize seed germination. Encapsulated
398 TEB provided better protection against maize head smut than the conventionally used Raxil (Yang et al 2014).
399 Zhang et al (2015) reported a study in which cells of cyanobacterium *Synechocystis* sp. *strain PCC* 6803 used to
400 encapsulate tebuconazole were coated with the rubber-like urea/formaldehyde material. That formulation remained
401 80% effective against powdery mildew for 12 days, as TEB was slowly and steadily released from microgranules,
402 while the efficacy of Raxil was 52.25%. In another study (Asrar et al 2004), TEB was encapsulated into
403 microcapsules prepared from poly(methyl methacrylate) (PMMA) and poly(styrene-co-maleic anhydride) (PSMA)
404 by using different techniques and used to spray plants. The bio-effectiveness of microparticles against wheat rust
405 *Puccinia recondita* in the wheat varied depending on the form and production technique employed; however, all
406 forms of microparticles provided better protection against wheat rust than commercial foliar-applied tebuconazole,
407 Raxil.

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

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

418 though, increased in later stages of the experiment. In the early stage (between Days 10 and 20), the percentage of
419 rot-damaged roots in the soil with TEB embedded in the slowly degraded P(3HB) matrix was similar to that in the
420 soil with Raxil. However, the efficacy of P(3HB)/TEB formulations lasted longer, and in later stages (between Days
421 20 and 30), the percentage of rot-damaged roots in that group did not grow, in contrast to the group with the soil
422 treated with Raxil and in the group with the pre-treated seeds.

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

428

429 **Conclusions**

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

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441

442 **Conflict of interest**

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

445

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536 **Figure legends**

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

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

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

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

545 Fig. 5 The effect of TEB delivery mode on the percentage of wheat roots damaged by rot in the experiment
546 with *Fusarium moniliforme* spores added to the soil: *1* – negative control, *2* – positive control (Raxil applied to soil),
547 *3* – P(3HB)/TEB films, *4* – P(3HB)/TEB granules.

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

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

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