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TOXIC EFFECTS OF THE FUNGICIDE TEBUCONAZOLE ON THE ROOT SYSTEM OF FUSARIUM-INFECTED WHEAT PLANTS

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Abstract

The study investigates toxic effects of the fungicide tebuconazole (TEB) on *Fusarium*-infected wheat (*Triticum aestivum*) plants based on the morphological characteristics of root apices and changes in the integrated parameters of redox homeostasis, including the contents of free proline and products of peroxidation of proteins (carbonylated proteins, CP) and lipids (malondialdehyde, MDA) in roots. In two-day-old wheat sprouts infected by *Fusarium graminearum*, the levels of proline, CP, and border cells of root apices are higher than in roots of uninfected sprouts by a factor of 1.4, 8.0, and 3, respectively. The triazole fungicide tebuconazole (TEB) at the concentrations of 0.01, 0.10, and 1.00 µg ml^{-1} of medium causes a dose-dependent decrease in the number of border cells. The study of the effects of TEB and fusarium infection on wheat plants in a 30-day experiment shows that the effect of the fungicide TEB on redox homeostasis in wheat roots varies depending on the plant growth stage and is significantly different in ecosystems with soil and plants infected by *Fusarium* phytopathogens. The study of the morphology of root apices shows that the toxic effects of TEB and fusarium infection are manifested in the destructive changes in root apices and the degradation of the root tip mantle.

Key words: Fusarium, tebuconazole, free proline, carbonylated proteins, malondialdehyde, border cells

1.   Introduction

Fusarium infection is one of the most common diseases affecting cereal crops. This disease is caused by soil pathogenic fungi of the genus *Fusarium*. The crop losses due to fusarium infection in case of maize, wheat, and rice affected by fusarium infection are economically important, as they are the major sources of plant protein and their yields constitute over 55% of the total yield of cereal crops. The crop losses may range between 5 and 30%. Many *Fusarium* species produce mycotoxins: deoxynivalenol (vomitoxin), zearalenone, and T-2 mycotoxin (Binder et al., 2007). The fusarium infection may damage the ear and result in the reduced grain yield. Mycotoxin-contaminated grain is unsuitable and even unsafe food and feed. The application of fungicides decreases the incidence of fusarium infection and reduces the levels of mycotoxins in commercial grain (Schmale and Bergstrom, 2003; SANCO, 2013). The triazole fungicides now constitute 30% of the marketed fungicides. One of them is tebuconazole (TEB). TEB is an effective multifunctional systemic fungicide used to protect a number of cereal crops. TEB rapidly penetrates the plants through both their vegetative organs and roots. However, triazole fungicides, including TEB, are phytotoxic (Ahemad and Khan, 2012a, b). The mode of action of the triazole group is to suppress ergosterol biosynthesis, preventing the formation of cell membranes, causing the death of pathogens (Lamb et al., 2001; Hartwig et al., 2012). Thus, *Fusarium*-infected crops treated with triazole fungicides are adversely affected by two factors: *Fusarium* infection and fungicide.

At the systemic level, the toxic effects of triazoles lead to hormonal imbalance (Yang et al., 2014), nitrogen imbalance, lower seed germination rates, disorders of root growth and development (Serra et al., 2013, 2015), and the appearance of chromosomal abnormalities (Wandscheer et al., 2017). The fungal sterol-14-α-demethylase – the effector target of triazole fungicides in cells of mycopathogens – belongs to the evolutionarily ancient cytochrome-450(CYP)-superfamily, which has also been detected in plants and animals (Lamb et al., 2001). The phytotoxicity (and toxicity of triazole fungicides for humans and animals) has been associated with the effect of fungicides on the activity of sterol demethylases and disturbance of the sterol dependent signaling (Hartwig et al., 2012). At the systemic level, sterol dependent signaling determines the activity of such processes as proliferation, differentiation, and the production of reactive oxygen species (Wassmann et al., 2001; Park et al., 2008).

The cause-effect chain "inhibition of sterol demethylases → sterol dependent signaling deficiency → inhibition of generation of reactive oxygen species" can be used as the basis for evaluating myco- and phytotoxicity of fungicides and for assessing plant resistance to mycopathogens. The regulated hyperproduction of reactive oxygen species (ROS) in response to pathogen invasion is one of the major protective responses of plants. In addition to being highly toxic, ROS trigger specific signaling systems, which cause changes in the gene expression patterns and induce the development of host plant resistance or sensitivity to pathogens (Frederickson and Loake, 2014; Swarupa et al., 2014).

76 Over the course of evolution, the pathogenic fungi have developed scavenging systems that allow them to

77 neutralize cytotoxic effects of the oxidative burst of the host plant. The pathogenic fungi use ROS generated in cells of

78 their host plant to regulate the expression of their own genes that control cell differentiation and hyphal growth in plant

79 tissues (Takemoto et al., 2007). Thus, ROS signaling simultaneously determines 1) activation of plant defense response

80 against invasion of mycopathogens, 2) the stimulation of growth and the differentiation of the mycopathogen in plant

81 tissues after invasion, and 3) myco- and phytotoxicity of fungicides. In order to produce consistently high crop yields,

82 ROS-dependent readjustment of these three systems should lead to the most complete suppression of mycoinfection

83 with the minimal phytotoxic effects.

84 In addition to the peroxidation products, there is another important indicator of the state of the plant root

85 system: a free amino acid proline, which is an integrated indicator of the activity of root antioxidant and defense

86 systems. Proline is a low-molecular-weight scavenger of free radicals (Signorelli et al., 2014), which also increases the

87 gene expression in antioxidant enzymes (de Carvalho et al., 2013). The activation of the synthesis of proteins with high

88 proline contents is an important factor in the functioning of mechanisms of the root defense against pathogen invasion

89 (Cecchini et al., 2011; Plancot et al., 2013; Qamar et al., 2015).

90 Pathogen invasion occurs through the plant roots, and, therefore, the state of the roots of infected plants can be

91 characterized by a system of border cells (Berrocal-Lobo and Molina, 2008). The border cells constitute a specific

92 population of metabolically active cells localized in the root apex and play a fundamental role in the root interactions

93 with symbiotic and pathogenic organisms of the rhizosphere (Gunawardena and Hawes, 2002; Bais et al., 2006; Wen et

94 al., 2007, 2009; Cannesan et al., 2011, 2012). The gel mantle is an excretory product of border cells, which encloses

95 them (Cannesan et al., 2011, 2012; Hawes, 2012). The invasion of root pathogens elicits the production of border cells

96 and increases their secretory activity – as a defense response (Plancot et al., 2013). Thus, the number of border cells can

97 be regarded as an integrated indicator of the activity of defense systems in pathogen-infected roots.

98 The present study investigated toxic effects of the fungicide tebuconazole in *Fusarium*-infected wheat

99 (*Triticum aestivum*) stands by examining the state of the root apices and changes in the integrated parameters of redox

100 homeostasis, including free proline content and the contents of protein and lipid peroxidation in roots.

101 **2. Materials and methods**

102 *2.1. Materials*

103 Fungicide: tebuconazole (TEB) is a multifunctional systemic fungicide, which is effective against a very wide

104 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.

105 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

106 and 9; it is stable upon the exposure to light and elevated temperature. The time of degradation in soil is 177 days. A

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107 commercial formulation Raxil Ultra (Bayer Crop Science, Russia), with tebuconazole (TEB) as the active ingredient

108 was used.

109   Wheat: experiments were performed in the communities of soft spring wheat cv. Altaiskaya 70.

110   *2.2. Wheat cultivation*

111   Fusarium-infected and uninfected wheat seeds were used. Toxic effects of tebuconazole and fusarium infection

112 were studied in the experiments with two-week-old wheat sprouts and in the long-duration experiment with fusarium-

113 infected wheat stands in the laboratory soil system. The wheat sprouts were grown as follows: the seeds were washed in

114 running water for 5-6 h and soaked in distilled water for 24 h at room temperature. Different doses of the aqueous

115 solutions of tebuconazole were added to Petri dishes containing distilled water (7 ml) to achieve end concentrations of

116 the fungicide in the medium of 0.001, 0.01, and 0.1 μg ml^{-1}. The seeds were sprouted at room temperature under

117 continuous light.

118   In the other experiment, wheat plants were grown in the laboratory soil microecosystems. The soil

119 microecosystems were prepared as follows. The agrogenically-transformed soil (collected at the village of Minino, the

120 Krasnoyarsk Territory, Siberia, Russia) was placed into 500-cm^3 plastic containers (500 g soil per container). The wheat

121 seeds were sown into the soil, at a planting density of 100.45 g seeds per 1 m^2. Plants were grown in a Conviron A1000

122 growth chamber (Canada) for 30 days under stable conditions: at an irradiation of 100-300 μmol. m^{-2} s^{-1}, under the

123 12L:12D photoperiod, at a temperature of 18-25°C and the humidity of 65%; conditions of the experiment and soil

124 ecosystems are described in detail elsewhere (Volova et al., 2017). The experiment consisted of the following

125 treatments and controls: 1) in the negative control (k-), the infected wheat seeds were sown into the soil, with no

126 fungicide applied; 2) in the positive control (k+), the seeds and the commercial formulation Raxil Ultra were buried in

127 soil simultaneously, with Raxil Ultra applied at a concentration corresponding to 3 μg TEB g^{-1} soil, and 3) in the

128 treatment, the seeds were soaked in a Raxil Ultra solution for 10 min before sowing, with no more TEB added to the

129 soil, i.e. seeds were pretreated before sowing (P).

130   *2.3. A biochemical study*

131   The toxic effects of TEB were evaluated by measuring changes in the integrated parameters of redox

132 homeostasis: the contents of proline, malondialdehyde, and carbonylated proteins in the roots of two-day-old wheat

133 sprouts and the wheat plants grown in soil-based systems – at Days 10, 20, and 30 of the experiment. The root samples

134 were prepared by cutting 1-cm-long terminal portions of the roots with apices. Then, the root biomass was homogenized

135 in a 0.05 M Tris-HCl buffer solution, pH=7.4, in a hand-held homogenizer, at T=4°C. To remove coarse debris, the

136 homogenates were centrifuged at 5000 *g*, for 45 min, at T=4°C. The supernatant fluid was collected and used to

137 determine the contents of carbonylated proteins – by the method of Carty et al. (Carty et al., 2000), malondialdehyde –

4

138   by the method of Bailly et al. (Baily et al., 1996), and proline – by the method of Bates et al. (Bates et al., 1973). The

139   contents of carbonylated proteins, malondialdehyde, and proline were calculated per 1 mg of root homogenate protein.

140   *2.4. A morphological study of root apices*

141   Prior to microscopic analysis, the root apices were fixed in 2.5% glutaric aldehyde in 0.1 M phosphate buffer,

142   pH=7.2. The root apices were rinsed in distilled water to remove the fixative and stained with 0.01% methylene blue.

143   Using a light microscope, we counted the number of free border cells that had detached from the surface of the root and

144   measured the size of the gel mantle (whose color had changed to blue due to the presence of a large amount of

145   polysaccharides). Sixty to seventy root apices were analyzed in each treatment and in the control.

146   *2.5. A study of the contamination of seeds and soil by phytopathogenic fungi*

147   The intrinsic contamination of wheat seeds with phytopathogens was determined by sprouting the seeds in

148   Petri dishes on sterile nutrient medium MEA (Russian Federal Standard 12044-93). In the experiment with wheat

149   stands, the number of phytopathogenic fungi, including *F. moniliforme*, in soil was counted at Days 10, 20, and 30 of

150   the experiment. The counting of the total microscopic fungi was performed by plating soil suspension onto Petri dishes

151   with malt extract agar, which was supplemented with chloramphenicol (100 µg L^{-1} of the medium) to suppress the cell

152   growth. All platings were performed in triplicate from 10^2-10^5 dilutions of soil suspension. The dishes were incubated at

153   a temperature of 25 °C for 7-10 days. Microscopic analysis of the colonies was done using an AxioStar microscope

154   (Carl Zeiss). Microscopic fungi were identified by their cultural and morphological properties, with the identification

155   guides (Sutton et al., 2001; Watanabe, 2002).

156   The counting of phytopathogenic fungi *Fusarium* and the total microscopic fungi in soil samples was

157   performed by plating soil suspension onto Petri dishes with malt extract agar, which was supplemented with

158   chloramphenicol (100 µg L^{-1} of the medium) to suppress the cell growth. All platings were performed in triplicate from

159   10^5 dilutions of soil suspension. The dishes were incubated in thermostat at a temperature of 25 °C for 7-10 days.

160   Microscopic analysis of the colonies was done using an AxioStar microscope (Carl Zeiss). Soil microscopic fungi were

161   identified by their cultural and macro-morphological properties (structure and color of colonies, structure of mycelium

162   and spore-bearing organs), which are objective parameters for identifying these microorganisms (Sutton et al., 2001;

163   Watanabe, 2002). The species of *Fusarium* were determined by the presence of purple-pink pigmentation of colonies

164   and by the formation of typical micro- and macroconidia on mycelium (Pradeep et al., 2013).

165   *2.6. Statistical analysis*

166   Statistical analysis of the results was performed using the standard software package of Microsoft Excel,

167   STATISTICA 8. The arithmetic means and standard deviations were determined using Student's t test. Results are

168   given as X±m.

169   *3.   Results and Discussion*

170   *3.1. The effect of phytopathogenic infection on wheat seed germination and the biochemical parameters and*

171   *morphology of roots of sprouts*

172   The phytosanitary analysis of wheat seeds grown on the nutrient medium showed the presence of infections

173   caused by the fungi of the genera *Fusarium* Link, *Alternaria* Nees, and *Bipolaris* Shoem. The wheat seeds infected by

174   plant pathogens constituted 9.5±1.2%, 5.6±0.2% of which (over 50%) were infected by *Fusarium* species. Thus, the

175   natural infections of the seeds were caused not only by the predominant *Fusarium* species, but also by the

176   phytopathogenic microscopic fungi that developed when the seeds containing internal infection were germinated.

177   The germination rate of the uninfected *Triticum aestivum* seeds reached 90±3%. The roots of two-day-old

178   sprouts contained 1.03±0.09 nM carbonylated proteins (CP) mg^{-1} protein, 10.59±0.26 µg proline mg^{-1} protein, and

179   0.300±0.035 nM malondialdehyde (MDA) mg^{-1} protein. MDA, as a product of the peroxidation of membrane lipids, can

180   be involved in the regulation of the activity of cell membranes (via rearranging of the lipid bilayer and changing of the

181   activity of membrane-bound proteins) (Ansari et al., 2015; Antosik et al., 2015). The levels of CP, MDA, and proline

182   revealed in the experiment characterize the redox homeostasis in normally developing roots of uninfected *Triticum*

183   *aestivum* sprouts.

184   The germination rate of the infected wheat seeds was lower (77±7%). The CP content in roots was 8 times

185   higher than in the roots of uninfected sprouts (Fig. 1). The level of proline in the roots of the infected sprouts was 1.4

186   times higher than in the roots of uninfected sprouts. These results are consistent with the notion of pathogen invasion

187   inducing the activation of the system for production of free radicals as a major defense mechanism of a plant cell

188   exposed to biotic and abiotic stresses (Sham et al., 2014; Chanclud and Morel, 2016). An increase in the activity of

189   protein peroxidation results from the regulated activation of generation of ROS in plant tissues as a response to the

190   invasion of pathogenic fungi; it is necessary for inducing ROS-dependent signaling of defense systems (Frederickson

191   and Loake, 2014; Swarupa et al., 2014). The level of MDA in the roots of infected sprouts was not significantly

192   different from the MDA level in uninfected roots (Fig. 1). That may be attributed to the involvement of MDA in the

193   oxidative modification of proteins (Augustyniak et al., 2015). Thus, the proportions of CP, MDA, and proline in roots

194   of infected sprouts differed from those in roots of uninfected sprouts, suggesting a transition of the redox systems to

195   another level of homeostasis.

196   In the experiment with the fungicide tebuconazole (TEB) added to the culture medium at the concentrations of

197   0.01, 0.10, and 1.00 µg ml^{-1}, the germination rate of the infected wheat seeds was similar to that of the infected seeds in

198   the experiment without TEB addition (75±8%). None of the TEB concentrations tested in this study affected CP, MDA,

199   and proline levels in the roots of infected sprouts (Table).

200   The morphological dissimilarities between the root apices of uninfected and infected sprouts, with pronounced

201   differences in the contents of carbonylated proteins, are shown in Figure 2. The root apex of an uninfected sprout is

202   ensheathed in a small gel mantle containing border cells (BC) that have detached from the surface of the apex (Fig. 2a);

203   there are 15±3 border cells/apex. The infected sprouts contain considerably more BC (47±7 cells/apex). Thus, ROS

204   signaling may be involved in the activation of BC production in response to the invasion of pathogenic fungi. As the

205   number of BC in the root apices of infected sprouts increased, the size of the gel mantle increased, too (Fig. 2b, c, d).

206   The scattering of the border cells around the root apex corresponds to the size of the gel mantle. In the root apices of the

207   infected sprouts, the border cells inside the gel mantle may form large aggregates (Fig. 2b), and layers of border cells

208   may peel off the lateral surface of the root apex (Fig. 2d).

209   The formation of the gel mantle is caused by the activation of the excretory function of border cells, which

210   defends the root apex from the pathogen invasion (the root apex is the most "protected" part of the root). The

211   effectiveness of defense is determined by not only an increase in the size of the gel mantle but also changes in its

212   composition (Baetz and Martinoia, 2014). In the infected roots, the border cells secrete xylogalacturonans, which are

213   resistant to the effects of pectolytic enzymes of pathogenic fungi, and arabinogalactan proteins (Cannesan et al., 2011,

214   2012).

215   Differentiation of border cells is associated with rearrangement of the cell wall, which eventually leads to the

216   loss of physical contact of border cells with the root apex surface and release of border cells into rhizosphere (in

217   hydroponic culture, this is mucilage cap). In our study, we counted border cells of this type (we analyzed 60-70 apices

218   in each experimental point). In infected sprouts (point 0, the medium containing no fungicide, Fig. 3a), the number of

219   free border cells was the greatest: interaction between root systems and the pathogen caused more border cells to

220   separate from the root apex surface. The addition of different concentrations of the fungicide to the culture medium

221   slowed down the release of border cells and led to a decrease in the number of free cells in the mucilage cap of the root

222   apex. The effects of fungicide concentrations of 0.01 and 0.1 μg ml^{-1} did not differ significantly: the number of free

223   border cells decreased by a factor of 2 on average compared to the control point (point 0, Fig. 3a). In the medium

224   containing a fungicide concentration of 1 μg ml^{-1}, the number of free border cells was the lowest – 4.5 times lower than

225   in the medium containing no fungicide.

226   As the BC number in the root apex decreased, the gel mantle grew smaller, too. At the highest tebuconazole

227   concentration in the medium (1.00 μg ml^{-1}), the majority of root apices were "bare", with no noticeable gel mantle and

228   free BC (Fig. 3b). The uninfected sprouts had no "bare" root apices. The "bareness" of the apices could be caused by

229   fungicide phytotoxicity. As TEB diffuses through the gel mantle into the root apex, it may inhibit BC production

230   through the sterol dependent signaling, which is involved in the regulation of cell proliferation activity (Roy et al.,

231   2011). The dramatic decrease in the number of free BC – major producers of molecular components of the gel mantle –

232   results in the occurrence of "bare" apices. This most probably weakens the defense systems of plant tissues subjected to

233   the invasion of phytopathogens.

234   Thus, in contrast to fusarium infection, the fungicide tebuconazole at concentrations used in the experiments

235   did not affect the content of carbonylated proteins in the roots of infected sprouts but caused a dramatic decrease in the

236   number of border cells and the size of the gel mantle, which eventually disappeared completely. These results, as well

237   as detection of products of peroxidation of proteins and lipids, suggest that border cell population can be regarded as

238   one of the effector targets of the fungicide tebuconazole, which can be used to evaluate the phytotoxicity of fungicides.

239   *3.2. Morpho-biochemical parameters of the root system of Fusarium-infected wheat grown in laboratory*

240   *experiments with variously applied tebuconazole*

241   The microbiological study of the structure of the initial soil microbial community showed that the microscopic

242   fungi were mainly represented by *Penicillium* species (58-65%). Fungi of the genera *Fusarium, Trichoderma*, and

243   *Aspergillus* constituted 8-11% of the fungal population. *Fusarium* species isolated from the initial soil samples were

244   represented by *F. solani* and *F. lateritium*. No *F. moniliforme* was detected in the initial microbial community. Results

245   demonstrating fungicidal activity of TEB in soil are shown in Figure 4. Dynamics of the total abundance of *Fusarium* in

246   soil is shown under different experimental conditions: with infected seeds sown into the soil containing no TEB

247   (negative control, k-) and with seeds and TEB buried in the soil simultaneously (positive control, k+) (Fig. 4a).

248   *Fusarium* species in the initial soil constituted 3.1×10^3 CFU·g^{-1}. Over the course of the experiment, *Fusarium* counts in

249   the negative control corresponded to the natural abundance of the fungi in soil, varying between 2.6×10^3 and 3.2×10^3

250   CFU·g^{-1}. In the soil supplemented with TEB, *Fusarium* counts gradually decreased, dropping to 1.8×10^3 CFU·g^{-1} after

251   10 days and to 1.3×10^3 and 0.8×10^3 CFU·g^{-1} after 20 and 30 days, respectively.

252   Raxil Ultra produced a fungistatic effect not only on phytopathogenic fungi but also on all saprotrophs. The

253   microbiological investigation of the structure of microbial communities showed that Raxil Ultra added to the soil

254   suppressed the growth of indigenous fungi such as *Penicillium*, *Alternaria*, and *Aspergillus*, decreasing their abundance

255   by a factor of 1.5-2.3 compared to the soil containing no TEB (negative control).

256   In experiments with TEB added to the soil, the number of the colonies with purple-pink pigmentation, which is

257   a marker of physiological activity of *Fusarium* species, was considerably greater than in the experiments with TEB-free

258   soil (Fig. 4b).

259   Figure 5 shows the contents of free proline and the products of peroxidation of proteins (carbonylated proteins,

260   CP) and lipids (malondialdehyde, MDA) in wheat roots at different time points of the 30-day experiment.

261   At Day 10, the contents of proline, MDA, and CP in the control did not differ significantly between the three

262   groups, reaching 0.189-0.232 10^{-6} M mg^{-1} protein, 0.265-0.41 10^{-9} M mg^{-1} protein, 0.586-0.78 10^{-8} M mg^{-1} protein,

263   respectively; these values were 2-3 times higher than those in the experiment with uninfected seeds. Thus, at that time

264   point (Day 10), the application of Raxil Ultra to soil and the pretreatment of the seeds did not produce any significant

265   effect on the contents of proline, CP, and MDA in wheat roots as compared to the control (Fig. 5a). The proportions of

266   proline, MDA, and CP revealed in the experiment characterize the level of redox homeostasis resulting from the

267   interaction between the host plant and pathogen under the experimental conditions.

268   At Day 20, the contents of proline and MDA in roots of the control plants increased dramatically (by a factor

269   of 19 and by a factor of 8.5) compared to Day 10 while CP decreased slightly (by a factor of 1.8) (Fig. 5b). These

270   changes may be associated with the tillering stage – underground branching of the stem and the development of the

271   secondary root system, which occurred between Days 10 and 20 of wheat plant growth. As root biomass grew rapidly,

272   the rates of cell proliferation and cell wall synthesis increased, requiring considerable energy expenditure. The reason

273   for the dramatic increase in proline content is that proline is a proteinogenic amino acid involved in synthesis of

274   arabinogalactans – glycoproteins forming cell wall matrix. The arabinogalactans are also excreted to the rhizosphere

275   (Gong et al., 2012; Nguema-Ona et al., 2013; Kishor et al., 2015). Since large amounts of proline are used in the

276   biogenesis of cell walls and the synthesis of root exudates, it is synthesized in larger quantities during the rapid growth

277   of root biomass. In addition, proline metabolism in mitochondria is accompanied by synthesis of FADH2 and NADH

278   (Deuschle et al., 2001), which supply electrons to the mitochondrial respiratory chain. Oxidative phosphorylation

279   causes generation of ATP molecules. The high demand of the rapidly growing root system for energy equivalents

280   during the tillering stage causes a sharp rise in proline content in wheat roots. On the other hand, the high level of

281   proline and active oxidation of proline in mitochondria increase not only the activity of oxidative phosphorylation but

282   also the production of free radicals, which is related to this process (Kishor et al., 2005). The increase in the MDA level

283   in wheat roots at Day 20 may be caused by the high rate of oxidative phosphorylation.

284   In the treatments with Raxil Ultra both applied to the soil and used to pretreat seeds, at Day 20, the content of

285   proline in wheat roots also increased dramatically but to levels somewhat lower than those in the control. MDA contents

286   in these treatments and in the control increased to similar levels. However, the number of the *Fusarium* cells in soil in

287   the treatment with tebuconazole supplementation was lower than in the control by a factor of almost two. These results

288   suggest targeted effects of the fungicide on phytopathogens and lightening of the load on the defense system of wheat,

289   including changes in the level of redox homeostasis of the roots.

290   At Day 30, proline content in roots of the control wheat plants decreased considerably (by a factor of 16

291   relative to Day 20) while MDA and CP contents did not change significantly (Fig. 5). In the treatment with Raxil Ultra

applied to the soil, proline, MDA, and CP contents in the roots did not differ significantly from the control, suggesting

that phytotoxic effects of the fungicide were softened as soil contamination with phytopathogens decreased. However,

in the treatment with seeds pretreated with Raxil Ultra, proline, MDA, and CP contents in the roots were higher than in

the control by a factor of 2.2, 2.0, and 1.7, respectively. That was indicative of the activation of free radical processes

and phytotoxic stress, as the fungicidal effect of TEB used to pretreat the seeds before sowing must have been

exhausted by Day 30.

This study showed that the effect of the fungicide TEB on redox homeostasis in wheat roots varied depending

on the plant growth stage and was significantly different in ecosystems with soil and plants infected by *Fusarium*

phytopathogens. At Day 20 of plant growth, during the tillering stage, tebuconazole produced the strongest phytotoxic

effect on wheat plants.

The morphological properties of wheat root apices in ecosystems invaded by phytopathogens, at different

levels of the fungicide, are shown in Figure 6. As rhizosphere population of border cells was lost when roots were

pulled out of the soil and then rinsed in water, only root apices were analyzed. The microscopic analysis of root apices

did not show any age-related morphological dissimilarities between the apices at different time points of the experiment

(at Days 10, 20, and 30); at the same time, the morphological characteristics of root apices differed considerably

between experimental groups. The roots of the wheat plants grown from the seeds initially contaminated by *Fusarium*

had either undamaged apices (Fig. 6a) or apices with loosened cells at the tip (Fig. 6b). Most of the wheat plants grown

in the soil supplemented with the fungicide Raxil had root apices with root tip mantles showing obvious signs of the

degradation (Fig. 6d). We assumed that the fungicide TEB had a strong effect on the steroid metabolism of the host

plant, the functions of cell walls (Schrick et al., 2004; Höfte, 2015), and hormonal homeostasis (Lin et al., 2015). Cell

wall damage, membrane system dysfunction, and the disorders of hormonal homeostasis may cause a decrease in the

activity of plant defense systems and help the pathogens invade the root system. The damage of the root ultrastructure

of *Pennisetum americanum* seedlings treated with atrazine was shown by Jiang et al. (Jiang et al., 2017).

The pretreatment of the seeds with the fungicide also caused the development of apices with clear signs of

degradation of the root tip mantle. The lateral surfaces of the root tip mantle were most degraded (Fig. 6c, shown by

arrows). The pre-emergence treatment of seeds affects the functional systems of the germinating seeds more than

fungicide application to soil. Penetrating through the seed coating, the fungicide can induce disorders of sterol

metabolism at very early germination stages. The sterol metabolism determines morphogenesis processes in the early

stages of development of the sprout (Closa et al., 52; Peng et al., 2015). The disorders of the sterol-dependent stages of

morphogenesis may cause various structural and functional defects in the developing root. Thus, the morphology of root

apices of wheat plants reflects the effects of stresses caused by both the phytopathogen and the fungicide.

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323   The results of biochemical and morphological investigations of wheat root apices suggest that these parameters

324   can be used as endpoints to evaluate the toxic effects of the fungicide tebuconazole. Another finding is that the effect of

325   TEB on redox homeostasis in wheat roots depends on the plant growth stage.

326   **4.   Conclusion**

327   We studied the toxic effects of the fungicide tebuconazole and fusarium infection on wheat roots in the

328   experiments with two-day-old wheat sprouts and 30-day experiments with wheat stands based on the changes in the

329   morphology of root apices and integrated parameters of redox homeostasis: the contents of proline and products of

330   peroxidation of proteins (carbonylated proteins) and lipids (malondialdehyde) in roots. In the experiments with two-

331   day-old wheat sprouts infected by Fusarium, the content of carbonylated proteins in the roots dramatically increased,

332   which was accompanied by an increase in the number of border cells and the size of the gel mantle of the root apex

333   (compared to uninfected sprouts). The fungicide tebuconazole did not influence the content of carbonylated proteins in

334   the roots of infected sprouts at the concentrations studied, but led to a sharp decrease in the number of border cells and

335   the size of the gel mantle (until complete disappearance) in the root. The study of the effects of TEB and fusarium

336   infection on wheat plants in a 30-day experiment showed that the effect of the fungicide TEB on redox homeostasis in

337   wheat roots varied depending on the plant growth stage and was significantly different in ecosystems with plants

338   infected by fusarium infection. The results of biochemical and morphological investigations of wheat root apices

339   suggest that these parameters can be used for the evaluation of biological action of fusarium infection and fungicides.

340   **Conflict of interest**

341   No conflict of interest to declare.

347

348   **References**

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504   Table Contents of carbonylated proteins (CP), malondialdehyde (MDA), and proline in roots of two-day-old *T.*

505   *aestivum* sprouts infected by *F. graminearum.*

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Content in roots	Fungicide concentration in the medium, $\mu g\ ml^{-1}$			
	0	0.01	0.1	1.0
CP ($nM\ mg^{-1}$ protein)	8.31±1.22	8.98±0.96	8.91±1.10	9.05±1.53
MDA ($nM\ mg^{-1}$ protein)	0.261±0.031	0.284±0.024	0.281±0.028	0.297±0.033
Proline ($\mu g\ mg^{-1}$ protein)	15.05±1.85	15.61±1.01	16.82±1.65	15.51±1.32

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557 **Figure Legends**

558 Fig. 1 The contents of carbonylated proteins (nM mg^{-1} protein), malondialdehyde (nM mg^{-1} protein), and

559 proline (µg mg^{-1} protein) in roots of two-day-old *T. aestivum* sprouts: along the X-axis: 1 – uninfected sprouts; 2 –

560 sprouts infected by *F. graminearum*. Asterisks denote values of 2 significantly different from values of 1, $p > 0.05$.

561 Fig. 2 Morphology of the root apices of two-day-old wheat (*T. aestivum*) sprouts: a – the root apex of

562 uninfected wheat sprouts; b, c, d – root apices of *F. graminearum*-infected sprouts.

563 Fig. 3 The effect of tebuconazole concentration on the number of border cells (BC) (a) and "bare" apices (b) in

564 the root apices of two-day-old wheat (*T. aestivum*) sprouts infected with *F. graminearum*. Asterisks denote values

565 significantly different from values of the test with no tebuconazole added to the medium, $p > 0.05$.

566 Fig. 4 Dynamics of *Fusarium* abundance in soil at different days of the experiment (a) and photographs of the

567 colonies with purple-pink pigmentation at Day 30 (b): (k-) - negative control (infected seeds sown to the soil without

568 Raxil Ultra), (k+) - positive control (infected seeds sown to the soil with Raxil Ultra).

569 Fig. 5 The contents of proline (10^{-6} M mg^{-1} protein), malondialdehyde (10^{-9} M.mg^{-1} protein), carbonylated

570 proteins (10^{-8} M mg^{-1} protein) in roots of wheat *T. aestivum* plants infected with *F. graminearum* at different days of the

571 experiment: (k-) - negative control (infected seeds sown to the soil without Raxil Ultra), (k+) - positive control (infected

572 seeds sown to the soil with Raxil Ultra), and P – seeds pretreated with Raxil Ultra.

573 Fig. 6 Morphology of root apices of *Fusarium*-infected wheat plants: a, b – control, infected seeds, with no

574 TEB applied to the soil: a – healthy apex and b – damaged apex; c – seeds pretreated with Raxil Ultra before sowing –

575 insignificant degradation of the mantle on the tip of the apex and destroyed lateral surface (shown by arrows); d – soil

576 application of Raxil Ultra – damaged apices with loosely packed cells at the tip
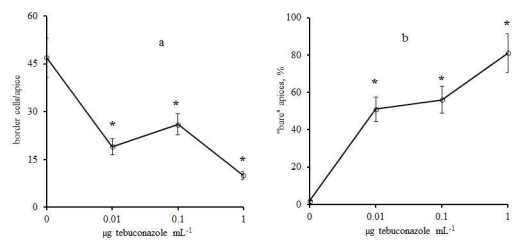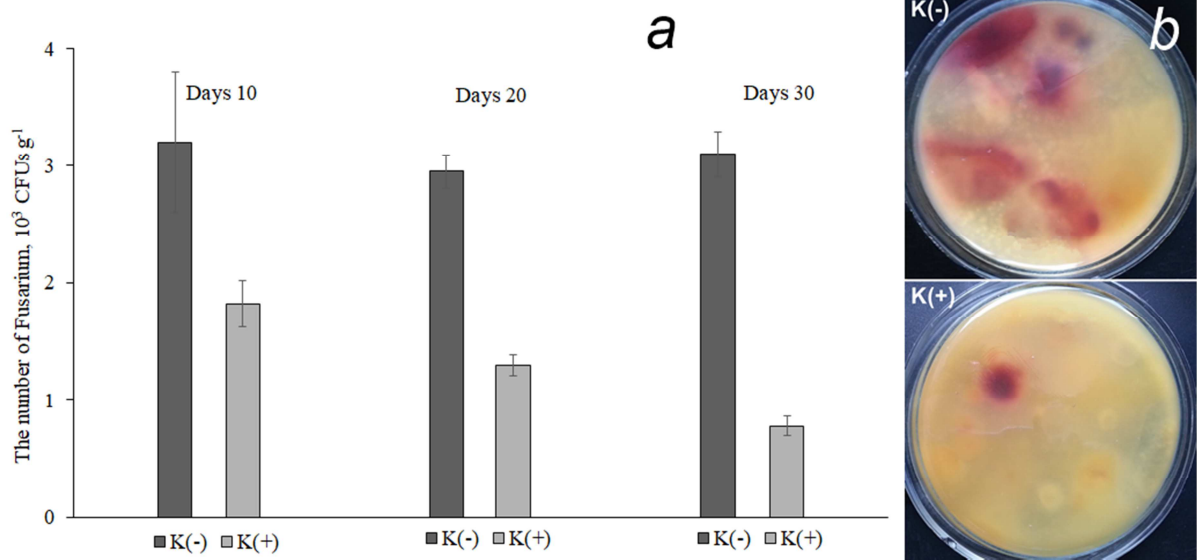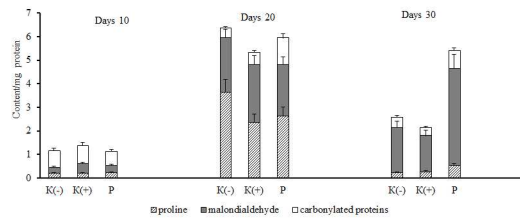
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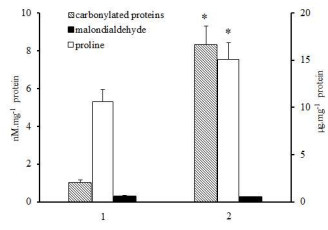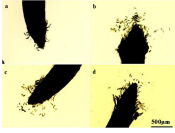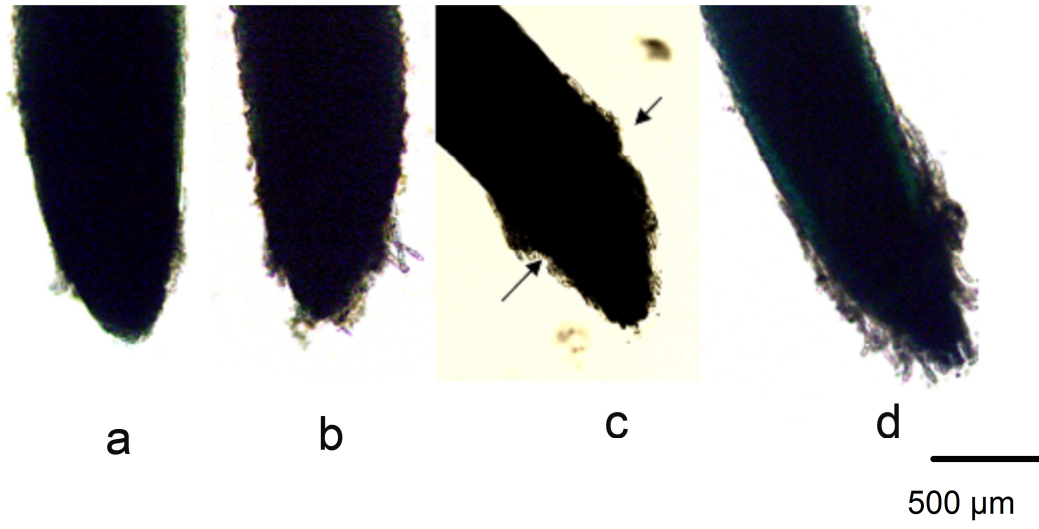
1. Toxic effects of tebuconazole (TEB) on *Fusarium*-infected wheat were studied.

2. The content of free proline, carbonylated proteins and malondialdehyde was determined

3. TEB causes a dose-dependent decrease in the number of border cells of root apices

Authors' contribution to the article:

Ekaterina Shishatskaya – biochemical study: measuring of the contents of proline, malondialdehyde, and carbonylated proteins in roots

Natalia Menzianova – morphological study of root apices; wheat cultivation

Natalia Zhila – a study of the contamination of seeds and soil by phytopathogenic fungi

Svetlana Prudnikova – a study of effect of TEB on *Fusarium* in soil

Volova Tatiana – analysis of results obtained, article preparation

Sabu Thomas – general leadership, article preparation