https://doi.org/10.17221/127/2022-PSE

Soil enzyme activities after application of fungicide Quadris^{R} at increasing concentration rates

Silvena Boteva Boteva[1], Anelia Evgenieva Kenarova[1], Michaella Roumenova Petkova[2], Stela Stoyanova Georgieva[3], Christo Dimitrov Chanev[3], Galina Simeonova Radeva[2]*

[1]*Deparment of Ecology and Environmental Protection, Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria*
[2]*Roumen Tsanev Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia, Bulgaria*
[3]*Department of Organic Chemistry and Pharmacognosy, Faculty of Chemistry and Pharmacy, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria*
**Corresponding author: sbboteva@biofac.uni-sofia.bg*

Citation: Boteva S.B., Kenarova A.E., Petkova M.R., Georgieva S.St., Chanev Ch.D., Radeva G.S. (2022): Soil enzyme activities after application of fungicide Quadris^{R} at increasing concentration rates. Plant Soil Environ., 68: 382–392.

Abstract: The study aimed to assess the effects of fungicide Quadris^{R} on activities of soil enzymes contributed to soil nutrient turnover. A batch laboratory experiment with Quadris^{R}-amended (0 mg/kg ds (dry soil) – 35.00 mg/kg ds) loamy sand soil was conducted, and shifts in soil physical environments and enzyme activities (beta-glucosidase, urease, acid and alkaline phosphatases, arylsulfatase and dehydrogenase) were evaluated on experimental days 1, 30, 60, 90 and 120. The results indicated that Quadris^{R} changed both soil properties and enzyme activities. The most sensitive environmental parameter to fungicide input was soil pH. The most suscaptable to Quadris^{R} enzymes were dehydrogenase and arylsulfatase, and the most resistant – urease. The mean overall dehydrogenase activity decreased by 33%, whereas the profile of arylsulfatase activity tended to permanent decrease over time. The general pattern of enzyme responses to Quadris^{R} was an immediate-early (days 1 – 30) decline of enzyme activities after fungicide application, except that of arylsulfatase. Beta-glucosidase manifested a temporal profile of steady-state stimulation under the lowest (2.90 mg/kg ds) and low sensitivity to the higher (14.65 mg/kg ds and 35.00 mg/kg ds) fungicide concentrations.

Keywords: azoxystrobin; Cambisols; soil mesocosms; soil hydrolases; soil oxidoreductase

Pesticides represent the backbone of the contemporary agrifood sector in its endeavour to ensure the growing human population with food and other goods (Baćmaga et al. 2015). Fungicides are the most applied category of pesticides for disease management in crop production, affecting fungi by damaging their cell membranes, inactivating critical enzymes or proteins, or by interfering with key processes such as energy production, respiration, sterols' or chitin's synthesis. Despite the intensive use of fungicides and the associated potential ecotoxicological risks for non-target organisms, the environmental fate and the effects of fungicides have received far less attention compared to the other pesticide categories. For instance, Köhler and Triebskorn (2013) published that only 13% of the studies on pesticide effects between 1991 and 2013 focused on fungicides, compared to 62% and 24% for insecticides and herbicides, respectively. Earlier studies recorded negative side effects of fungicides on soil non-pathogenic fungi (Howell et al. 2014),

Supported by the National Research Fund of the Bulgarian Ministry of Education and Science, Grant No. DN 11/6 – Dec 2017).

https://doi.org/10.17221/127/2022-PSE

bacteria (Wang et al. 2004), archaea (Puglisi et al. 2012), protozoa (Bending et al. 2007), nematodes (Howell et al. 2014) and soil enzymes (Bending et al. 2007, Muñoz-Leoz et al. 2011, Wang et al. 2016).

Soil is a reservoir of many enzymes, and therefore it is regarded as an indicator of microbiological activity (Baćmaga et al. 2015). Enzymes which indicate soil quality include oxydoreductases and hydrolases due to their participation in a range of biochemical reactions which take place in the environment (Utobo and Tewari 2015). The most tested enzymes for fungicide effects on soil biochemistry were soil dehydrogenase (Bending et al. 2007, Muñoz-Leoz et al. 2011, Wang et al. 2016) and acid phosphatase (Muñoz-Leoz et al. 2011, Wang et al. 2016). Far less attention was paid to beta-glucosidase, cellulase, amylase, invertase, arylsulfatase and alkaline phosphatase (Floch et al. 2011, Muñoz-Leoz et al. 2011, Wang et al. 2016).

Strobilurins (azoxystrobin, pyraclostrobin, fluoxastrobin, kresoxim-methyl, trifloxystrobin, picoxystrobin, mandestrobin, metominostrobin and others) are fungicides designed to block fungal mitochondrial respiration (Bartlett et al. 2002), and they present the largest category by market value, which accounts for 23– 25% of the global fungicide sales (Anonymus 2016). One of the most worldwide used strobilurins is azoxystrobin (methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate) (Wang et al. 2018), being discovered and developed by ICI (now Syngenta). Azoxystrobin is marketed by Syngenta as a single active substance product under several trade names, the major ones for crop protection being: Amistar, Abound, Priori, Quadris, Dynasty for seed treatments, and Heritage for turf. Data concerning azoxystrobin's non-target effects on soil organisms and enzyme activities are still insufficient and, in some cases, contradictory. Most of the researchers who tested azoxystrobin effects on soil enzymes used primarily dehydrogenase (Sopeña and Bending 2013, Baćmaga et al. 2015, Guo et al. 2015, Álvarez-Martín et al. 2016, Wang et al. 2018) and urease (Baćmaga et al. 2015, Guo et al. 2015, Wang et al. 2018) as indicators of soil health. Additionally, the pure substance azoxystrobin was used for toxicity tests in most of the studies. However, commercial products consist not only of active but also of other ingredients, some of which may influence negatively or positively on soil organisms and soil biochemistry.

The aim of this study was to assess the changes that occurred in the activities of five soil hydrolases (beta-glucosidase, urease, alkaline and acid phosphatases, arylsulfatase) and dehydrogenase (oxidoreductase) under a single application of fungicide Quadris^{R} at increasing concentrations. The enzymes were selected to identify the fungicide effects on: (1) the main pathways of carbon (beta-glucosidase), nitrogen (urease), phosphorous (acid and alkaline phosphatases), and sulphur (arylsulfatase) cycle in soil, and (2) the cell energy (dehydrogenase) of soil microorganisms (bacteria and fungi) as the main producers of soil enzymes.

MATERIAL AND METHODS

Sampling site and procedure. Soil samples were collected from a grassland near Gabra village (Sofia region, Bulgaria – 42°31'48.36"N and 23°37'28.20"E). This land location is a point of the Bulgarian soil quality monitoring network. It is regularly monitored by the Bulgarian Executive Environment Agency (EEA) for pesticides and heavy metal residues. Data received by EEA for this point indicated a lack of soil contamination (personal communication). Five subsamples were pooled randomly from a 0–20 cm soil depth, sieved through 2 mm mesh, and mixed in aliquots after determining the dry weights of 1 g samples at 105 °C in an oven for 24 h.

Fungicide. The study was conducted with fungicide Quadris^{R} (Qs) containing azoxystrobin (Az) as an active substance at 250 mg/L. Qs consists of 22.9% Az, and 77.1% other ingredients, most of which are trade secret, except 1,2-propanediol and bentonite. With its xylem-mobile systemic activity, Qs is a broad-spectrum fungicide and provides complete plant protection, inhibiting fungal spore germination. Qs offers effective control of all four classes of fungi (ascomycetes, basidiomycetes, deutoromycetes and oomycetes), and it is used on grape vines, cereals, potatoes, apples, bananas, citrus, tomatoes and other crops, as well as it optimises crop yield and quality by helping the plant to use resources efficiently. Qs was applied to soil mesocosms in concentrations of 0.00 mg/kg dry soil (ds) (Qs0), 2.90 mg/kg ds (Qs1), 14.65 mg/kg ds (Qs2) and 35.00 mg/kg ds (Qs3) calculated towards Az. The fungicide mentioned above concentrations noted soil Az residues identified by gas chromatography immediately after fungicide application to soil mesocosms (Alexova et al. 2021). These concentrations reflected half (Qs1) of the maximum recommended field dose of Az (Bending et al. 2007), and three (Qs2) and seven (Qs3) times

https://doi.org/10.17221/127/2022-PSE

higher than Qs1. Qs2 and Qs3 were in a concentration gradient, which has been previously shown to alter the soil fungal community structure (Bending et al. 2007), and used to investigate the outcomes of accidental overexposure to the fungicide due to inappropriate use.

Design of mesocosm experiment. Soil mesocosms were prepared in triplicates. Each contained 2 000 g of dry weight equivalent soil amended with azoxystrobin (Az) under the form of commercial product Quadris^{R}. The fungicide was dissolved in 30 mL acetone and uniformly mixed into a separate soil sample (200 g wet soil) derived from the respective mesocosm. Then, acetone was allowed to volatilise, and the sample was added to the rest of the soil by mixing during a 10-min period. An equal volume of acetone was added to the control soil, following the procedure mentioned above. Soil water content was adjusted to 40% of the maximum water holding capacity. The soil moisture was maintained by weighting soils every 3 days and using sterile distilled water in order to compensate for any moisture loss. The mesocosms were incubated at 22 ± 1 °C in the dark to prevent the Az physical degradation by light. Soil samples were collected randomly in triplicates from each mesocosm on the 1st (D1), 30th (D30), 60th (D60), 90th (D90) and 120th (D120) day after fungicide application.

Soil physico-chemical properties. The soil was classified as Cambisols (FAO) with loamy sand texture (83% sand, 2% clay, and 15% silt) determined according to the sedimentation method in 0.1 mol/L KCl (https://www.nrcs.usda.gov/wps/portal/nrcs/main/soils/edu/). Organic carbon was 21.92 ± 1.41 g/kg ds, Kjeldahl nitrogen – 2.20 ± 0.21 g/kg ds, and potassium – 131.16 ± 4.40 mg/kg ds. Soil pH was measured potentiometrically (HANNA Instruments, Woonsocket, USA) after suspending soil in 0.01 mol/L $CaCl_2$ solution (1:5; weight:volume) and shaking for 30 min. Soil bioavailable forms of nitrate nitrogen (NO_3^--N), ammonium nitrogen (NH_4^+-N) and inorganic phosphates (P) were determined spectrophotometrically by the methods of Keeney and Nelson (1982) and Olsen (1982), respectively.

Azoxystrobin soil residues. Gas chromatography was used to determine the concentrations of Az soil residues on experimental days. Detailed Az extraction procedure and gas chromatography settings were described in Aleksova et al. (2021).

Enzyme assays. The general approach was to cultivate a soil sample (1 g wet soil) with the respective enzyme's substrate at 22 °C in the dark for 1 h, followed by spectrophotometry of soil supernatant/extract and calculation of enzyme activity according to a standard curve. Enzyme activities were calculated as micrograms per gram of dry soil per hour. Dehydrogenase (EC 1.1; Dha) activity was determined, followed by Friedel et al. (1994), measuring (λ = 405 nm) the reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium to iodonitrotetrazolium formazan. Beta-glucosidase (EC 3.2.1.21; BGl) activity was measured according to Eivazi and Tabatabai (1988), determining (λ = 405 nm) the amount of released p-nitrophenol during the incubation of soil samples with *p*-nitrophenyl glycoside. Urease (EC 3.5.1.5; Ur) activity was determined according to Kandeler and Gerber (1988), and ammonia concentration resulting from the urea hydrolysis was evaluated (λ = 690 nm) based on a modified indophenol reaction. Alkaline (EC 3.1.3.1; AlP) and acid (EC 3.1.3.2; AcP) phosphatases were assayed by the method of Tabatabai and Bremner (1969), which involves the determination (λ = 420 nm) of p-nitrophenol released after sample incubation with p-nitrophenyl phosphate in buffer with pH 11.0 (AlP) and pH 6.0 (AcP). Arylsulphatase (EC 3.1.6.1; Ars) activity was measured (λ = 400 nm) according to a modified method of Tabatabai and Bremner (1970) after the release of p-nitrophenol as a final product of p-nitrophenyl sulphate hydrolysis.

Data analysis. Each data point in the paper represented the mean value of the respective Qs soil amendment ± standard deviation. One-way ANOVA followed by Tukey's test was performed to examine the differences in the means of soil (pH, NO_3^--N, NH_4^+-N, P, Az) and enzyme (Dha, BGl, AcP, AlP, Ur and Ars) values. Linear discriminant analysis (LDA) was used to distinguish the effects of Qs application on soil physical environments and enzyme activities in relation to the applied fungicide concentration. The above statistics were performed with the packages PAST (Hammer et al. 2001) at a significance of $P < 0.05$.

RESULTS AND DISCUSSION

Soil environments. Qs application did not significantly affect soil organic carbon, Keljdal nitrogen and potassium, the opposite to the induced significant changes in the values of soil pH, NO_3^--N, NH_4^+-N and P. The mean overall values of Qs0's soil metrics were 5.87 ± 0.4 (pH), 84.39 ± 31.5 mg/kg ds (NO_3^--N), 3.83 ± 3.8 mg/kg ds (NH_4^+-N), and 12.13 ± 7.8 mg/kg ds (P). The

https://doi.org/10.17221/127/2022-PSE

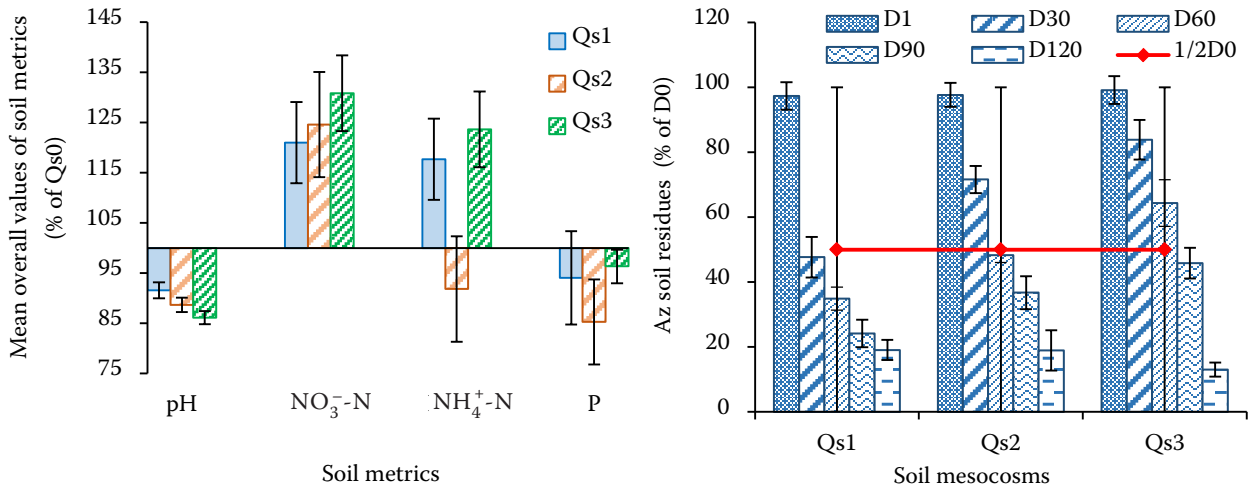

Figure 1. Soil physical environment expressed by (A) mean overall values of pH, nitrate nitrogen (NO_3^--N), ammonium nitrogen (NH_4^+-N), phosphates (P) at Qs1 (2.90 mg/kg ds), Qs2 (14.65 mg/kg ds) and Qs3 (35.00 mg/kg ds) mesocosms calculated as a percent of Qs0 (0 mg/kg ds), and (B) azoxystrobin (Az) soil residues on days of sampling (D1, D30, D60, D90 and D120). The symbols indicate mean values; the bars indicate standard errors. D1 – 1, D30 – 30, D60 – 60, D90 – 90, D120 – 120 days after fungicide application

fungicide decreased the values of pH (by 8–14%) and P (by 4–15%) and increased these of NH_4^+-N (by 17–24%, except Qs2) and NO_3^--N (by 21–31%) (Figure 1A). Detailed temporal dynamics of soil metrics were given in Aleksova et al. (2021). Over time, Az soil residues decreased in an inverse relationship with the applied fungicide concentrations (Figure 1B).

The profiles of Az dissipation indicated possible inhibitory effects on Az degradation at higher fungicide concentrations, being prolonged by one (Qs2) and two (Qs3) months compared to that of Qs1. Similar environmental changes after the Az application were detected by many authors. For example, soil acidification was explained by Ghosh and Singh (2009) as a result of azoxystrobin acid accumulation – the main end product of Az degradation; increasing of soil NO_3^--N's concentrations was related to fungicide stimulation on soil microbial

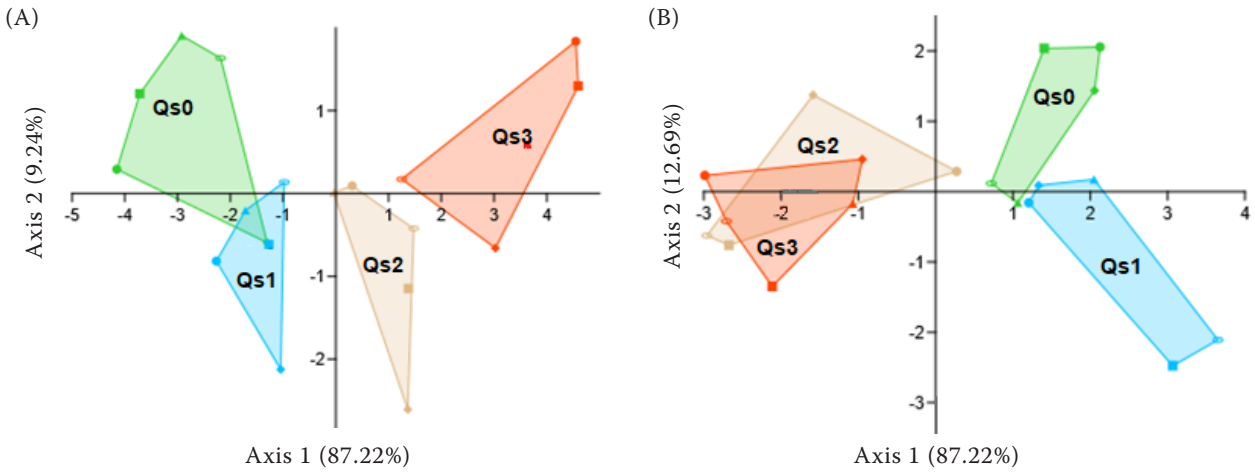

Figure 2. Linear discriminant score plots of (A) soil physical environments and (B) enzyme activities, both in Quadris^R un-amended (Qs0) and fungicide amended with 2.90 mg/kg ds (Qs1), 14.65 mg/kg ds (Qs2) and 35.00 mg/kg ds (Qs3) soil mesocosms. Symbols represent soil properties on D1 (dot), D30 (square), D60 (triangle), D90 (diamond) and D120 (oval); D1 – 1, D30 – 30, D60 – 60, D90 – 90, D120 – 120 days after fungicide application

https://doi.org/10.17221/127/2022-PSE

nitrification (Muñoz-Leoz et al. 2011), or nitrogen input by fungicide adjuvants (Mijangos et al. 2009).

In order to assess the overall effects of Qs on soil physical environments, a linear discriminant analysis was conducted (Figure 2A).

The first two functions provided discrimination among the soil's physical environments accounting for more than 96% of the total variance (Figure 2A). The scores were clustered in relation to the applied fungicide concentrations, discriminating different soil physical environments (except the overlap of Qs1 and Qs0 on D30). The discriminant model provided a correct classification of 90% of the variability among soils, in which 80% were correct for each of Qs0 and Qs3, and 100% for each of Qs1 and Qs2. The strongest discriminators among analysed soil variables were NO_3^--N and Az soil residues.

Soil enzyme activities. Soil enzyme activity is a result of complex processes of synthesis and regulation. In this term, Qs could affect soil microorganisms by killing non-target fungi or inhibiting the activity of certain microbial groups, or changing the values of soil properties. Additionally, the dead fungal biomass or the fungicide inputs could serve as nutrients, mitigating fungicide impacts on living microorganisms or reducing the interspecific microbial competition. In this study, soil enzymes were used as indicators of Qs effects on soil quality and health of microbial communities, as the main producers of soil enzymes.

Qs application induced changes in the overall enzyme activities and these changes were concentration-independent (Table 1).

In general, Qs negatively affected soil enzymes, except at Qs1, where the overall mean activities of BGl, AlP and Ur were slightly stimulated by 17.7, 9.5 and 2.7%, respectively. Qs1 was toxic for Dha, Ars and AcP, reducing the mean overall enzyme activities by 33.0, 13.0 and 4.0%, respectively.

The temporal data of enzyme activities were used to construct the LDA model in order to distinguish the biochemistry of soil mesocosms (Figure 2B). After the analysis, three discriminant functions were obtained. The first and the second functions provided discrimination among the soil mesocosms, accounting for 87.22% (Axis 1) and 12.69% (Axis 2) of the total variance.

The scores calculated from the first two discriminant functions were visualised in Figure 2B. Each symbol in the convex hull represented the soil enzyme activities from the respective sampling day. The scores were clustered in relation to the applied fungicide concentration, discriminating both the enzyme activities of Qs0 from that of the fungicide amended soil mesocosms and the enzyme activities of Qs0 and Qs1 from that of Qs2 and Qs3. The convex hulls of Qs2 and Qs3 overlapped, indicating an insignificant difference in enzyme dynamics at fungicide concentrations equal to and higher than 14.65 mg/kg ds. The overlapping of convex hulls at higher fungicide concentrations could be interpreted as evidence for induction of microbial community tolerance to Qs by the selection of resistant species (Álvarez-Martín et al. 2016, Wang et al. 2020) and thus unification of microbial responses. In this term, Qs could be considered a microbial structuring factor at concentrations equal to and higher than 14.65 mg/kg ds. A future study will investigate such effects in the fungicide concentration range from 2.90 mg/kg ds to 14.65 mg/kg ds.

The first two discriminant scores in Figure 2B were loaded by the variability of Dha and BGl (Axes 1

Table 1. Overall enzyme activities (means ± standard deviation) of control (Qs0) and Qs-amended soil mesocosms with 2.90 mg/kg ds (Qs1), 14.65 mg/kg ds (Qs2) and 35.00 mg/kg ds (Qs3)

Enzyme	Enzyme activity (μg/g ds/h)			
	Qs0	Qs1	Qs2	Qs3
Dehydrogenase (Dha)	20.11 ± 9.2^{a}	13.51 ± 4.2^{b}	12.25 ± 4.3^{b}	11.36 ± 4.4^{b}
Beta-glucosidase (BGl)	48.4 ± 14.0^{a}	57.03 ± 16.5^{b}	43.71 ± 13.6^{a}	45.76 ± 16.7^{a}
Urease (Ur)	8.89 ± 5.3^{a}	9.14 ± 5.1^{a}	8.05 ± 4.7^{a}	8.12 ± 4.8^{a}
Acid phosphatase (AcP)	57.01 ± 10.2^{a}	55.12 ± 8.2^{a}	53.59 ± 4.2^{ab}	58.39 ± 8.0^{ac}
Alkaline phosphatase (AlP)	5.94 ± 5.4^{a}	6.50 ± 4.7^{ab}	5.10 ± 2.1^{a}	4.57 ± 1.4^{ac}
Arylsulfatase (Ars)	19.12 ± 5.9^{a}	16.96 ± 9.2^{a}	15.86 ± 10.1^{a}	16.68 ± 9.4^{a}

Mean values marked by the same letters per enzyme are not significantly different ($P < 0.05$) from each other according to Tukey's pairwise post-hoc test

https://doi.org/10.17221/127/2022-PSE

and 2) and AcP and Ars (Axis 2). The discriminant model provided a correct classification of 65% of the enzyme variability among the soil mesocosms, in which 100% were correct for Qs0, 60% for each Qs1 and Qs3, and 40% for Qs2. Single LDAs were conducted to determine the relevance of each enzyme in expressing the Qs impact on soil biochemistry (Figure 3).

The eigenvalues obtained from LDA indicated that the first discriminant factor (Axis 1) captured most of the variance among the enzyme activities at the respective sampling days and contributed from

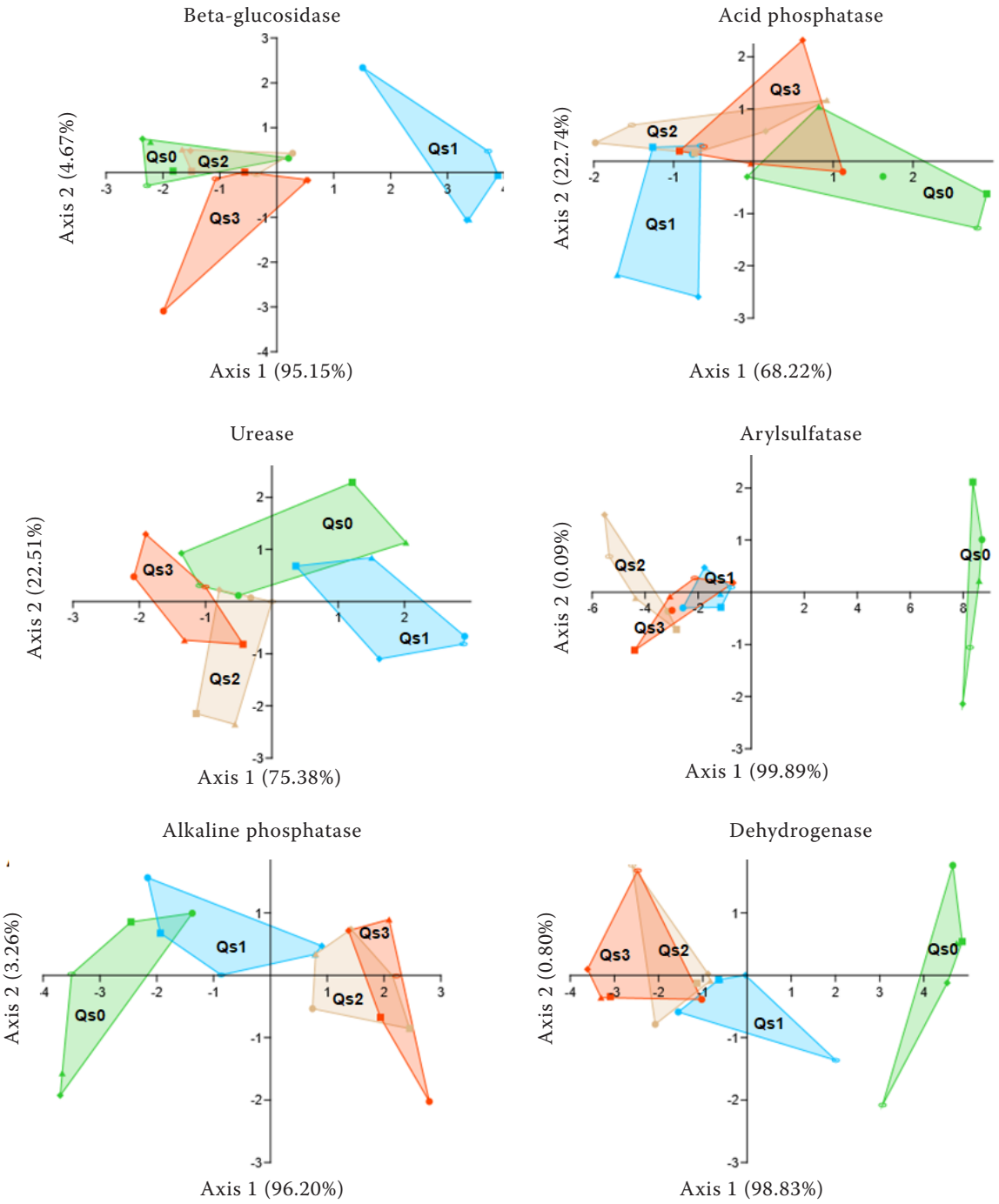

Figure 3. Score plots of the first two discriminant functions of enzyme activities in Quadris^{R} un-amended (Qs0) and amended with 2.90 mg/kg ds (Qs1), 14.65 mg/kg ds (Qs2) and 35.00 mg/kg ds (Qs3) soil mesocosms. Symbols represent the respective enzyme activity on D1 (dot), D30 (square), D60 (triangle), D90 (diamond) and D120 (oval). D1 – 1, D30 – 30, D60 – 60, D90 – 90, D120 – 120 days after fungicide application

https://doi.org/10.17221/127/2022-PSE

68% (AcP) – 75% (Ur) to 95–99% (BGl, AlP, Dha and Ars) of the total data dispersion (Figure 3). Axis 2 explained much lower temporal enzyme variance, contributing from 0.09% (Ars) to 22.7% (AcP) of the total dispersion. Individual discriminant models indicated: (1) clear segregation of Qs-amended from Qs-unamended soil mesocosms concerning the fungicide effects on Dha and Ars; (2) fungicide effects on AlP could discriminate Qs0 from both Qs2 and Qs3, but not from Qs1; (3) Qs concentration of 2.90 mg/kg ds stimulated BGl and differentiated Qs1 from the other soil mesocosms concerning enzyme responses to the fungicide; (4) activities of Ur and AcP did not change significantly under Qs; (5) LDA was not powerful to discriminate well the enzyme responses of Qs2 and Qs3 (2 × Az2), indicating threshold effect, already discussed in section "Overall enzyme activity".

It was shown that the most sensitive to Qs were Dha and Ars. Qs inhibited their overall enzyme activities by 33–44% (Dha) and by 11–17% (Ars), and the Qs harmful effect increased by increasing the fungicide concentrations. Ars's inhibition was not so dramatic as that of Dha, but the temporal profiles of enzyme activities showed the permanent trend of deepening of enzyme inhibition with a minimal value on D120 (60% from Qs0, on average). Az inhibition on Dha was reported by other authors (Bending et al. 2007, Sopeña and Bending 2013, Guo et al. 2015, Wang et al. 2018), although a lack of significant effects was also reported by Baćmaga et al. (2015), and Álvarez-Martín et al. (2016). For example, Baćmaga et al. (2015) worked with Az concentrations ranging from 0.075 mg/kg ds to 22.5 mg/kg ds and identified an insignificant decrease (by 7%) of Dha activity only at fungicide concentration of 22.5 mg/kg ds. On the other hand, Guo et al. (2015) and Wang et al. (2018) worked with fungicide concentrations from 0.1 mg/kg ds to 10.0 mg/kg ds, and both of them reported a high decrease of enzyme activity, although the authors tested different soil types. Wang et al. (2018) reported a 16–69% decrease of Dha activity depending on applied fungicide concentration. Comparing the results, we supposed that the data discrepancies come from both fungicide concentrations used and soil types tested by the authors. Furthermore, other fungicides like benomyl and captan (Chen et al. 2001), mefenoxam and metalaxyl (Monkiedje et al. 2002), and carbendazim (Burrows and Edwards 2004), mancozeb and dimethomorph (Cycoń et al. 2010) were also proved to have negative effects on soil Dha. It was shown in all these studies that the decline rate of Dha activity related linearly to the fungicide chemistry, concentration, and exposure time (Bending et al. 2007, Sopeña and Bending 2013, Guo et al. 2015, Wang et al. 2018). In the context of our results, we proved that Qs affected soil respiration (Dha), even at a concentration of 2.90 mg/kg ds (Az), probably by killing or inhibiting the non-target microorganisms like soil non-pathogenic fungi. Fungi are an important part of soil microbiology. They dominate in soils, especially acidic soils (Lavelle and Spain 2005), breaking down the organic residues so that many different types of microbes can start to decompose and process the residues into usable products.

LDA well segregated the Ars responses of Qs-impacted soil mesocosms from that of the control, in contrast to ANOVA and Tukey's test, which were not powerful to distinguish this difference. Formerly, a few studies were interested in fungicide effects on Ars, and none of them studied Az or its commercial products. Muñoz-Leoz et al. (2011) studied the influence of tebuconazole on Ars in a concentration range from 5 mg/kg ds to 500 mg/kg ds. They recorded harmful effects on enzyme activity even at the lowest applied concentration. On the opposite, Muñoz-Leoz et al. (2013) found that difenoconazole, another triazole fungicide, affected Ars just at a concentration of 500 mg/kg ds. Fungicides mancozeb (Floch et al. 2011) and metalaxyl (Sukul 2006) inhibited Ars at 100 mg/kg ds and 160 mg/kg ds, respectively. Comparing our results with the above-shown data, we could conclude that Quadris^{R}, respectively its active ingredient (Az), is highly toxic to Ars along with the tebuconazole. The gradually decrease of Ars activity over fungicide exposure time (more than 4 months) may significantly inhibit the biochemical mineralisation of organic sulfur moieties in soils and thus reduce bioavailable sulfur for plant growth. The deficiencies in plant available sulfur have long been recognised as a cause of delayed maturity, stunting of plants, and interveinal chlorosis in crop productions worldwide (Siwik-Ziomek et al. 2016). Our results supported the findings of Ruske et al. (2004) that the application of strobilurin fungicides reduced the concentration of proteins and sulfur in winter wheat grain (cv. Malacca).

LDA also segregated the BGl activity of Qs1 from that of the other mesocosms. Qs stimulated BGl permanently during the incubation time, increasing its overall mean activity by 17.7%. Similar effects on the

https://doi.org/10.17221/127/2022-PSE

enzyme were recorded for fungicides metalaxyl (Sukul 2006) and boscalid (Xiong et al. 2014). According to Riah et al. (2014), the high BGl resistance to pesticides might be related to the enzyme's high functional redundancy since a large number of microbial species is able to express it. Increased activity of BGl may stimulate the hydrolysis of cellobiose residues in soil and release of simple sugars (glucose) that are subsequently incorporated by the soil microbiota as an energy source (Waldrop et al. 2000). Stimulation of soil microorganisms by increasing the number of bioavailable energy sources may partially overcome the negative impact of fungicide on other enzymes. On the other hand, stimulated microbial activity may increase organic matter degradation and soil depletion of it. In this case, the soil may turn into a source instead of the carbon sink for the atmosphere, contributing to global warming.

Time of enzyme responses and temporal dynamics. Most of the tested soil enzymes responded to Qs in a similar manner, showing the lowest activity on D1 (Dha) or D30 (BGl, Ur, AcP, AlP), and the highest one on D120 (Dha, BGl, Ur, AlP) (Figure 4). The exception of this response model were the activities of AcP and Ars with maximum values on D1 (AcP and Ars) and minimum – on D120 (Ars). Comparing to Qs0 the lowest and the highest values of enzyme activities (on average) were 45.8% (Dha), 97% (BGl), 59.6% (Ur), 76.7% (AcP), 62.2% (AlP) and 60% (Ars), and 75.5% (Dha), 120.1% (BGl), 118.9% (Ur), 131.1% (AcP), 151.2% (AlP) and 111% (Ars), respectively.

Our results confirmed the findings of some authors about the "immediately-early" inhibition of Dha (Bending et al. 2007, Söpena and Bending 2013, Baćmaga et al. 2015, Guo et al. 2015, Wang et al. 2018), Ur (Baćmaga et al. 2015, Guo et al. 2015, Wang et al. 2018, 2020), and AlP (Baćmaga et al. 2015) after Az application (0.075–50 mg/kg ds). Similar pattern of "immediate-early" responses of AcP were detected to fungicides boscalid (Xiong et al. 2014), tebuconazole (Muñoz-Leoz et al. 2011), difenoconazole (Muñoz-Leoz et al. 2013), and metalaxyl (Sukul 2006). Tebuconasole (Helicur 250 EW) caused "immediate-early" inhibition (on 40 days) and late stimulation (on 60 days) of Ars (Baćmaga et al. 2020) – inverse temporal profile of enzyme activity compared to that under Qs.

Temporal dynamics of enzyme activities were studied, and they demonstrated enzyme-specific profiles (Figure 5), although some common trends could be summarised: (1) enzyme temporal dynamics were not fungicide concentration-dependent, except for BGl and Dha; (2) temporal profiles demonstrated enzyme inhibition of one/two months longevity, followed by recovery (BGl: Qs2–Qs3; Ur: Qs2–Qs3; and AcP: Qs1–Qs3) and stimulation (Ur: Qs1 and AcP:

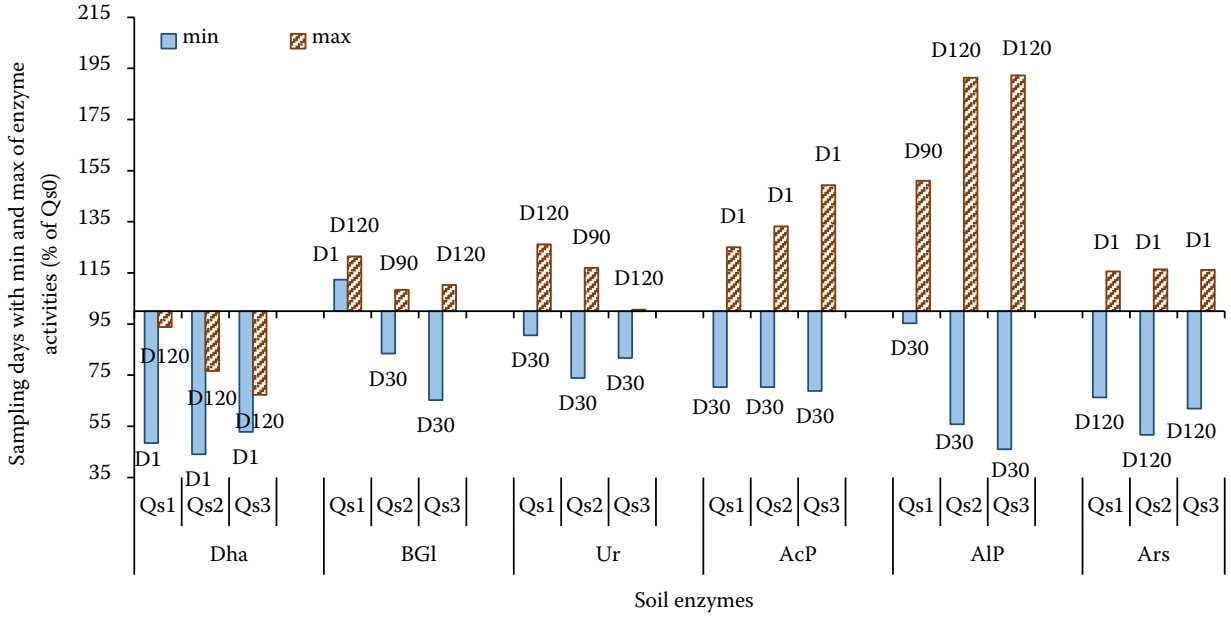

Figure 4. Days (D) of minimum and maximum of enzyme activities at Quadris^{R} (Qs)-amended soil mesocosms in concentrations of 2.90 mg/kg ds (Qs1), 14.65 mg/kg ds (Qs2) and 35.00 mg/kg ds (Qs3) calculated towards the control (Qs0). Dha – dehydrogenase; BGl – beta-glucosidase; Ur – urease; AcP – acid phosphatase; AlP – alkaline alkaline; Ars – arylsulfatase; D1 – 1, D30 – 30, D60 – 60, D90 – 90, D120 – 120 days after fungicide application

https://doi.org/10.17221/127/2022-PSE

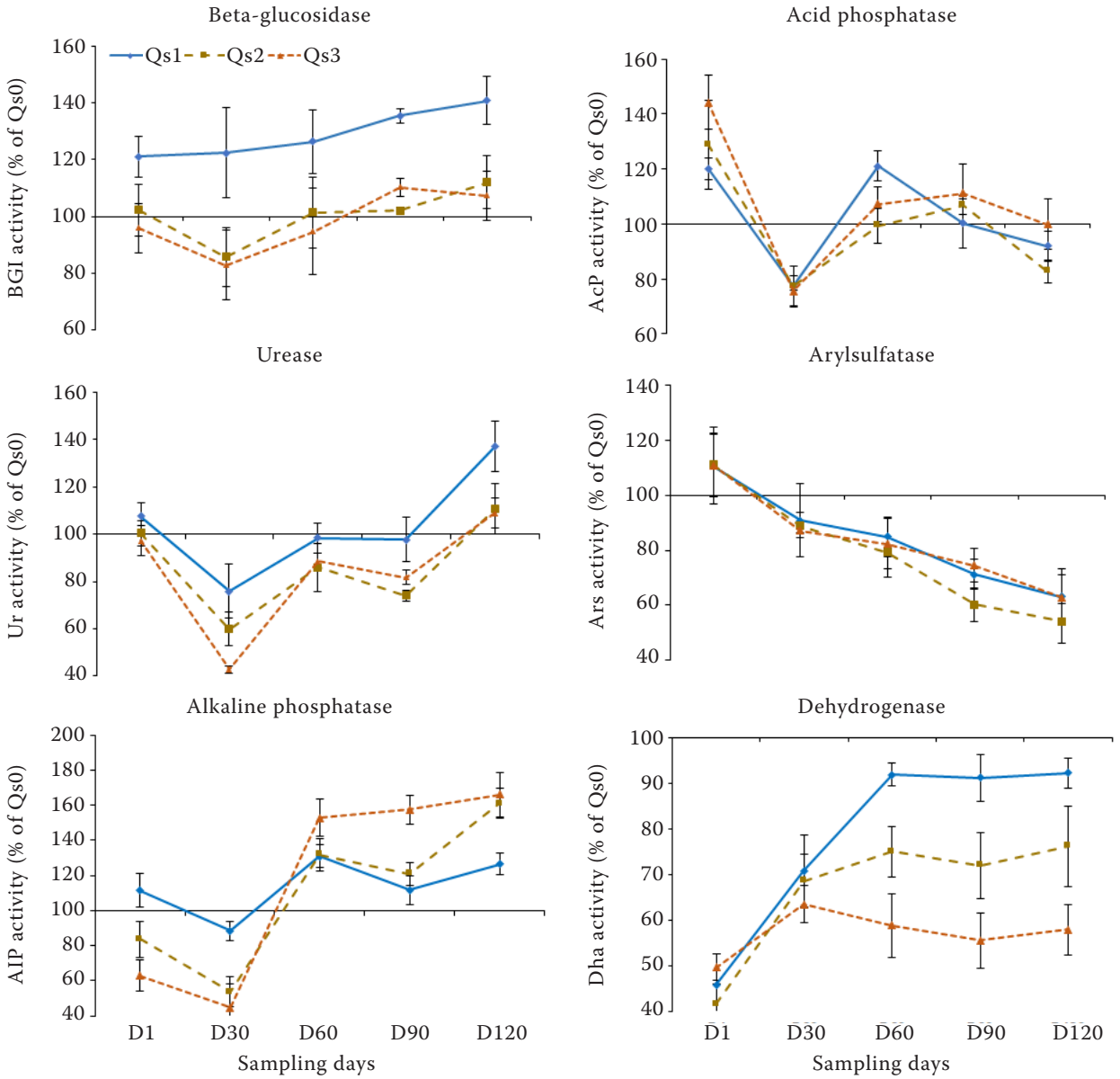

Figure 5. Temporal dynamics of soil enzyme activities under increasing concentrations of Quadris^{R} (Qs1 – 2.90 mg/kg ds; Qs2 – 14.56 mg/kg ds; Qs3 – 35.00 mg/kg ds) expressed as a percent of the control (Qs0). Dha – dehydrogenase; BGl – beta-glucosidase; Ur – urease; AcP – acid phosphatase; AlP – alkaline alkaline; Ars – arylsulfatase; D1 – 1, D30 – 30, D60 – 60, D90 – 90, D120 – 120 days after fungicide application

Qs1–Qs3). More distinct than the others were the temporal profiles of BGl (Qs1) and Ars (Qs1–Qs3), showing the stable-state trend of stimulation (BGl) and downward trend of inhibition (Ars) of enzyme activities. Additionally, Dha's temporal profiles demonstrated uncompleted recovery after the stage of inhibition, reaching up to 92% (Qs1), 74% (Qs2) and 57% (Qs3) of the control enzyme activity (Qs0). We supposed that temporal changes in enzyme activities resulted from mixed effects of changes that occurred in physical environments and soil microbiota. A more profound study should demonstrate the intrinsic nature of these relationships, giving important knowledge for agroecosystem functions driven by soil enzymes under fungicide application.

In general, the soil enzymes of Cambisols were influenced by the application of Quadris^{R}, and the most sensitive to the fungicide were Ars and Dha. The longevity and the significance of Qs impact were enzyme-specific, time-dependent and concentration-independent (except BGl and Dha), although changes were deeper at the higher fungicide concentrations.

https://doi.org/10.17221/127/2022-PSE

Although azoxystrobin is considered to have low toxicity, our results suggest that it should be used carefully and according to the manufacturer's recommendations. We concluded that Quadris^R might influence on crop growth, impacting the rates of nutrient cycling and energy flow in soil even at lowest applied concentration. Its use in increased concentrations unbalanced the soil homeostasis based on the activity of soil microorganisms, and it may have a strong impact on plant growth and yield.

However, it must be noted that this study was focused on Cambisols; thus, fungicide impacts on other soils may vary depending on microbial communities present and other specific soil parameters. Future work should focus on quantifying the soil nutrient impacts of Quadris^R (Az) on other soil types in order to determine both common and soil-specific trends in enzyme activities under fungicide stress.

REFERENCES

Aleksova M., Kenarova A., Boteva S., Georgieva S., Chanev Ch., Radeva G. (2021): Effects of increasing concentrations of fungicide Quadris^R on bacterial functional profiling in loamy sand soil. Archives of Microbiology, 203: 4385–4396.

Álvarez-Martín A., Hilton S.L., Bending G.D., Rodríguez-Cruz M.S., Sánchez-Martín M.J. (2016): Changes in activity and structure of the soil microbial community after application of azoxystrobin or pirimicarb and an organic amendment to an agricultural soil. Applied Soil Ecology, 106: 47–57.

Anonymous (2016): China Strobilurin Fungicides Market Report 2016 Edition – Research and Markets. Available at: https://www.businesswire.com/news/home/20161123005338/en/China-Strobilurin-Fungicides-Market-Report-2016-Edition---Research-and-Markets. (accessed 31. 1. 2022)

Baćmaga M., Kucharski J., Wyszkowska J. (2015): Microbial and enzymatic activity of soil contaminated with azoxystrobin. Environmental Monitoring and Assessment, 187: 615.

Baćmaga M., Wyszkowska J., Kucharski J. (2020): Response of soil microorganisms and enzymes to the foliar application of Helicur 250 EW fungicide on *Horderum vulgare* L. Chemosphere, 242: 125163.

Bartlett D.W., Clough J.M., Godwin J.R., Hall A.A., Hamer M., Parr-Dobrzanski B. (2002): The strobilurin fungicides. Pest Management Science, 58: 649–662.

Bending G.D., Rodríguez-Cruz M.S., Lincoln S.D. (2007): Fungicide impacts on microbial communities in soils with contrasting management histories. Chemosphere, 69: 82–88.

Burrows L.A., Edwards C.A. (2004): The use of integrated soil microcosms to assess the impact of carbendazim on soil ecosystems. Ecotoxicology, 13: 143–161.

Chen S.K., Edwards C.A., Subler S. (2001): Effects of the fungicides benomyl, captan and chlorothalonil on soil microbial activity and nitrogen dynamics in laboratory incubations. Soil Biology and Biochemistry, 33: 1971–1980.

Cycoń M., Piotrowska-Seget Z., Kozdrój J. (2010): Responses of indigenous microorganisms to a fungicidal mixture of mancozeb and dimethomorph added to sandy soils. International Biodeterioration and Biodegradation, 64: 316–323.

Eivazi F., Tabatabai M.A. (1988): Glucosidases and galactosidases in soils. Soil Biology and Biochemistry, 20: 601–606.

Floch C., Chevremont A., Joanico K., Capowiez Y., Criquet S. (2011): Indicators of pesticide contamination: soil enzyme compared to functional diversity of bacterial communities *via* Biolog® Ecoplates. European Journal of Soil Biology, 47: 256–263.

Friedel J.K., Mölter K., Fischer W.R. (1994): Comparison and improvement of methods for determining soil dehydrogenase activity by using triphenyltetrazolium chloride and iodonitrotetrazolium chloride. Biology and Fertility of Soils, 18: 291–296.

Ghosh R.K., Singh N. (2009): Effect of organic manure on sorption and degradation of azoxystrobin in soil. Journal of Agricultural and Food Chemistry, 57: 632–636.

Guo P., Zhu L., Wang J., Wang J., Xie H., Lv D. (2015): Enzymatic activities and microbial biomass in black soil as affected by azoxystrobin. Environmental Earth Sciences, 72: 1353–1361.

Hammer Ø., Harper D.A.T., Ryan P.D. (2001): PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electronica, 4: 1–9.

Howell C.C., Semple K.T., Bending G.D. (2014): Isolation and characterization of azoxystrobin degrading bacteria from soil. Chemosphere, 95: 370–378.

Kandeler E., Gerber H. (1988): Short-term assay of soil urease activity using colorimetric determination of ammonium. Biology and Fertility of Soils, 6: 68–72.

Keeney D.R., Nelson D.W. (1982): Nitrogen-inorganic forms. In: Page A.L., Miller R.H., Keeney D. (eds.): Methods of Soil Analysis, Part 2, Agronomy Monograph 9. Madison, American Society of Agronomy and Soil Science Society of America, 643–698.

Köhler H.R., Triebskorn R. (2013): Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond? Science, 341: 759–765.

Lavelle P., Spain A.V. (2005): Chapter 3: Soil Organisms. In: Lavelle P., Spain A.V. (eds.): Soil Ecology. Dordrecht, Springer, 201–356. ISBN: 9780306481628

Mijangos I., Becerril J.M., Albizu I., Epelde L., Garbisu C. (2009): Effects of glyphosate on rhizosphere soil microbial communities under two different plant composition by cultivation-dependent and -independent methodologies. Soil Biology and Biochemistry, 41: 505–513.

Monkiedje A., Ilori M.O., Spiteller M. (2002): Soil quality changes resulting from the application of the fungicides mefenoxam and metalaxyl to a sandy loam soil. Soil Biology and Biochemistry, 34: 1939–1948.

https://doi.org/10.17221/127/2022-PSE

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

Muñoz-Leoz B., Garbisu C., Charcosse J.Y., Sánchez-Pérez J.M., Antigüedad I., Ruiz-Romera E. (2013): Non-target effects of three formulated pesticides on microbially-mediated processes in a clay-loam soil. Science of the Total Environment, 449: 345–354.

Olsen S.R. (1982): Phosphorus. In: Page A.L., Miller R.H., Keeney D. (eds.): Methods of Soil Analysis, Part 2, Agronomy Monograph 9. Madison, American Society of Agronomy and Soil Science Society of America, 1040–1042.

Puglisi E., Vasileiadis S., Demiris K., Bassi D., Karpouzas D.G., Capri E., Cocconcelli P.S., Trevisan M. (2012): Impact of fungicides on the diversity and function of non-target ammonia-oxidizing microorganisms residing in a litter soil cover. Microbial Ecology, 64: 692–701.

Riah W., Laval K., Laroche-Ajzenberg E., Mougin Ch., Latour X., Trinsoutrot-Gattin I. (2014): Effects of pesticides on soil enzymes: a review. Environmental Chemistry Letters, 12: 257–273.

Ruske R.E., Gooding M.J., Dobraszczyk B.J. (2004): Effects of Triazole and Strobilurin fungicide programmes, with and without late-sea son nitrogen fertilizer, on the baking quality of Malacca winter wheat. Journal of Cereal Science, 40: 1–8.

Siwik-Ziomek A., Lemanowicz J., Koper J. (2016): Arylsulphatase activity and sulphate content in relation to crop rotation and fertilization of soil. International Agrophysics, 30: 359–367.

Sopeña F., Bending G.D. (2013): Impacts of biochar on bioavailability of the fungicide azoxystrobin: a comparison of the effect on biodegradation rate and toxicity to the fungal community. Chemosphere, 91: 1525–1533.

Sukul P. (2006): Enzymatic activities and microbial biomass in soil as influenced by metalaxyl residues. Soil Biology and Biochemistry, 38: 320–326.

Tabatabai M.A., Bremner J.M. (1969): Use of p-nitrophenylphosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry, 1: 301–307.

Tabatabai M.A., Bremner J.M. (1970): Arylsulfatase activity of soils. Soil Science Society of America Journal, 34: 225–229.

Utobo E.B., Tewari L. (2015): Soil enzymes as bioindicators of soil ecosystem status. Applied Ecology and Environmental Research, 13: 147–169.

Waldrop M.P., Balser T.C., Firestone M.K. (2000): Linking microbial community composition to function in a tropical soil. Soil Biology and Biochemistry, 32: 1837–1846.

Wang Y.S., Wen C.Y., Chiu T.C., Yen J.H. (2004): Effect of fungicide iprodione on soil bacterial community. Ecotoxicology and Environmental Safety, 59: 127–132.

Wang C., Wang F., Zhang Q., Liang W. (2016): Individual and combined effects of tebuconazole and carbendazim on soil microbial activity. European Journal of Soil Biology, 72: 6–13.

Wang F., Li X., Zhu L., Du Z., Zhang C., Wang J., Wang J., Lv D. (2018): Responses of soil microorganisms and enzymatic activities to azoxystrobin in Cambisol. Polish Journal of Environmental Studies, 27: 2775–2783.

Wang X., Lu Z., Miller H., Liu J., Hou Z., Liang S., Zhao X., Zhang H., Borch T. (2020): Fungicide azoxystrobin induced changes on the soil microbiome. Applied Soil Ecology, 145: 103343.

Xiong D., Li Y., Xiong Y., Li X., Xiao Y., Qin Z., Xiao Y. (2014): Influence of boscalid on the activities of soil enzymes and soil respiration. European Journal of Soil Biology, 61: 1–5.

Received: April 13, 2022
Accepted: July 7, 2022
Published online: August 24, 2022