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# *The Resistance and Resilience of Soil Enzymes after the Application of Fungicide Azoxystrobin to Loamy Sand Soil*

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**Abstract. T**he use of fungicides in crop protection effectively eliminates fungal pathogens of plants. However, they may cause changes in soil microorganisms concerning microbial ability to mediate soil functions. The aim of the study was to evaluate the changes in soil environment, and soil enzyme resistance and resilience (beta-glucosidase, urease, acid and alkaline phosphatases and arylsulphatase) in a response to the increasing concentrations of azoxystrobin (Az), applied under the trade form Quadris[R]. A laboratory study was carried out for 120 days on soil mesocosms, amended with Az in concentrations from 0.00 mg $kg^{-1}$ to 35.00 mg $kg^{-1}$. Az soil amendment caused changes in soil physico-chemical properties and microbial activity. Microbial responses immediately (day 1) after Az application, showed that more resistant to the fungicide were urease, beta-glucosidase and arylsulphatase in the opposite to the acid phosphatase, which demonstrated high sensitivity to the chemical stress. One month later, the resistance of beta-glucosidase, urease and acid phosphatase decreased even more compared to day 1, the resistance of alkaline phosphatase remained unchangeable, whereas the resistance of arylsulphatase slightly increased. The calculated resilience on day 120 manifested that enzymes were not able to recover within four months after fungicide application to soils. Pearson correlation analysis demonstrated significant linear relationships between Az soil residues and enzyme resistance/resilience. Our results highlighted that the application of Quadris[R] altered soil enzyme system for more than four months, which might reflect the speed of organic matter turnover in soil, especially that of organophosphates.

**Key words:** fungicide azoxystrobin, Quadris[R], soil, chemical stress, enzyme resistance, enzyme resilience.

## Introduction

Fungicides are chemical compounds used in crop protection to control the development of fungal pathogens. Strong worldwide demand owing to increase in agricultural activities is expected to be a key driver for the growth of the fungicide market in future. Primarily, the use of fungicides is aimed at controlling target

organisms. Despite this fact, it is not possible to predict their environmental fate. The highest quantities of fungicides are accumulated in soil, which may cause changes in the terrestrial environments, often manifested by decreasing soil fertility.

Fungicides are classified by chemical type such as triazoles, benzimidazoles, chloronitriles, dithiocarbamates, phenylamides and strobilurins. Among the fungicides with a natural origin, strobilurins accounts for the largest share of the global fungicide market. Strobilurins form part of the group of quinone-outside inhibitors (QoI), inhibiting mitochondrial respiration at the Qo site of cytochrome b, part of the cytochrome bc1 complex (Complex III), and thus preventing spore germination and mycelial growth in fungal pathogens (Bartlett et al., 2002). Azoxystrobin, (methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate), is one of the strobilurin members, and can be used to control diseases caused by pathogenic fungi such as ascomycetes, basidiomycetes, oomycete, and imperfect fungi (Bartlett et al., 2002). Because of its broad-spectrum activity, this fungicide has become the leader in the world fungicide market (Bai, 2007). However, many authors evidenced that azoxystrobin (Az) negatively affected soil microorganisms, disturbing the structure of soil fungal communities (Bending et al., 2007; Sopeña & Bending, 2013), inhibiting soil respiration (Guo et al., 2015; Wang et al., 2018), and changing the activity of soil enzymes (Baćmaga et al., 2015; Guo et al., 2015; Sopeña & Bending, 2013; Wang et al., 2018). Soil enzymes catalyze an extensive number of biological processes in soil and provide a unique assessment of soil function mediated mainly by soil microbiota (Yang et al., 2013). The major authors' interest was referred to the Az influence on the activity of soil dehydrogenase, and less to the activity of soil urease (Alvarez-Martin et al., 2016; Baćmaga et al., 2015; Bending et al., 2007; Guo et al., 2015; Sopeña & Bending, 2013; Wang et al., 2018). In some cases the information is contradictory as most of the authors reported slight to strong negative effects of Az applied at low and high concentrations, respectively. In both cases, the effects were manifested at a later stage of Az exposure. Unlike the other authors, Alvarez-Martin et al. (2016) reported no significant effect of Az on dehydrogenase activity at both low (0.2 mg $kg^{-1}$) and high (25 mg $kg^{-1}$) fungicide concentrations.

Considering this fact, the aim of our study was to evaluate the effect of Az on soil health by determining the resistance and resilience of soil enzymes (beta-glucosidase, urease, phosphatase acid phosphatase, alkaline phosphatase, and arylsulphatase) under chemical stress.

## Material and Methods

*Soil sampling and soil properties*

Soil was collected from a grassland located near Gabra village (Sofia region, Bulgaria) - 42°31´48.36´´N and 23°37´28.20´´E. Five subsamples were pooled randomly from a 0 – 20 cm soil depth, sieved through a 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. Soil was classified as loamy sand with texture of 83 % sand, 2 % clay, and 15 % silt. Total organic carbon was 15.7 g $kg^{-1}$, and nitrogen Kjeldahl - 1.67 g $kg^{-1}$. Soil pH ($H_2O$) was acidic with original value of 5.67 (Executive Environment Agency, personal communication). During the experiment, values of soil pH, inorganic nitrogen ($NO_3$ and $NH_4$), inorganic phosphates ($HPO_4$) and Az soil residues were followed. Soil pH was measured potentiometrically (HANNA Instruments) after mixing soil in 0.01 mol $l^{-1}$ $CaCl_2$ solution (1:5; weight : volume), and shaking it for 30 min. Soil bioavailable forms of inorganic nitrogen ($NO_3$-N and $NH_4$-N) and phosphates ($HPO_4$) were determined spectrophotometrically according to the methods of Keeney and Nelson (1982), and Olsen (1982), respectively. A gas chromatography was used to assess the Az soil residues extracted by methanol: ethylacetate (75 : 25, v/v) solution after 1 h sonification of samples in an ultrasonic bath (35 kHz and 285 W) (Aleksova, 2019).

*Design of mesocosm experiment*

Soil mesocosms were prepared as each of them contained 2000 g of dry weight equivalent soil amended with Az under the

form of commercial substance Quadris$^R$ (Syngenta). Quadris$^R$ was applied in concentrations of 2.90 mg kg$^{-1}$ (Az1), 14.65 mg kg$^{-1}$ (Az2) and 35.00 mg kg$^{-1}$ (Az3) calculated towards the active ingredient methyl(E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate, and indicated by gas-chromatography method as Az soil residues a day after soil treatments. A mesocosm without fungicide was used as a control (Az0). In the study was used the name of the active principle (Az) although it was tested the commercial formulation containing multiple (active principle plus adjuvants) ingredients. Each mesocosm was prepared in triplicates. Soil water content was adjusted to 40% of the maximum water holding capacity. The soil moisture was maintained by weighting soils every 3 days using sterile distilled water in order to compensate for any moisture loss. The mesocosms were incubated at 22 ± 1 °C in dark to prevent physical degradation of Az by light. Soil samples were collected randomly in triplicates from each mesocosm on the 1$^{st}$ (D1), 30$^{th}$ (D30), and 120$^{th}$ (D120) day after Az application.

*Soil enzyme activities*

The method of enzyme activity determination was based on 1 g soil cultivation with the respective enzyme substratum, extraction and colorimetric determination of the enzyme products. Beta-glucosidase (BGl), urease (Ur), alkaline phosphatase (AlP), acid phosphatase (AcP) and arylsulphatase (Ars) activities were determined following the method of Eivazi & Tabatabi (1988), Kandeler & Gerber (1988), Tabatabi & Bremner (1969), Browman & Tabatabi (1978), and Tabatabi & Bremner (1970), respectively. Soil enzyme activity was measured on a Cecil CE 3021 spectrophotometer (Cecil Instruments, Cambridge, England) at λ = 405 nm (BGl), λ = 690 nm (Ur), λ = 420 nm (AlP and AcP), and λ = 400 nm (Ars). The following substrates were used to determine enzyme activity: p-nitrophenyl β-D-glucopyranoside for BGl, urea for Ur, 4-nitrophenyl phosphate disodium for AlP and AcP, and p-nitrophenyl sulfate for Ars (Sigma - Aldrich).

Formulas proposed by Orwin & Wardle (2004) were used to determine the soil resistance index (RS) on days 1 (D1) and 30 (D30), and the soil resilience index (RL) on day 120 (D120) after Az application to soils:

$$RS(t_0) = 1 - \frac{2|D_0|}{(C_0 + |D_0|)} \quad \text{and}$$

$$RL\ at\ t_x = \frac{2|D_0|}{(|D_0 + |D_x||)} - 1$$

where: $D_0 = C_0 - P_0$, $D_x = C_x - P_x$, $C_0$ is the enzyme activity under natural conditions over time $t_0$, $P_0$ is the enzyme activity of Az disturbed soil over time $t_0$, $C_x$ is the enzyme activity under natural conditions over time $t_x$, $P_x$ is the enzyme activity of Az disturbed soil over time $t_x$.

*Data analysis*

Each data point in the paper represented the results from the three replicates of each soil mesocosm, and each value was expressed as a mean value for the respective Az treated soil. One-way ANOVA followed by Tukey's test were performed to examine the differences in the means of soil (pH, $NO_3$-N, $NH_4$-N, $HPO_4$, Az) and microbial (RS and RL) parameters among the studied soil mesocosms. Person product moment correlation analysis was performed to examine the linear correlations between the Az soil residues and enzyme resistance/ resilience. Principal component analysis (PCA) was performed using the data matrix of RS and RL to ordinate the enzyme responses to Az. The above statistics were performed with the package PAST (Hammer et al., 2001) at a level of significance $p<0.05$.

**Results and Discussion**

*Soil environment*

Different trends of changes in soil parameters were recorded during Az exposition (Table 1).

The Az0's soil variables are shown as actual values, whereas those of Az1, Az2 and Az3 were calculated as percentages of Az0. For each sampling day, values of each soil variable (Az1 – Az3) followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's HSD test. Comparing with Az0, soil content of ammonium nitrogen and phosphates in Az amended soils increased and that of Az residues and nitrate nitrogen decreased over time. Soil acidification also increased along the soils' incubation. The higher soil content of $NH_4$-N and $PO_4$ at the end of the experiment might be related to the accumulation and biodegradation of death fungal biomass in Az soils. pH gradual decrease over time might be related to the process of $NH_4$-N accumulation in soils and/or Az degradation and release of azoxystrobin acid as a main end product of the fungicide transformation in soils (Singh et al., 2010). Az application (D1) increased dramatically soil concentration of nitrate nitrogen (50 % - 78 %) at the beginning of the experiment. Nitrate increase in soils after pesticide application has been reported also in other studies. The authors suggested that this effect might be a result of soil pre-treatment activities (Franzluebbers, 1999) or nitrogen input by fungicide adjuvants (Devare et al., 2007; Mijangos et al., 2009). For example, according to Syngenta, Quadris$^R$ consists of 22.9% Az and 77.1% of other ingredients. In general, it was found that Az application caused changes in soil environment, and we supposed that it might moderate the fungicide influence on soil enzyme activities (secondary effects of Az).

*Resistance and resilience of soil enzymes*

Microorganisms are the key players of many soil functions such as biogeochemical cycling and plant productivity, and are essential for the integrity of terrestrial ecosystems. Given the crucial importance of maintaining soil functions, we aimed to investigate the resistance and resilience of soil/ microbial enzymes to Az application. In general, resistance is commonly defined as the ability of a system to withstand a disturbance, while resilience is considered as an ability of a system to recover as soon as possible after the end of the perturbation (Griffiths & Philippot, 2013). In this aspect, the resistance and resilience are the two components of system stability (Loreau et al., 2002). The resistance and resilience of soil enzymes were calculated and they are shown on Fig. 1.

Az application to soils caused immediately (D1) microbial response, manifested by the relatively high resistance (on average, Az1 – Az3) of Ur (0.942), followed by that of BGL (0.840), Ars (0.723), AlP (0.667) and AcP (0.650). High resistance of Ur to Az, especially at concentrations lower than 10 mg $kg^{-1}$, was recorded also by Baćmaga et al. (2015) and Guo et al. (2015), and both of them mentioned that enzyme inhibition was manifested at longer exposure - 14 days (Guo et al. 2015) and 30 days (Baćmaga et al. 2015) after fungicide amendment of soil. Since the effects of Az was not tested on the other soil enzymes except Ur, we cannot compare our results with earlier findings in this aspect. There are some results concerning the effects of triazoles, acylalanines and mancozeb on Ars (Floch *et al.*, 2011; Saha *et al.*, 2016; Sukul, 2006), but these fungicides have different mechanisms of action on target organisms comparing to Az, making the comparison inaccurate.

Pearson correlation coefficients were calculated on D1 and they evidenced that the RSs of BGl, Ur and Ars did not relate significantly with Az soil residues, in the opposite to that of AcP and AlP (Table 2), indicating again the relatively high resistance of BGl, Ur and Ars to the chemical stress. The next enzyme resistance determination was a month after Az application (D30), when different trends of changes in enzyme resistances were recorded (Fig. 1). Comparing the enzyme RSs to that of D1, the resistance of AlP remained unchangeable, the

resistances of BGL, AcP and Ur decreased by 15 % - 25 %, and the resistance of Ars increased by 10 %. On D30, the number of significant relationships between Az soil residues and enzymes increased, indicating increased influence of Az on soil functioning. Considering the negative direction of the above mentioned correlations, we assumed that Az might slowdown the nutrients' transformation in fungicide impacted light textured soils (sandy soils).

Even if enzyme activities are sensitive to Az, the microbial community might still be resilient and able to return quickly to its pre-disturbance functioning. A number of features of microorganisms suggest that the resilience could be their common property, because microorganisms 1) have fast growth rates and they have the potential to recover quickly after disturbance and 2) have a high degree of physiological flexibility (Griffths & Philippot, 2013). In our study, the RL indices calculated on D120 would indicate whether the effect of Az on soil microorganisms has disappeared four months after fungicide application, whether the fungal communities (main contributors to the soil enzyme pool) have been recovered, and whether the soil functions are completely carried out.

The RL values (D120) clearly showed that four months after soil treatment with Az, soil enzyme activities were still not recovered and moreover they were far from their stable natural state (pre-treatment state) (Fig. 1). For example, the values of RLs (on average for Az1 – Az3) were for BGL: - 0.39, Ur: 0.09, AcP: - 0.15, AlP: 0.14, and Ars: - 0.21. Calculating the mean value of overall enzyme resilience per mesocosm, it was recorded negative RL values for Az1 (-0.19) and Az2 (-0.28), and positive RL value for Az3 (0.16). In fact, at Az3 more resilient to Az was Ur, having relatively high (0.86) and positive value of the index. Considering that the metabolic capacity of microbial communities likely reflect the abundance and bioavailability of nutrients (Orwin et al., 2006), we assumed that Az influenced on enzyme RLs by changing the size of soil nutrient pool.

**Table 1.** Soil parameters at different levels of azoxystrobin (Az) amended soil mesocosms. The Az0's soil variables are shown as actual values, whereas those of Az1, Az2 and Az3 were calculated as percentages of Az0. For each sampling day, values of each soil variable (Az1 – Az3) followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's HSD test. Legend: *Az concentrations on D1 were taken as 100% and they were used for the calculation of the Az soil residues on D30 and D120.

| Mesocosm | Day of sampling | Az | $HPO_4$ | $NH_4$-N | $NO_3$-N | pH |
|---|---|---|---|---|---|---|
| **Az0** | D1 | - | **2.83 mg kg$^{-1}$** | **2.24 mg kg$^{-1}$** | **16.48 mg kg$^{-1}$** | **5.63** |
| Az1 | | 100* | 96.11$^{a}$ | 99.65$^{a}$ | 176.30$^{a}$ | 93.43$^{a}$ |
| Az2 | | 100* | 86.57$^{b}$ | 57.29$^{a}$ | 151.37$^{b}$ | 91.12$^{b}$ |
| Az3 | | 100* | 113.78$^{c}$ | 92.36$^{a}$ | 178.14$^{a}$ | 86.68$^{c}$ |
| **Az0** | D30 | - | **7.80 mg kg$^{-1}$** | **0.67 mg kg$^{-1}$** | **47.04 mg kg-$^{1}$** | **6.04** |
| Az1 | | 48.96$^{a}$ | 120.64$^{a}$ | 109.30$^{a}$ | 123.28$^{a}$ | 88.24$^{a}$ |
| Az2 | | 73.31$^{b}$ | 88.59$^{a}$ | 145.35$^{b}$ | 123.72$^{a}$ | 81.46$^{b}$ |
| Az3 | | 84.51$^{b}$ | 96.02$^{a}$ | 147.67$^{c}$ | 131.26$^{b}$ | 83.28$^{c}$ |
| **Az0** | D120 | - | **23.08 mg kg$^{-1}$** | **10.47 mg kg$^{-1}$** | **146.73 mg kg$^{-1}$** | **5.72** |
| Az1 | | 10.34$^{a}$ | 110.57$^{a}$ | 154.98$^{a}$ | 111.06$^{a}$ | 85.14$^{a}$ |
| Az2 | | 19.38$^{b}$ | 112.56$^{a}$ | 76.37$^{b}$ | 103.64$^{a}$ | 84.79$^{a}$ |
| Az3 | | 13.14$^{ab}$ | 119.84$^{a}$ | 113.08$^{c}$ | 112.16$^{b}$ | 84.09$^{a}$ |

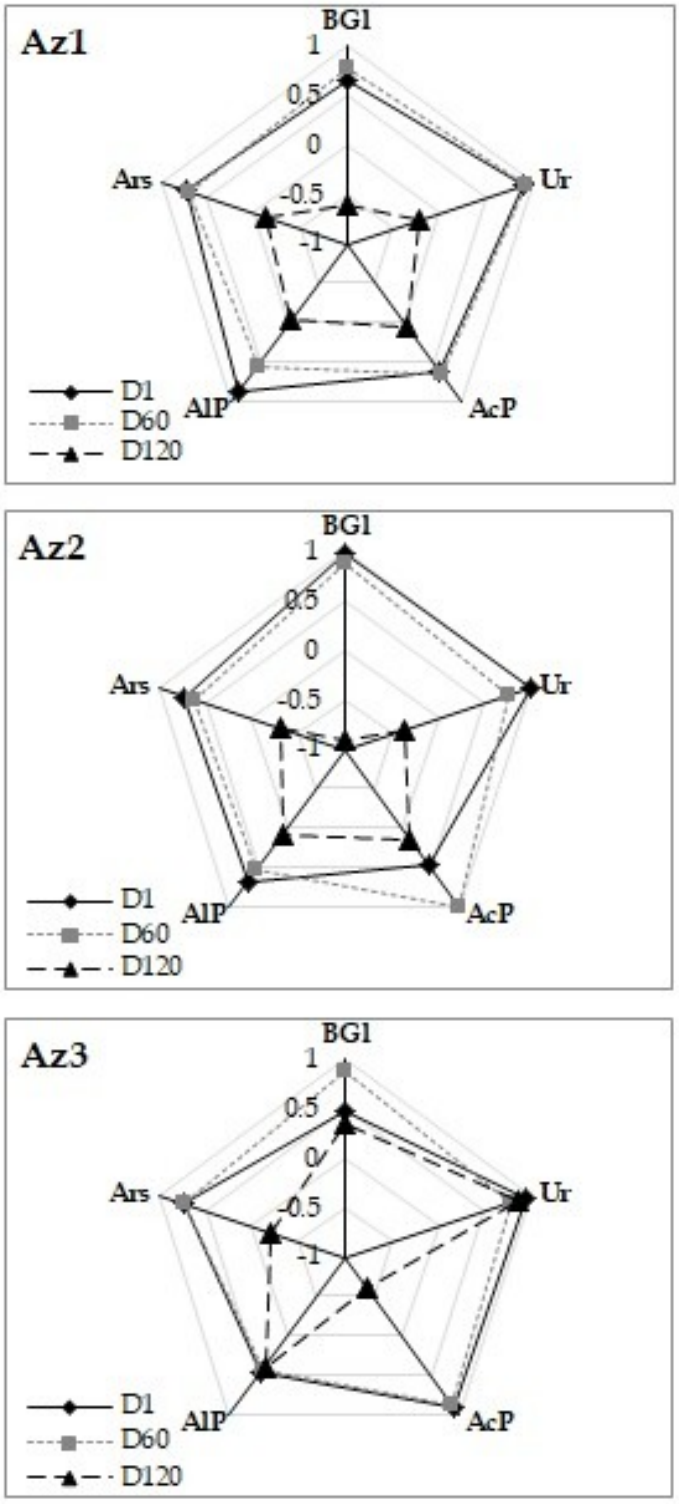


**Fig. 1.** Resistance (D1 and D30) and resilience (D120) of soil enzymes beta-glucosidase (BGl), urease (Ur), acid (AcP) and alkaline (AlP) phosphatases and arylsulphatase (Ars) after application of Az to soils in increasing concentrations (Az1 – Az3).

We assumed that the highest Az concentration caused 1) the highest death and accumulation of fungal biomass (proteins) in soil, and 2) the highest synthesis of detoxification agents (mainly proteins) - molecules that can later be metabolized by the same microbiota (Degens et al., 2000; Hall, 2002). Accumulation of microbial proteins (easy degradable substrates) in soil could stimulate Ur activity (Tabatabai, 1982) and increase its recovery rate.

On D120, significant relationships were calculated between Az soil residues and the resiliences of Ur, AlP, and AcP (Table 2), indicating that these enzymes were still under the influence of Az. Although, the correlation between Az and BGl was not significant (0.64, p=0.06), it was considered as strong.

Summarizing the RL results, we could conclude that four months after Az application soil enzyme activity is still unrecovered, probably due to the Az toxicity on soil fungi, which are considered to be the main drivers of nutrient turnover in soils (Güsewell & Gessner, 2009).

Principle component analysis (PCA) was used to examine how soil microbial communities from Az amended soils differ with respect to the resistance and resilience of their enzyme activities (Fig. 2).

**Table 2.** Pearson correlation analysis between Az soil residues and soil enzyme responses in the respective day of sampling. The significant correlation coefficients are in bold.

| **Soil enzymes** | **Corr. indices between Az soil residues and enzyme resistance** | | **Corr. indices between Az soil residues and enzyme resilience** |
|---|---|---|---|
| | **D1** | **D30** | **D120** |
| BGl | -0.49 (0.18) | **-0.99** (0.00) | 0.64 (0.06) |
| Ur | 0.42 (0.26) | -0.39 (0.29) | **0.74** (0.02) |
| AcP | **0.76** (0.02) | **0.79** (0.01) | **-0.74** (0.02) |
| AlP | **-0.99** (0.00) | **-0.98** (0.00) | **0.93** (0.00) |
| Ars | -0.58 (0.10) | **-0.98** (0.00) | -0.39 (0.29) |

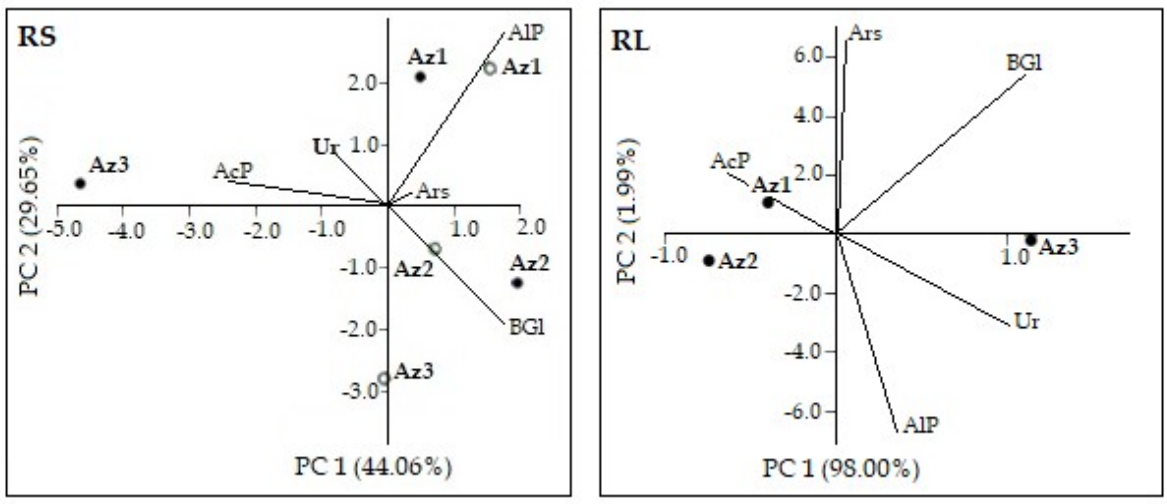


**Fig. 2.** Principal component analysis (PCA) plots showing similarity in soil enzyme resistance (RS; filled dot – D1 and open dot – D30) and resilience (RL) responses to the soil application of Az in increasing concentrations (Az1 – Az3).

PC 1 and PC 2 together were able to constrain more than 83 % of the variation in the soil enzyme RS and RL. Relationships of RS and RL with the respective primary axes indicated strong correlations of PC 1 with AlP, BGl and AcP (RS), and BGl and Ur (RL). PC 2 correlated strongly with AlP and BGl (RS), and Ars and AlP (RL).

Az1 and Az2 were separated by each other (PC 1), and both of them differed from Az3 (PC 2) due to the resistance of BGl and phosphatases (AcP and AlP). BGL and Ur differentiated the resilience between Az1 and Az2, whereas the resilience of AlP and Ars were the main factors contributed to the differences between Az3, and both Az1 and Az2.

On the PC plots, increasing distance between soil mesocosms equates to higher dissimilarity in enzyme responses (RS and RL) towards Az stress. The distances between the RSs of Az1 on D1 and D30 and between that of Az2 on D1 and D30 were relatively low, indicating minor changes within one month after Az application to soils. It was not the case of Az3, where the distance of RSs between the two sampling events (D1 and D30) was much higher than that between the mesocosms amended with lower Az concentrations. The same pattern of segregation of RLs was manifested for Az amended soil mesocosms on D120.

## Conclusions

The effect of increasing concentrations of Az on soil functioning was assessed through the calculation of the resistance and resilience of soil enzymes involved into the turnover of organic carbon (BGl), nitrogen (Ur), phosphorus (AcP and AlP) and sulfur (Ars). The results evidenced that the most sensitive (less resistant) to Az stress was AcP. AcP was also the soil enzyme which recovered (low resilience) most slowly, which assumed that the soil cycling of organophosphates would be slowed down by Az for much more than four months. The other tested enzymes demonstrated high (Ur) to medium (BGL and Ars) resistance to soil chemical perturbation, but their ability to recover was low (Ur and Ars) to extremely low (BGl). Most of the enzyme responses were dependent on Az residues in soils. Summarizing the results, we could conclude that Az application caused stress on soil enzymes, and the recovery of enzyme activities can be moderated in different rates by soil properties, including Az residues and fungicide adjuvants. Considering the fact that fungicides are frequently introduced into soils, their long-term effects on microbially-mediated soil functions should be a subject of a more detailed further research.

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