DOI: 10.2428/ecea.2018.25(1)9 ECOL CHEM ENG A. 2018;25(1):81-92

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EVALUATION OF AZOXYSTROBIN TOXICITY TO SAPROPHYTIC FUNGI AND RADISH IN THE EARLY STAGES OF GROWTH

OCENA TOKSYCZNOŚCI AZOKSYSTROBINY DLA SAPROFITYCZNYCH GRZYBÓW ORAZ RZODKIEWKI WE WCZESNYCH STADIACH WZROSTU

Abstract: The aim of the study was to assess the toxicity of azoxystrobin, a fungicide belonging to the strobilurin class, for selected saprophytic fungi (*Saccharomyces cerevisiae* and *Penicillium* sp.) and for radish (*Raphanus sativus* L.) The parameters of fungi growth and the early development stages of radish were analysed. Based on the sensitivity of the organisms and their physiological processes to azoxystrobin, they can be arranged in the following order: growth of *sod1 S. cerevisiae* mutant, growth of wild-type *S. cerevisiae*, growth of *Penicillium* sp., respiration of germinating radish seeds, early seed germination, elongation of roots and seedlings, late seed germination. The mechanism of azoxystrobin toxicity seems to be associated with cellular antioxidant status.

Keywords: azoxystrobin, yeast, superoxide dismutase, filamentous fungi

Azoxystrobin (methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate) is a synthetic compound in the strobilurin class of fungicides. It was first produced in 1996, there are now ten major strobilurin fungicides commercially available, which account for 23–25 % of global fungicide sales [1]. Of all strobilurin preparations, azoxystrobin can be demonstrated to have the broadest spectrum of activity.

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Of particular importance is their activity against all four classes of plant pathogenic fungi, i.e. Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes [2].

Studies on their mode of physiological action have demonstrated that spore germination and zoospore motility are stages of fungal development that are particularly sensitive to strobilurins [3, 4]. This can be explained by the disruption of energy production caused by inhibition of complex III (ubiquinol-cytochrome c oxido-reductase, EC1.10.2.2) in the mitochondrial respiratory chain by strobilurins [5]. For this reason they are particularly effective against these highly energy-demanding stages of fungal development.

In addition to their anti-fungal effect, strobilurins have been reported to produce simultaneous effects in plant physiology, that lead to: better cereal quality and yield [6], increased area of photosynthetic green tissues, improvement of the assimilation rate, delayed senescence, enhanced concentrations of nitrogen, chlorophyll and protein [7, 8] and changes in hormone status [9]. The exact mechanism explaining this phenomenon is unknown. It has been suggested that the effects observed may be the result of direct biochemical/physiological plants response to azoxystrobin.

Another hypothesis is based on specific action of strobilurin against rhizosphere and phyllosphere pathogenic, non-pathogenic and saprophytic fungi, which leads to their elimination from the environment, thereby preventing the initiation of energy-intensive host defence responses.

The literature contains a great deal of information on the effects of strobilurin (including azoxystrobin) on phytopathogenic fungi, under both field and laboratory conditions [10–12]. However, there are relatively few data regarding the response of saprophytic fungi, in particular yeasts living in association with various plant organs, to these preparations. It has been suggested that they could be bioindicators of environmental pollution [13, 14].

The aim of the study was therefore to assess the effect of azoxystrobin on the growth of two non-pathogenic strains of laboratory fungi belonging to the Ascomycetes class: *Saccharomyces cerevisiae* yeast and the filamentous fungus *Penicillium* sp. These fungi represent species that occur frequently in the rhizosphere and the phyllosphere of various wild and cultivated plants. The response of radish *Raphanus sativus* L. to this fungicide was examined as well.

Radish is an important vegetable of the Brassicaceae family, grown and consumed all over the world, due to its wide adaptation, high yield, and high nutritional content. This plant is also an important source of medicinal compounds [15] and a model organism in short-term ecotoxicological studies (American Society for Testing and Materiale, 1991). As a model organism, it has been used in research on the effects of triazole fungicides [16], pyrethroid insecticides [17], herbicides and phytohormones [18], heavy metals such as lead [19], cadmium, and mercury [20] and recently nanoparticles based on essential metals [21] and ferrocene derivatives [22]. The results of these studies showed that radish seedlings and their germination process are particularly sensitive to these xenobiotics. Therefore, the effect of azoxystrobin on the early stages of radish development was analysed in this study.

Materials and methods

Azoxystrobin was obtained from the Institute of Industrial Organic Chemistry (Warszawa, Poland). Culture media components were obtained from BD Difco (Becton Dickinson and Company, Spark, USA), except for glucose (POCh, Gliwice, Poland).

Seeds of radish *Raphanus sativus* L., cv. Rowa from Przedsiebiorstwo Nasiennictwa Ogrodniczego i Szkolkarstwa in Ozarow Mazowiecki were used in the experiments.

Fungal cultures used in the experiment came from the collection of the Department of Biochemistry and Environmental Chemistry, Department of Agricultural Sciences in Zamosc, University of Life Sciences in Lublin. Two different fungus species were used: *Saccharomyces cerevisiae* yeast (SP4 wild strain (*wt*) of genotype Mat α leu1 arg4 [23] and the DSCD1-1C strain devoid of the cytoplasmic activity of superoxide dismutase of genotype Mat α leu1 arg4 *sod1* and *Penicillium* sp. The yeast cultures were grown in liquid YPD medium containing peptone (1 %), yeast extract (1 %) and glucose (2 %) in order to obtain a culture of cells at the early stationary stage of growth. *Penicillium* sp. was grown on the same medium but solidified with agar (2 %), in the form of colonies.

The effect of azoxystrobin on yeast growth was tested with plate-test assays. The plate test involved plating the yeast suspension onto solid media and determining the number of cells capable of proliferation and colony formation. Briefly, an acetone-water solution of azoxystrobin was added to the liquid YPD with agar medium (50 ^{o}C) to final concentrations of 0.0005, 0.001, 0.003, 0.007 [%]. The yeast suspension (0.1 cm^3) at a cell density of $1–5 \cdot 10^3$ was plated on Petri dishes with solid medium and incubated 48 h at 30 ^{o}C. Then, the colonies were counted using an automatic counter (aCO-Lyte; Synbiosis) and associated software (version 2.0.8). The number of colonies obtained for the control samples, i.e. those that were not exposed to azoxystrobin, was assumed to be 100 %.

The effect of azoxystrobin on *Penicillium* sp. growth was investigated based on colony diameter measurements after point inoculation on solid YPG 2 % media supplemented with azoxystrobin. Concentrations of azoxystrobin were the same as described above. Culture incubation was carried out at 25 ^{o}C. After 120 hours, measurements of the growing colony were made (diameter, in cm). The mean diameter of fungal colonies on a medium without fungicide was assumed to be 100 %.

Growth parameters of whole seedlings and their organs (roots and hypocotyls) and the germination rate in the presence of azoxystrobin were assayed according to a previous work [24]. In these assays, higher concentrations of azoxystrobin were used than in the tests with fungi, i.e. 0.003, 0.007, 0.018, 0.037 and 0.076 %. The mean length of root, hypocotyl and whole seedling obtained for the control samples, i.e. those that were not exposed to azoxystrobin, was assumed to be 100 %. The number of germinated seeds were expressed in relation to the number of seeds sown.

The release of CO_2 by the radish seeds was determined with a Pettenkofer apparatus according to Mendes et al. [25], with a minor modification. Radish seeds in the amount of 3.5 g were covered with distilled water or acetone-water solutions of azoxystrobin in concentrations as described above. After 24 hours of incubation, the amount of CO_2 released was determined by titration with 1 M HCl.

Respiratory activity was calculated using the following equation: N · D · 22, where N = normality of acid used (HCl 0.1N), D = difference between the volume of spent HCl titration proof of blank and the volume of spent HCl titration sample, and 22 = normality of CO_2. The result was expressed as the quantity of carbon dioxide released per gram of seed per hour [μg $CO_2 \cdot (g^{-1}$ seed $h^{-1})$]. The value obtained for the control samples, i.e. those that were not exposed to azoxystrobin, was assumed to be 100 %.

The inhibition rate, *IR*, of physiological processes (estimated in fungi and radish) was calculated using a formula:

$$IR = (a - b) / a \cdot 100$$

where: *IR* – inhibition rate [%];
a – *S. cerevisiae* survival / mean diameter of *Penicillium* sp. colonies / length of radish seedlings and their organs / number of germinated radish seeds in the control group;
b – *S. cerevisiae* survival/ mean diameter of *Penicillium* sp. colonies / length of radish seedlings and their organs / number of germinated radish seeds in the treatment group.

Statistical analysis: Unless stated otherwise, the data presented are means ± confidence intervals from at least three independent experiments. Confidence intervals were calculated assuming the Student's distribution of data for $p = 0.05$.

Results and discussion

Yeast growth was determined as the ability to produce colonies on a solid medium in the presence of azoxystrobin, which reflects their ability to proliferate. The wild strain (SP4) was more resistant to this fungicide than the *sod1* mutant devoid of activity of cytoplasmic superoxide dismutase, an enzyme which removes superoxide anion-radical from the cytoplasmic space. The survival rate of the *sod1* mutant cells versus wild-type cells at 0.0005 %, 0.001 % and 0.003 % azoxystrobin concentrations was two, three and over eight times lower, respectively. In the presence of 0.007 % azoxystrobin, *sod1* mutant cells were not able to proliferate at all (Fig. 1).

The growth of *Penicillium* sp. mycelium was assessed by means of colony diameter measurements 5 days after point inoculation. In this case, in the presence of 0.0005 % azoxystrobin the size of the colonies was reduced by half, while further increases in the concentration of this fungicide had no significant effect on the morphology of the colonies (Fig. 1).

The results presented in this work indicate that azoxystrobin inhibits the growth of vegetative cells of both unicellular yeast and mycelial fungi. Similar results for saprophytic fungi have been obtained by Bertelsen et al. [4], who observed that azoxystrobin delayed mycelial growth of *Alternaria alternata* and inhibited growth of *Cladosporium macrocarpum* in the growth chamber. At the same time, they observed severe inhibition of spore development in these fungi, which suggests that azoxystrobin

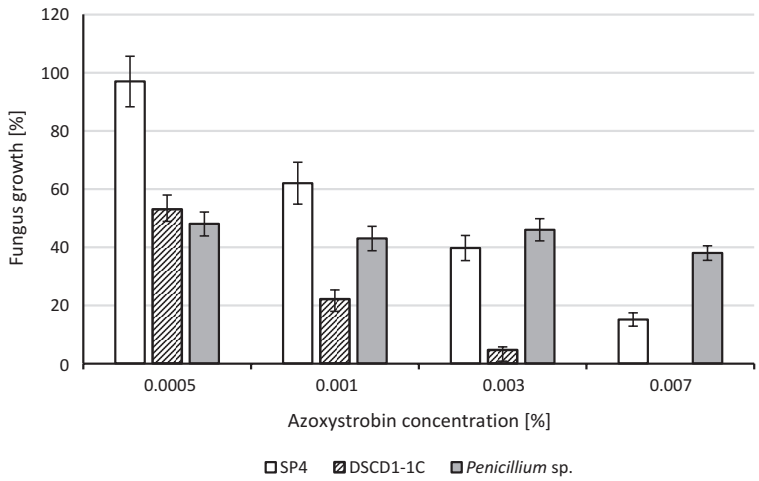

Fig. 1. Growth of yeast strains SP4 and DSCD1-1C and mycelium of *Penicillium* sp. in the presence of azoxystrobin

exerts a strong inhibitory effect on the early development of saprophytic fungi. Similarly high susceptibility of early developmental stages to azoxystrobin has been reported in fungal pathogens of wheat, grape and citrus [26–28].

Comparison of the susceptibility of the two fungal species (wild-type yeast and the filamentous fungus *Penicillium* sp.) to this fungicide revealed different responses depending on the concentration of the fungicide. At a low azoxystrobin concentration of 0.0005 %, the wild-type yeast strain was more resistant than the *Penicillium* mycelium to the inhibitory effect of the fungicide, while at the highest concentration, i.e. 0.007 %, the reverse pattern was observed.

It has been suggested that the presence of yeasts in the phyllosphere of crop plants may have a beneficial effect on yield by acting antagonistically against leaf plant pathogens [29, 30]. In this context, elimination of yeast from this environment following the application of high levels of fungicides may be an unfavourable phenomenon.

The use of yeast mutants in this type of research not only makes it possible to investigate the potential toxicity of xenobiotics, but also provides insight into their mechanism of action [18, 31]. The greater sensitivity of the *sod1* mutant cells to azoxystrobin indicates oxidative stress as the mediator of this toxicity. There is much evidence that blocking the mitochondrial electron flow leads to oxidative stress caused by the release of electrons from the respiratory chain in the form of reactive oxygen species. [32, 33]. Accordingly, Kim et al. [34, 35] found that yeast mutants lacking the activity of mitochondrial superoxide dismutase (MnSOD), an enzyme removing superoxide anion radical from the mitochondrial matrix, showed greater sensitivity to azoxystrobin. Similar effects were observed in the case of the *S. cerevisiae* wild-type yeast strain treated with azoxystrobin in combination with selected phenolic compounds which reduce the activity of MnSOD. Thus it seems that the efficiency of the antioxidant response system determines the level of sensitivity to azoxystrobin of saprophytic fungi.

Radish is not a target organism for fungicides, so based on preliminary study (data not shown), a higher dose of azoxystrobin was used than for determination of fungal sensitivity (Fig. 2).

Azoxystrobin applied in low concentrations (0.001, 0.003 %) does not significantly affect the germination process (Fig. 2). The strong inhibitory effects of azoxystrobin is expressed at higher concentrations of azoxystrobin, i.e. 0.018, 0.037 and 0.0076 % and only in the initial period of germination (24 and 48 hours after sowing). Later (72 and 96 hours after sowing), the germination rate increases, which indicates the temporal nature of the action of azoxystrobin in this case.

The length of the seedlings and organs was measured 96 hours after sowing (Fig. 3). Azoxystrobin inhibited elongation of radish seedlings and their organs. At the lowest

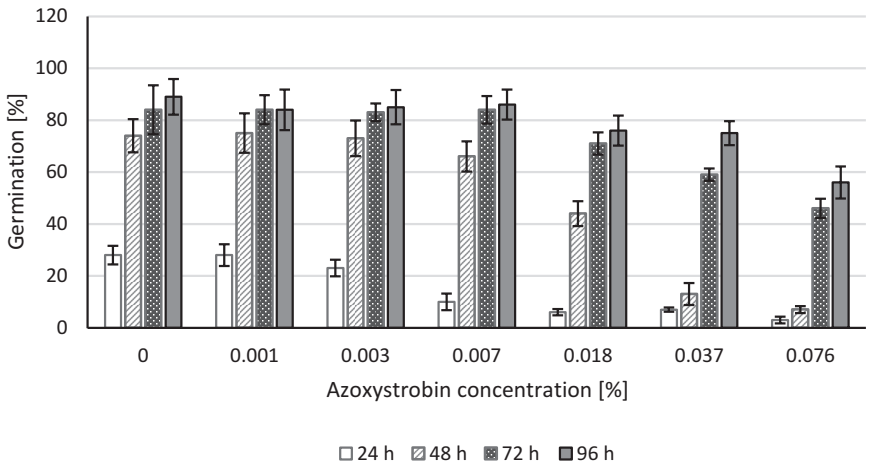

Fig. 2. Dynamics of radish seed germination in the presence of azoxystrobin

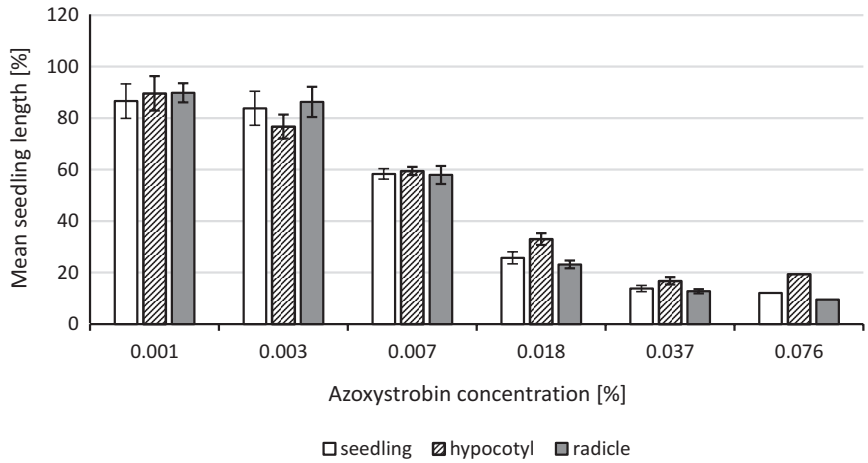

Fig. 3. Length of radish seedlings and their organs in the presence of azoxystrobin

concentration of azoxystrobin (0.001 %), the length of the seedlings and their organs was about 10 % lower than that of the control seedlings, while the level of inhibition of elongation of seedlings and their organs was more or less the same. However, the two highest concentrations (0.037 and 0.076 %) led to a reduction in the length of the seedlings and their organs by about 80 %. In this case elongation of the root was more severely inhibited than that of the hypocotyl.

Azoxystrobin inhibited germinated seed respiration in concentration dependent manner (Fig. 4). The level of respiration of the germinated seeds in the presence of the lowest concentration of azoxystrobin (0.001 %) was one third lower than in the case of the control sample, and at the two highest concentrations (0.037 and 0.076) it was about 10 times lower. The results are in line with data on the germination level of seeds evaluated at the same time, i.e. after 24 hours of incubation with a fungicide.

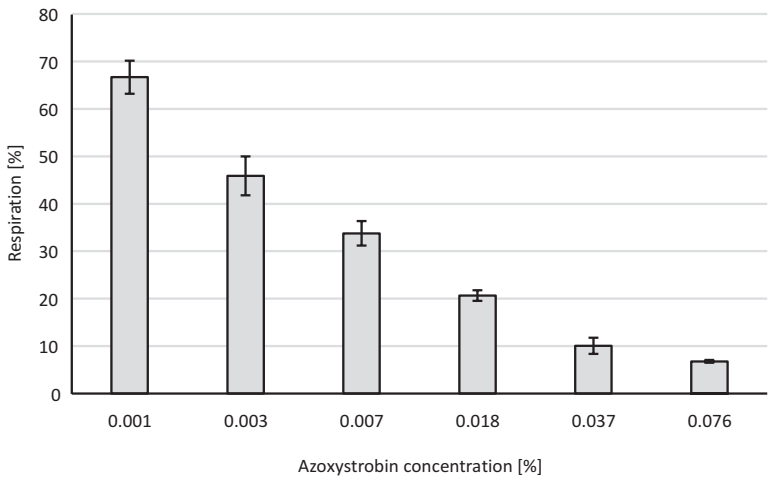

Fig. 4. Germinated radish seeds respiration in azoxystrobin presence

The increase in the respiration rate during seed germination indicates the activation of catabolic pathways aimed at obtaining the pool of ATP and intermediates necessary for biosynthesis of the cellular macromolecules required for the development of the plant embryo. Inhibition of seed respiration (Fig. 4) is in this case most likely the result of a specific interaction of azoxystrobin with mitochondrial respiratory chain enzymes.

The radish was more resistant to azoxystrobin than the fungi tested. The results are consistent with the observations of other authors. Sudisha et al. noted an inhibitory effect of three commercial formulations of strobilurins, i.e. trifloxystrobin, kresoxim--methyl, and azoxystrobin, applied in a concentration range from 0.0002 % to 0.0005 %, on sporangial sporulation, zoospore release, and motility of the fungal pathogen *Plasmopara halstedii* [36]. The same preparations were effective at a concentration 10 times higher in germinating sunflower seeds, but in this case their effect was found to be stimulatory. The highest seed germination was recorded at 0.001 % for kresoxim-methyl, and maximum seedling vigour was noted for trifloxystrobin at

Table 1

Inhibition rate, *IR*, of physiological processys in radisch and saprophitic fungi under azoxytrobin treatment

Phisiological process	Time of analyse [h]	Azoxystrobin concentration [%]							
		0	0.0005	0.001	0.003	0.007	0.018	0.037	0.076
SP4 *wt* growth	48	0	3.1	38.0	60.3	84.9	—	—	—
DSCD1-1C *sod1* growth	48	0	47.2	77.9	95.4	100.0	—	—	—
Penicillium sp. growth	120	0	52.5	57.4	54.9	62.4	—	—	—
Radisch seeds germination	24 96	0 0	— —	0.0 5.6	17.9 4.5	64.3 3.4	78.6 14.7	75.6 15.7	89.3 37.1
Seeds respiration	96	0	—	33.3	54.1	66.2	79.4	89.9	93.2
Seedlins elongation	96	0	—	1.8	16.9	41.6	74.3	86.2	87.9
Radicles elengation	96	0	—	10.2	13.7	42.1	76.8	87.2	90.5
Hipocotyl elongation	96	0	—	10.4	23.3	40.5	67.0	83.2	80.6

— means lack of date.

0.003 %. The differences in the response of plants to these fungicides are most likely due to species sensitivity as well as to differences in the methods of fungicide application and evaluation of their effects.

In the present study, azoxystrobin, especially at high doses, negatively influenced the germination process and elongation of radish seedlings (Table 1). The most sensitive processes were respiration of germinating seeds determined 24 hours after sowing and seed germination determined at the same time, followed by elongation of the radicle and the whole seedlings. The harmful effects of the fungicide observed during the study are not isolated. Reductions or delays of plant emergence, inhibition of shoot growth, and a reduction in subcrown internode length are commonly observed in cereals following application of triazoles and imidazoles. Physiological effects of this fungicides type are often accompanied by metabolic changes such as increased resistance to environmental stress, which may have a positive effect on yield [37, 38]. This type of nonspecific effect of fungicides on cereals when applied as a seed dressing is known as plant growth regulator (PGR) activity. The results of our study and those of other scientists confirm that strobilurins are PGR-like compounds affecting the growth of organisms via a mechanism associated with the antioxidant status of the cel [39, 40].

Conclusions

1. Azoxystrobin in a concentration range from 0.0005 % to 0.007 % inhibited the vegetative growth of yeast and the filamentous fungus *Penicillium* sp.

2. Yeast cells devoid of activity of cytoplasmic superoxide dismutase were hypersensitive to azoxystrobin at concentrations higher than 0.0005 %.

3. Radish, as an organism which is not a target of fungicides, was more resistant to azoxystrobin.

4. At concentrations higher than 0.003 %, azoxystrobin strongly inhibited respiration in the germinating seeds, the early stages of the germination process, and the development of the radicle and whole seedlings

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OCENA TOKSYCZNOŚCI AZOKSYSTROBINY DLA SAPROFITYCZNYCH GRZYBÓW ORAZ RZODKIEWKI WE WCZESNYCH STADIACH WZROSTU

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Abstrakt: Celem badań była ocena toksyczności azoksytrobiny, fungicydu należącego do klasy strobiluryn dla saprofitycznych grzybów (*Saccharomyces cerevisiae* i *Penicillium* sp.) oraz rzodkiewki (*Raphanus sativus* L.). Analizowano wzrost drożdży szczepu dzikiego i jego mutanta bezdysmutazowego *sod1*, grzyba strzępkowego *Penicillium* sp. oraz parametry biochemiczne i fizjologiczne kiełkujących nasion rzodkiewki i po-

wstałych z nich siewek. Najbardziej wrażliwym na azoksytrobinę okazał się wzrost drożdży (mutanta *sod1*, następnie szczepu dzikiego *wt*) w następnej kolejności: wzrost grzybni *Penicillium*, oddychanie kiełkujących nasion rzodkiewki, proces kiełkowania oznaczany po 24 godzinach od wysiewu nasion, wydłużanie korzeni, wydłużanie siewek, proces kiełkowania określany po 96 godzinach. Mechanizm toksyczności azoksytrobiny wydaje się być powiązany z aktywnością komórkowego systemu antyoksydacynego.

Słowa kluczowe: azoksystrobina, drożdże, dysmutaza ponadtlenkowa, grzyby strzępkowe