The Effects of Difenoconazole Treatment on Microorganism from Soil

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The present study quantified the effect of difenoconazole (DFC) on bacteria and mold communities grown in laboratory conditions, in treated soil microcosms difenoconazole, using the following concentrations: control, half dose (0.037 mg DFC / g soil, HD), normal dose (0.075 mg DFC / g soil, ND) and double dose (0.150 mg DFC / g soil, DD). The microbiological analysis included mesophilic bacteria involved in the nitrogen cycle: aerobic and anaerobic nitrogen-fixing, ammonifying, nitrifying and denitrifying bacteria. The mesophilic bacteria were incubated at 37°C and identified qualitatively on solid growth medium (Nutrient agar), whereas soil fungi were identified both quantitatively and qualitatively on solid growth medium (Potato-Glucose-Agar). In high doses, the DFC decreased the microbial communities involved in nitrogen cycle, namely aerobic and anaerobic bacteria and denitrifying bacteria. Noticeable was the response of two genera, namely Mucor sp. and Actinomucor sp., that showed resistance to the toxic effect of DFC, underlying their potential further practical use in the decontamination of polluted soils.

Keywords: difenoconazole, soil, bacteria

Difenoconazole or difenoconazol (DFC) is a chemical compound used as fungicide, insecticide, being also used as seed protection or seed treatment. The IUPAC name of difenoconazole is 1-[[2-[2-chloro-4-(4-chlorophenoxy) phenyl]-4-methyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole. DFC is a xenobiotic that can enter into the organism by inhalation, ingestion or dermal contact, when it is slowly absorbed and metabolized [1].

In agriculture, pesticides are applied to improve crop yield and quality and to maximize economic returns. Pesticides represent major inputs to agricultural production systems (i.e. agro-ecosystems). However, they comprise a wide variety of bioactive, toxic substances with direct negative impacts on soil productivity and agroecosystem quality [2, 3]. Their impact on non-target organisms in soils, microorganisms in particular (fungi and bacteria) has become a serious concern over the last decades [4].

The negative effects of pesticides on soil microflora are of great importance, because many microbial functions are critical to crop production, soil sustainability and environmental quality [5]. The structure and diversity of soil microbes, together with their metabolic activities, appear to be a crucial aspect within such a scenario. Since the soil microflora represents a major and mobile pool of life-essential elements, it plays a pivotal role in the biogeochemical cycling of nutrients [6, 7]. Hence, the structural and functional characteristics of microbial communities represent important indicators for monitoring the impact of pesticides on soil ecosystems and their biological status.

Numerous previous studies highlighted the effects of different groups of xenobiotics, such as pesticides and heavy metals, on water, soil, animals and soil microorganisms [8-12]. These groups of substances induce quantitative and qualitative changes of the soil microbial communities [13, 14]. Most studies up to date had a main focus on assessing the negative effects of herbicides on soil bacterial communities and their ability to biodegrade the herbicides [15, 16].

Several previous studies investigated the effects of difenoconazole (DFC) on soil bacterial communities, such as its biodegradation, the dehydrogenase and dimethyl-sulfoxid activities in soils, along with a certain fraction of microflora that not only became resistant to this product, but metabolized it as well. Other studies investigated the effects of DFC on dehydrogenase, phosphatase, urease and protease activities within soils and found a decreasing activity, but also an increased activity of protease at concentrations of 0.150 mg DFC / g soil [17]. It was equally showed that the DFC residues in rice plants (recorded within safe limits for human consumption) and in paddy soils (below the detection limit <0.01 mg/kg) [18].

However, data regarding the effects of DFC on diversity and functions of soil fungi are limited compared to those related to bacteria; therefore, we expect that further experiments on the effects of different fungicides on communities of soil fungi will improve the knowledge in this field. In this study the fungicide DFC was chosen [19]. It represents a broad-spectrum 1,2,4-triazole fungicide, used for the control of fungal diseases in fruits, vegetables, cereals and other field crops. Previous investigations regarding the effects of DFC on soil communities were undertaken using biochemical analyses (e.g. enzymes), underlining the indirect effects of this fungicide on these communities [14, 17, 20].

These previous results were sometimes contradictory in surprising the secondary effects of DFC, varying from any lack of response to various secondary effects, directly related with the dosage of this fungicide. The current study aims continuing this line of investigation, by reporting new effects of various concentrations of DFC on the soil

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microbial communities that play a crucial role in the biogeochemical cycle of nitrogen. Given the current high usage of this fungicide in agriculture, the importance of such investigations is obvious.

Experimental part

Materials and methods

The analyzed soil, of cambic chernozem type, was sampled from 0 to 20 cm depth in the fields of the Banat's University of Agricultural Sciences of Timisoara (Romania), from an area where insecticides, herbicides or chemical fertilizers were never used. The sampling was random, from a surface of 500 m^2, pooling a total sample of 15 kg. The collected soil sample was transported afterwards in the laboratory and treated with (DFC), which was purchased known as (3-chloro-4-[(2RS,4RS,2RS,4SR)-4-methyl-2-(1H-1,2,4-triazole-1-il-methyl)-1,3-dioxolan-2-il]phenyl4-chlorphenyl ether). DFC was purchased under the trade name *Score 250 EC,* which contains 250 g/L DFC active compound (Syngenta Corp Protection AG, Switzerland).

Soil treatment with fungicide. The soil was sieved (2-mm mesh size) and transferred in polyethylene bags. Different doses of fungicide were prepared with distilled water and then used to treat the soil samples, taking care that the soil humidity was maintained to a constant level of 40%. The fungicide dosage applied on dry soil was calculated assuming a uniform distribution in the sifted layer [21].

The following concentrations of fungicides were used: untreated control (CS), half dose (HD, 0.037 mg DFC / g soil), normal dose (ND, 0.075 mg DFC / g soil) and double dose (DD, 0.150 mg DFC / g soil). The correct amount of DFC for each treatment was prepared by following closely the indications from the product label, the used dosage being considered the one used in treatments of soils. The various DFC concentrations were applied only once, at the beginning of the experiment. Each treatment was applied in three replicates.

Afterwards, the samples were incubated in laboratory conditions for 7 days, at variable temperatures (25-28ºC). After incubation, the microbiological analysis of the samples was carried out. The control (untreated soil) was incubated within same conditions as the treatments. The bacteria and fungi communities were analyzed.

Ecophysiological groups of bacteria. Serial decimal dilutions were prepared from soil (10^1-10^6) for each sample (starting with 1 g of soil), using double-distilled water, and were inoculated in selective culture mediums. The number of bacteria was estimated according to the multiple-tube method. For each ecophysiologic group of bacteria, selective, autoclaved mediums were used.

The nitrogen fixing bacteria (NFB) were identified using a culture medium with the following chemical composition: sucrose (20.0 g); K_2HPO_4 (0.64g); KH_2PO_4 (0.16 g); $MgSO_4x7H_2O$ (0.20 g); NaCl (0.20 g); $CaSO_4x2H_2O$ (0.05 g); $Na_2MoO_4x2H_2O$ 0.05% (5.0 mL); $FeSO_4x7H_2O$ 0.3% (5.0mL) and distilled water (990 mL). The medium was sterilized by autoclaving. As part of NFB group, the species of interest were *Azotobacter vinelandii* and *Azotobacter chroococum* that can be identified by the type of veil formed at the surface of the liquid growing medium. Depending on species, the veil is either greenish-yellow (*A. vinelandii*) or brown (*A. chroococum*). From the group of anaerobic nitrogen fixing bacteria, (anNFB), the genus *Clostridium sp* was identified, using the growing medium with the following chemical composition: concentrated mineral salts solution (100 mL), phosphate solution (100 mL), iron sulfate (0.1% aqueous solution) (10 mL), biotin (0.002% aqueous solution) (0.25 mL), cysteine hydrochloride (0.5 g), glucose (10 g), agar (15 g), trimethoprim (16 mg), cycloserine (10 mg), polymyxin B sulfate (20 mg), distilled water (790 mL). The presence of genus *Clostridium sp.* is confirmed by the presence of gas bubbles within the growing medium [22].

The culture medium for ammonifying bacteria (AMB) had the following chemical composition: NaCl (0.5 g), peptone (2 g) and distilled water (1000 mL). The samples were incubated for 14 days, at 28°C. AMB number was assessed based on the reaction between ammonia and Nessler reagent [$K_2(HgI_4)$]; the Alexander table was used as screening benchmark [23].

The culture medium for nitrifying bacteria (NB) comprised standard saline traces (50 mL), $(NH_3)SO_4$ (0.5 g), $CaCO_3$ (1 g) and distilled water (950 mL). Samples were incubated for 20 days, at 28°C. Tubes containing nitrate were identified with diphenylamine-sulphuric acid. A blue color reaction shows that nitrite and nitrate are present, therefore the tube was scored positive [24].

The denitrifying bacteria (DNB) were grown in selective culture mediums which contained: standard saline solution (50 mL), KNO_2 (20 g), glucose (10 g), KCO_3 (5 g), oligoelements solution (1 mL), and distilled water (950 ml). The samples were incubated at 28ºC for 7-15 days and diphenylamine-sulphuric acid was added in each test tube. Positive samples were colorless due to nitrate metabolization by DNB [24].

Total number of bacteria CFU (colony forming units) / g soil. Dilutions were prepared from soil samples treated with fungicides and incubated for 7 days under laboratory condition. Key-samples of 10^{-1} and 10^{-6} dilutions were used, starting with one gram of soil. Equal volumes of 1 mL of every soil dilution were inoculated on selective nutrient culture mediums. Incubation was carried out at 37° C for 48 h, to count the total number of aerobic mesophilic bacteria (TNG). Bacterial colonies isolation was carried out on Plate-Count-Agars (Carl Roth GmbH, Germany) with soil extract. Then the sterile soil was poured into Petri dishes seeded with test samples (in 3 replicates). The steps of this seeding technique were the following: placing the culture medium on Petri dishes, seeding the medium with the test samples and inoculum dissemination with the help of an *L shaped* rod [23].

Total number of mold CFU (colony forming units) / g soil. Key-samples of 10^{-1} and 10^{-6} dilutions were used, starting with one gram of soil. Equal volumes of 1 ml of every soil dilution were inoculated on elective nutrient culture medium. The culture medium used for fungi growth and identification was Potato-Glucose-Agar (PGA) (Carl Roth GmbH, Germany). The steps of this seeding technique are: place the culture medium in Petri dishes, seed the medium with test samples, inoculate dissemination with the help of an *L shaped* rod and incubate the plates in a thermostat for 48 h at 28° C [23]. The determination of mold colonies was made up to the genus level based on the morphological characteristics of colonies at the surface of the culture medium [25].

Statistical analysis. The data was grouped into database tables in Microsoft Excel module of Microsoft Office 2007 software package, spreadsheet program Minitab statistical, version 14. All data were presented as means along with their standard deviation (X±SD). Both the experimental combinations of soils treated with DFC and the control samples were analyzed in three replicates. The difference between samples was determined using Mann-Whitney

tests and was considered significant for $p<0.05$, highly significant for $p<0.01$ and non-significant when $p>0.05$.

Results and discussions

Bacteria from different ecophysiological groups, involved in the biogeochemical cycle of nitrogen in soil, registered numerical variations in relation to the group and difenoconazole dose used.

Aerobic nitrogen-fixing bacteria *A. chroococcum* could not be detected. Aerobic nitrogen-fixing bacteria *A. vinelandii* registered numerical decrease with the increase of the DFC dose. The recorded values varied between 740±72.15 bacteria / g soil for DD and 2150±55.25 bacteria / g soil for CS, statistically significant ($p<0.05$).

The results are concurrent with other studies. In soils treated with the fungicides azoxystrobin and cyproconazole the bacterial community was drastically reduced, with ramifications on the NFB activity and reduced nitrogenase activity [26]. Anaerobic nitrogen-fixing bacteria of the genus Clostridium sp. recorded values between 200±80.75 bacteria / g soil at HD and 610±80.95 bacteria / g soil at ND. In control samples, 245±60.71 bacteria / g soil was determined. Normal dosage applications of DFC triggered an increase in an NFB community from genus Clostridium sp., consistent with previous findings. We suggest that the normal dose stimulated the growth NFB, mainly Clostridia, which are saccharolytic or proteolytic and can fix N_2, as observed previously for *Clostridium pasteurianum* [27].

In this experiment, ammonifying and nitrifying bacteria registered low values at variant HD, followed by a significant increase in their number ($p<0.05$) at variant ND, respectively CS and a slight decrease at the third dose of variant DD. The high values for the 0.075-0.150 mg DFC / g soil concentrations can be explained using DFC as a carbon and nitrogen source by some of the bacteria in these two ecophysiological groups (fig. 1).

Denitrifying bacteria registered a statistically significant decrease ($p<0.01$) with the increase of the DFC dose with values between 5500±111.57 bacteria / g soil at variant HD and 615±70.11 bacteria / g soil at variant DD, which points out the toxic effect of the fungicide upon this ecophysiological group of bacteria.

Microbial parameters provide information on the soil nitrogen cycle and previous findings reported that the bacterial communities involved are very sensitive to pesticide application. Nitrification and ammonification are closely related processes; the determination of chemical parameters, along with microbiological ones indicated the disruption in the nitrogen cycle within soil [28].

Studies carried out previously showed not only the inhibitory effect of pesticides on the nitrification process in soil, but also the increase of ammonification due to the stimulation of ammonifying bacteria development which can utilize pesticides as a food source [29-32].

Analyzing difenoconazole effect on communities of soil microorganisms and their enzymatic activities it was found that difenoconazole applied for short periods presents an inhibitory effect on enzyme activity [7].

The DFC may have a significant impact on microbial parameters in soils and can influence many soil microorganisms, including bacteria [32]. Studies showed the stimulatory effect of tebuconazole on populations of nitrifying soil bacteria, but tebuconazole had an inhibitory effect on the microorganism's nitrite that was sensitive to the fungicide treatment [29].

The increase in pesticide concentrations due to the incapacity of degradation and the accumulation in soils have negative effects both on soil microbial communities and soil quality. The recorded values for the major ecophysiological groups of bacteria involved in the nitrogen cycle in the analyzed soil samples showed that high concentrations of DFC had a negative effect, inducing stress on soil microbial communities involved in the nitrogen cycle.

In experimental combinations treated with DFC, the growth capacity of bacterial colonies showed quantitative variations based on the fungicide dose. After 24 h of incubation, the values recorded for the number of bacterial colonies varied between 1.66±0.120x106 CFU / g soil at variant HD and 6.66±0.275x106 CFU / g soil at variant DD. A similar situation was observed after 48 h of incubation: the recorded values varied between 3.33±0.150x106 CFU / g soil at variant HD and 9.33±x106 CFU / g soil at variant DD. In controls the numbers of bacterial colonies varied between 9.66±0.330 x106 CFU / g soil at 24 h incubation time and 12.33±0.455 x106 CFU / g soil at 48 h.

The Mann-Whitney test applied between control and experimental variants showed statistically significant differences ($p<0.05$) with the increase in DFC dose (fig. 2).

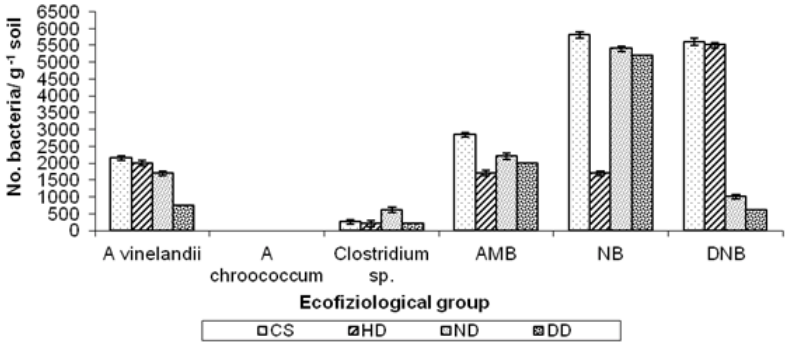

Fig. 1. Variation of ecophysiological groups of bacteria density (mean ± SD) involved in the nitrogen cycle, after DFC treatment in laboratory conditions (*Azotobacter vinelandii, Azotobacter chroococum* AMB-ammonifying bacteria, NB-nitrifying bacteria, DNB-denitrifying bacteria, CS-control sampling, HD-half dose, ND-normal dose, DD-double dose)

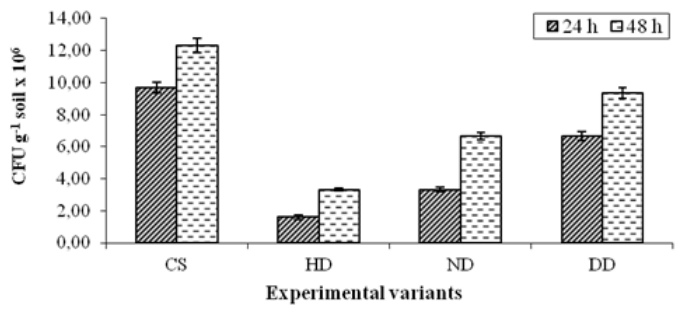

Fig. 2 .The variation of bacterial colonies from soils treated with DFC in laboratory conditions (CFU- Colony Forming Units, CS-control sampling, HD- half dose, ND- normal dose, DD- double dose)

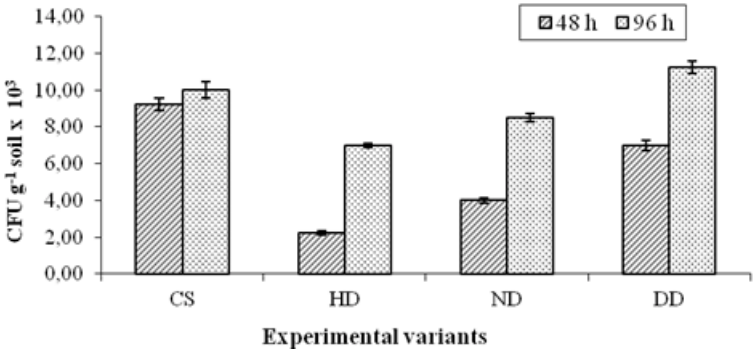

Fig. 3. The variation of number of mold colonies in soils treated with DFC in laboratory conditions (CFU-Colony Forming Units, CS-control sampling, HD- half dose, ND- normal dose, DD- double dose)

Microorganisms play a key role in many soil processes and the assessment of soil microbial diversity provides useful indicators of the impact of xenobiotics on soil quality [32, 33]. Fungicide application at soil level can have negative effects on soil microbial communities, with adverse consequences for soil quality [34, 35].

For samples treated with difenoconazole, the growth capacity of mold colonies also showed quantitative variations based on the DFC dose. After 48 h of incubation, the values registered for the number of mold colonies (developed on the surface of the culture medium Potato-Glucose-Agar) varied between 2.25±0.125x103 CFU / g soil at variant HD and 7±0.x103 CFU / g soil at variant DD. A similar situation was observed after 96 h of incubation: the recorded values varied between 7±0.120x103 CFU / g soil at variant HD and 11.25±x103 CFU / g soil at variant DD (fig. 3). In controls the numbers of mold colonies varied were between 9.25±0.333 x106 CFU / g soil after 48 h of incubation and 10±0.450 x106 CFU / g soil after 96 h of incubation. The Mann-Whitney test applied between the control and experimental variants incubated for 48 h, respectively 96 h showed statistically significant differences ($p<0.05$).

In experimental samples treated with DFC the main mold genera identified showed quantitative and qualitative variations based on the used dosage. In controls the following mold genera were identified: *Penicillium sp.*, *Actinomucor sp.*, *Aspergillus sp.*, *Rhizopus sp.* and *Mucor sp.*

The genus *Mucor* had a relatively uniform distribution, being present in all experimental combinations and decreasing directly with increasing the dose of DFC. The recorded densities for genus *Mucor* varied between 1.25±0.120x103 CFU / g soil at variant DD and 4±0.333x103 CFU / g soil at variant ND.

The Mann-Whitney test applied for the development of mold colonies showed statistically significant differences ($p<0.05$) based on the DFC dose, for some of the mold genera.

In the case of the genus *Aspergillus*, *Rhizopus* and *Penicillium*, no significant differences could be detected in terms of colony development with exposure to different doses of DFC. In addition, the genus *Rhizopus* showed the lowest values of CFU / g soil for all three experimental variants.

The results of this study indicated significant differences ($p<0.05$) for the *Mucor* genus, based on the different DFC concentrations. Other significant differences ($p<0.05$) were observed for the *Actinomucor* genus in all experimental combinations. In terms of abundance and sensitivity to the effects of difenoconazole, decreases of the CFU / g soil were registered as follows: *Mucor sp.>Actinomucor sp.>Penicillium sp.>Aspergillus sp.>Rhizopus sp.*

Several studies concerning the effect of fungicides (difenoconazole and chlorothalonil) on growth and germination of spores were undertaken previously. The results underlined that DFC and chlorothalonil fungicides had a detrimental effect on mycelium growth and spore germination in vitro for two species of *Altenaria. A. solani* had a higher sensitivity than *A. alternata* [36]. Furthermore, DFC was more effective than chlorothalonil on mycelium growth and conidia germination for *A. solani* and *A. alternata*.

One of the major findings of this study is that the DFC, applied in high dosage is detrimental for the envisaged microbiological communities, mainly for denitrifying bacteria. Moreover, the resistance of several genera of fungi to this product was envisaged, mainly the genera *Mucor* and *Actinomucor*.

Conclusions

The effects of DFC consisted in the disturbance of soil microbiology, negatively influencing both the quantity and the quality of microorganisms within soil. In high doses, the DFC had significant ($p<0.05$) negative effects on some microbial communities involved in the nitrogen cycle: *A. vinelandii, A. chroococcum* and denitrifying bacteria. After 24 and 48 h of incubation, a similar and significant increase of the CFU was noticed for all three combinations. Some mold colonies showed resistance to the toxic effect, which will allow their further isolation and use for decontamination of polluted soils. Difenoconazole caused the following decrease of mold colony abundance: *Mucor sp.>Actinomucor sp.>Penicillium sp.>Aspergillus sp.>Rhizopus sp.* Cluster analysis showed a similar behavior for the genera Aspergillus and Penicillium, when the standard dose of DFC was applied.

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