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# THE EFFECT OF HIGH DIFENOCONAZOLE CONCENTRATION ON SOIL MICROBIOTA ASSESSED BY MICROBIOLOGICAL ANALYSIS

**Marioara Nicoleta FILIMON[1,2], Paula MANIU[1,2*], Mihaela Georgiana BRAN[1,2]**

[1]West University of Timisoara, Faculty of Chemistry, Biology, Geography, Department Biology - Chemistry, Pestalozzi, 16, 300115, Romania
[2]Advanced Environmental Research Laboratories, Oituz, 4, Romania
**Corresponding author's e-mail address: paula.maniu@yahoo.com*
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**ABSTRACT**
*In order to improve crop quality and production efficiency, are widely used pesticides. Although they are beneficial in this respect it acts as a stressor on the soil, changing its microbiological balance. The study took into account the determination of physico-chemical parameters (temperature, pH, humidity and content of organic matter), biochemical (enzymatic activities of dehydrogenase, urease, phosphatase and protease) and microbiological (determining the total plate count aerobic mesophilic that develops at 37 ° C, determining the number of colonies of fungi and identifying genres). The determinations were made in a dynamic period of 21 days. Elevated difenoconazole had a deleterious effect on communities affecting soil microorganisms and soil quality.*

**KEY WORDS:** *soil, difenoconazole, enzymes, microorganisms, parameters*

## INTRODUCTION

The use of pesticides in increased amounts cause negative effects on soil biodiversity, but also to microorganisms, influencing biochemical processes in soil determined by microbial and enzymatic processes (Baxter & Cummings, 2008; Popescu *et al*, 2013). Application of pesticides disrupt the soil ecosystem by damaging the communities of microorganisms. The conducted studies highlight the negative effects of application of pesticides on farmland biodiversity, soil and the microorganisms (Littlefield-Wyer *et al*, 2008). The role of soil enzymes and their activities are defined by their relationships with soil and other environmental factors affecting their activities. The enzymatic activity of the soil reflects not only soils solution enzymes and living tissue enzymes, but also enzymes fixed on soil colloids and humic substances (Nannipieri *et al.,* 1996).

Pesticides that reach the soil can disrupt local metabolism or enzymatic activities (Liu *et al.,* 2008). The negative impact of pesticides on soil enzymes such as hydrolases, oxidoreductases and dehydrogenase has been reported in numerous studies in the literature (Monkiedje & Spiteller, 2002).

Soil dehydrogenase is an integral component of intact cells, alive in the soil, not extracellular identified in soil. Intervenes in the process of oxidative degradation, dehydrogenation of organic matter by transferring electrons and hydrogen from the acceptor substrate under the action of enzymes. Various factors affect the activity of dehydrogenase

enzymes in the soil, of which the incubation temperature and time are more important. Dehydrogenase activity decreased with increasing soil depth (Velmourougane *et al.,* 2013). Determining the effect of the application of pesticides in soil was based on the values recorded for dehydrogenase activity, stimulation or inhibition of dehydrogenase activity in soil has been reported in studies (Crouzet *et al,* 2010; Muñoz-Leoz *et al.,* 2011).

Urease activity of soil is another biochemical parameter, whose values may indicate the effects of xenobiotic chemicals applied to crops. Thus, increasing the incubation period influences the activity of urease, which is significantly higher on day 20 of incubation, after which the enzymatic activity gradually decreased with increasing incubation period at application of pesticide (Mekapogu *et al.,* 2011). Also, it has been determined that some urease activity is influenced by environmental parameters such as - the type of buffer, the pH of the buffer solution, the concentration of urea, the incubation period.

Soil phosphatase was investigated because of its importance in the phosphorus cycle of the soil; can catalyze the conversion of organic phosphorus in inorganic phosphorus. There are three types of phosphatases in soil: acidic, alkaline or neutral. Application of pesticides in soil can cause negative effect on phosphatase activity or a positive one, to stimulate the development of microorganisms and their involvement in the metabolism of the pesticide (Sukul, 2006; Omar *et al,* 2001; Tejada 2009). The existence of phosphorus can limit net primary production in tropical forests, where the soil derived from inorganic phosphate is, in geochemical terms, limited (Ianovici, 2010; Cleveland *et al.,* 2011).

Proteases can be sorted by various criteria, type of reaction catalysed, functional group of the active site, and evolutionary molecular structure (Landi *et al.,* 2011). In soil, they come from several different sources, including microorganisms, plants, animal droppings (feces and urine) (Kania *et al.,* 2010; Singh *et al.,* 2011). Potential protease activity, faster than the actual activity of the enzyme is often measured using potential activity tests under optimum conditions (temperature and pH) with the use of synthetic substrates (Nannipieri *et al.,* 2002).

A considerable amount of pesticide applied more frequently, end up in the soil, where they undergo biological and physico-chemical transformations. Once in soil, microbial degradation is the main way to remove them. Pesticide application can have harmful effects on non-target organisms and microorganisms, with adverse consequences on the quality of soil (Niemi *et al.,* 2009).

Depending on environmental factors, pesticides often have low rates of degradation in soil, but repeated application of pesticides may eventually lead to their accumulation in high concentrations to the detriment of soil microorganisms. In the study we tested the impact of pesticides on soil microbial communities, dependent on the type of pesticide, the pesticide concentration and incubation time (Nannipieri *et al.,* 2002).

Respiratory coeficient is an index which can reflect environmental stress in microbial communities. Indeed, an increased respiratory activity related to the microbial biomass size may reflect stressful conditions for microbial communities, forcing them to use a larger amount of their resources of energy for maintenance and survival, leading to a lower incorporation of organic C in microbial biomass. Studies have established that the application of pesticides can lead to an increase in ammonifying bacteria community. Nitrified bacteria were most sensitive to the application of pesticides (Sáez *et al.,* 2003).

The aim of this study was to highlight the effect of difenoconazole applied in high concentration, on the metabolic activity of microorganisms in the soil over a period of 21 days, finally determining the enzymatic indicator of soil quality (EISQ).

## MATERIALS AND METHODS

The soil sample used for experiments was randomized taken from areas where they were applied xenobiotic substances and were not reported sources of pollution, so that the soil types were mixed resulting in a single sample.

Difenoconazole solution was made from the recommended quantities of the product producing company Syngenta. It is an established concentration: 0.225 mg / 1g soil - 3x normal dose recommended by the producing company.

**Experimental variants.** In laboratory and field experimental variants were realized 4:

- A - control soil fungicide untreated, maintaining the sample temperature at 23º C, under laboratory conditions
- B - soil treated with 0.225 mg / 1 g of soil (3xND), maintaining the sample temperature at 23º C
- C - control soil fungicide untreated, maintaining the sample temperature at 17º C
- D - soil treated with 0.225 mg / 1 g of soil (3xND), maintaining the sample temperature at 17ºC.

**Physico-chemical analysis methods.** Determination of pH in analyzed soil samples were performed using multiparameter WTW pH 340i / SET presenting a specific sensor for each parameter. The pH determination involves the preparation of a soil solution: 10g soil in 100 ml distilled water. Temperature monitoring was performed daily using thermometers placed at the level of the soil samples from the four experimental variations. Determination of organic substances in soil samples was carried out by following the processes of weighing, drying and calcining (Gergen, 2004).

**Biochemical analysis methods.** Using the Casida methods we were able to determinte the actual and potential dehydrogenase activity. The reacting mixture consisted of 3 g soil, 0.5 ml 2,3,5 triphenyltetrazolium solution (TTC), 2 ml distilled water and 1 ml glucose solution, respectively, for potential dehydrogenase. The treated samples were incubated at a temperature of 37° C for 48 hours. Dehydrogenase activity was expressed as mg formazan/3 grams soil.

In conformity with Dragan-Bularda's method (2000) we determinated urease activity. Reaction mixtures consisted of 3 g soil, 2 ml toluen, 5 ml phosphate buffer, 5 ml solution of urea 3%. The mixture was incubated at 37° C for 24 hrs. Activity was expressed as mg $NH_4$/3 grams soil.

Phosphatase activity (PhA) was estimated based descompunerea phenyl phosphate disodic in produsii finali disodic phosphate and phenol. For each sample, about 2.5 g soil were put into a test tube containing 10 mL of 0.5% disodic phosphate solution. The mixture was incubated at 37° C for 48 hrs. The phenol with Gibbs reactive (2,6-dibromchinon-chloramide) resulting in a blue precipitate. Activity was expressed as mg phenol/ 1 gram of soil (Dragan-Bularda, 2000).

Protease activity (PA) was estimated by reaction of ninhydrin with the amino acids resulted after the hydrolysis of gelatin used as substrate. For each sample, about 3 g soil was

mixed with 7 mL of 2 % gelatin and 0.5 mL toluene. The mixture was incubated at 37 ºC for 24 h. PA was defined as milligrams amino-N/ 1 gram of soil (Dragan-Bularda, 2000).

The calculation of enzymatic indicator was based on the absolute values of the enzymatic activities from every sample analyzed. In order to di that we used the formula proposed by Muntean: $EISQ = 1/n \sum Vr(i) / Vmax(i)$, where: EISQ - enzymatic indicator of the soil quality, n - number of activities, Vr (i) - real individual value, Vmax (i) - maximal theoretical individual value.

**Microbiological analysis methods.** Total number of bacteria CFU (colony forming units) / 1 g soil.

Soil extract diluted at $10^{-6}$ was inoculated on nutrient culture medium.

Incubation was carried out at 37° C for 48 hrs, to determine the total number of aerobic mesophilic bacteria (TNG). Bacterial colonies isolation was carried out on Plate-Count-Agar with soil extract. Then the sterile soil was poured into Petri plates seeded with test samples (in 3 repetitions). The steps of this seeding technique were: placing the culture medium on Petri plates, seeding the medium with the test samples, inoculum dissemination with the help of an "L shaped" rod (Dragan-Bularda, 2000).

Total number of mold CFU (colony forming units) / 1 g soil

The culture medium used for fungi isolation was Potato-GlucoseAgar habitat (PGA). Then the sterile soil was poured into Petri plates seeded with test samples (in 3 repetitions). The steps of this seeding technique were: placing the culture medium on Petri plates, seeding the medium with the test samples, inoculum dissemination with the help of an "L shaped" rod. Seeding plates were kept for 48 h at 28°C (Dragan-Bularda, 2000).

## RESULTS AND DISCUSSIONS

The soil samples of the experimental variants, treated or untreated with difenoconazole were maintained in laboratory conditions, but also under field conditions with different values of temperature. After 7, 14 and 21 days from fungicide application enzymatic activities were determined, and then on the basis of their values was determined the soil quality enzymatic indicator.

In the variants treated with difenoconazole the values are smaller ($p < 0.05$) of the dehydrogenase activity compared to the values for control soil samples (Ml respectively Mc), which indicates the toxic effect of a high concentration of difenoconazole on the communities of soil microorganisms. Dehydrogenase activity is often used as a measure for assessing the factors that disturb soil like application of pesticides or work performed in soil. It can indicate the type and significance of soil pollution.

In the experimental variants treated with DFC were recorded higher values compared to the values of urease activity in the controls after 7 and 14 days with the fungicide. Values greater in the experimental variants compared to the control indicate the less toxic effect of the fungicide on this enzymatic activity, but another possible cause could be the use of the fungicide as a source of C and N for nutrition, growth and development because of the chemical structure of DFC.

The values decreased from 1.870 mg $NH_3$ / g soil (7 days) at 0.512 mg $NH_3$ / g soil (21 days) for the experimental variant 3xD l, and from 1.787 mg NH3 / g soil (7 days) at 0.351 mg

$NH_3$ / g soil (21 days) for experimental variant 3xD c. The decrease is statistically significant (p <0.05) (Figure 2).

In the experimental versions are recorded declines in the values of phosphatase activity from 2.748 mg phenol / g soil (7 days) to 0.316 mg phenol / g soil (21 days) for experimental variant 3xD l, and from 2,374 mg phenol / g soil (7 days) to 0.131 mg phenol / g soil (21 days) for experimental variant 3xD c. Declines at 14 days and 21 days compared to day 7th are statistically significant (p <0.05) (Figure 3).

Comparing the readings of the experimental variations in soil samples with the ones from the control, in both cases were obtained lower values of phosphatase activity, which highlights the inhibiting effect on the enzymatic activity by the fungicide applied.

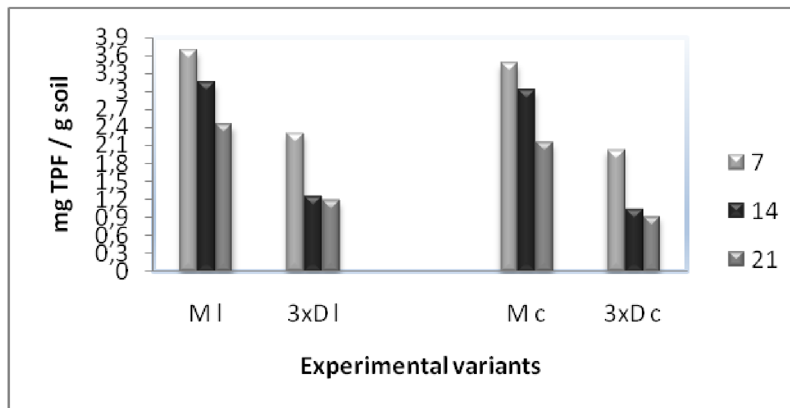


**FIG.1. The dehydrogenase activity evolution in the analyzed experimental variants (M l control sample in laboratory, 3x D l DFC treated soil in laboratory, Mc control sample in field, 3x D c DFC treated soil in field)**

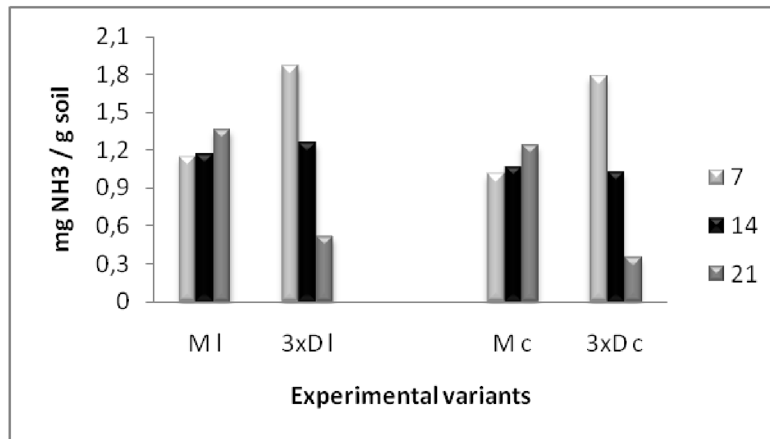


**FIG.2. The urease activity evolution in the analyzed experimental variants (M l control sample in laboratory, 3x D l DFC treated soil in laboratory, Mc control sample in field, 3x D c DFC treated soil in field)**

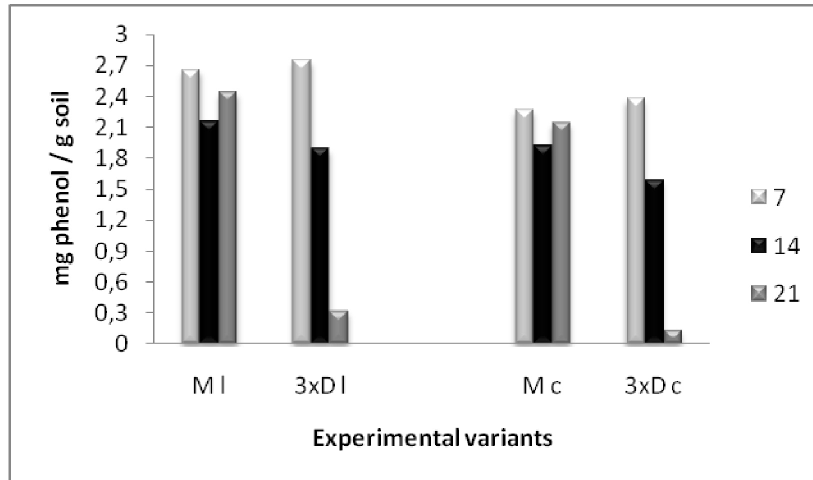


**FIG.3. The phosphatase activity evolution in the analyzed experimental variants (M l control sample in laboratory, 3x D l DFC treated soil in laboratory, Mc control sample in field, 3x D c DFC treated soil in field)**

Higher values for the protease activity on the 21st day indicates a restored balance in the soil after DFC application. Also, higher values in the experimental variants compared to the control indicates less toxic effect on soil microorganisms. Increases for protease activity from day 7 to day 21 are statistically significant ($p < 0.05$). While another possible cause of these high values is the use of the fungicide as a source of C and N.

It is noticed a decrease in the indicator value with increasing incubation period regardless of the experimental variant. EISQ values recorded decreases in experimental variants treated with DFC compared with the values from the controls. Increasing the dose of applied difenoconazole has reduced soil quality by reducing the number of microorganisms responsible for producing enzymes at soil level (Figure 5).

The maximum values for the number of colonies of bacteria in soil samples treated with difenoconazole were recorded on day 7th of analysis, values were $30 \times 10^6$ CFU bacteria in experimental variant 3xDl at 48 hours of incubation, respectively $17 \times 10^6$ CFU bacteria in experimental variant 3xDc at 48 hours of incubation. The maximum values of the number of bacterial colonies (CFU) in the control soil samples were recorded on day 7th of the test, the values were in the experimental variant M1 $46{,}5 \times 10^6$ bacteria CFU at 48 hours of incubation, respectively $45 \times 10^6$ CFU bacteria in the experimental variant Mc at 48 hours of incubation (Figure 6). Overall there was a decrease in soil bacterial communities, resulting in toxic effect of increased concentration of difenoconazole on soil micropopulation.

The populations of fungi in control soil samples incubated under different temperature, recorded according to incubation time a behavior thats is achieved with Gause's curve. Variations in the temperature at which the soil samples in the experimental variant Mc were incubated determined lower values in the number of colonies (CFU) compared to those incubated in laboratory under conditions of constant temperature (Figure 7).

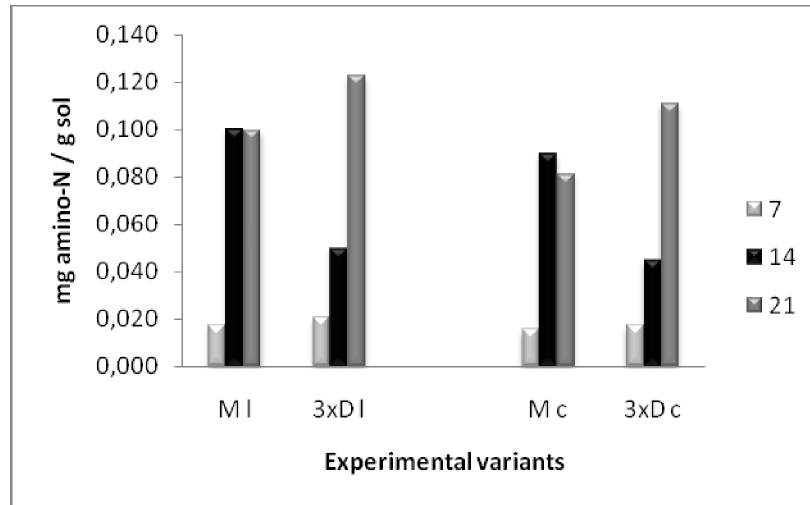


**FIG.4. The protease activity evolution in the analyzed experimental variants (M l control sample in laboratory, 3x D l DFC treated soil in laboratory, M c control sample in field, 3x D c DFC treated soil in field)**

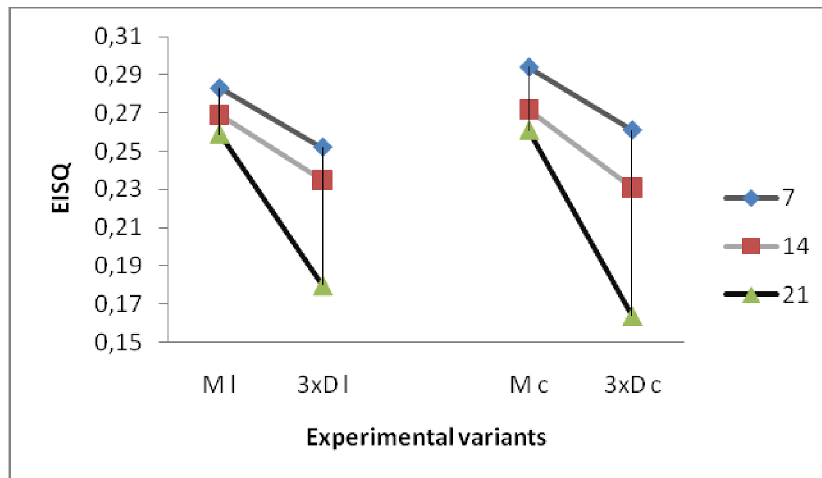


**FIG.5. The EISQ of the analysez soil samples (M l control sample in laboratory, 3x D l DFC treated soil in laboratory, Mc control sample in field, 3x D c DFC treated soil in field)**

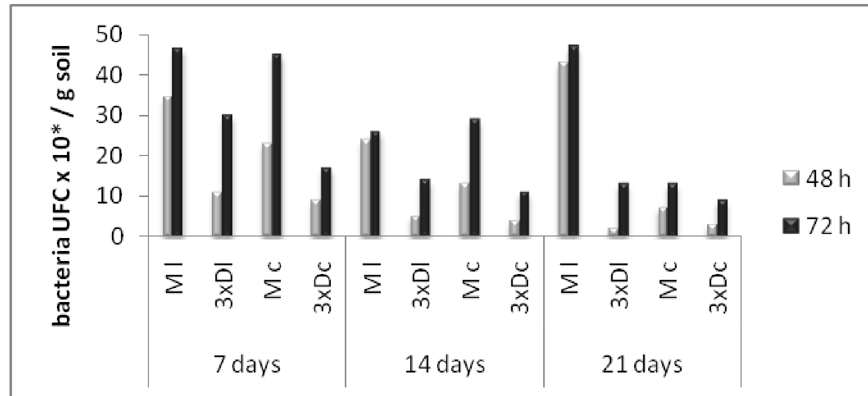


**FIG.6. The evolution of the bateria communities in the analyzed soil samples**

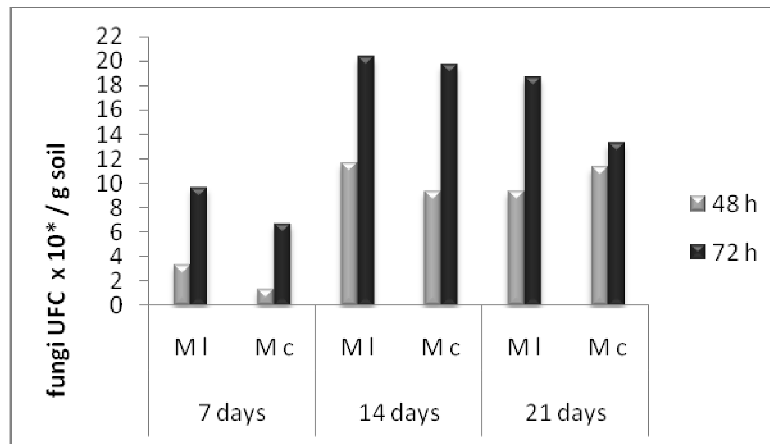


**FIG.7. The evolution of the fungi population in the analyzed soil samples**

In the soil samples treated with difenoconazole and incubated in the laboratory we find the number of colonies values lower than those determined for the control soil samples regardless of the time of incubation. The lower values are the result of the inhibitory effect of difenoconazole applied in high concentration on communities of fungi. However a significant increase in the number of colonies recorded with the increase in the application period difenoconazole which highlights the possibility of its use as a food source (Figure 8).

The main fungal genera and species identified in the experimental variants treated with difenoconazole were *Actinomucor spp*., *Mucor* sp., *Penicillium sp.*, *Humicola sp.*, *Rhizopus nigricans*.

The population of fungi exhibit a very heterogeneous behavior in the soil sample treated with difenoconazole and maintained under laboratory conditions. It may be noted the absence of genres at 48 h incubation (*Actinomucor*, *Mucor*) or extremely low values for *Humicola sp*. and *Rhizopus nigricans*. At 72 hours of incubation shows that the dominant genus of fungi is *Mucor*, followed by *Penicillium* and *Humicola*. This can be attributed to better

funcicide tolerance by genres mentioned so they are resistant to the toxic effect of the increased concentration of DFC.

Following the evolution of fungi population in most cases we find a behavior performed by Gause's curve. In the 7th day of the application of the fungicide the values determined for the number of colonies of fungi is relatively low, then follows the maximum value for most genera and species identified in the $14^{th}$ day of the test, and finally the number of colonies of the fungus to lower, but the values are higher compared to day 7 (Figure 9).

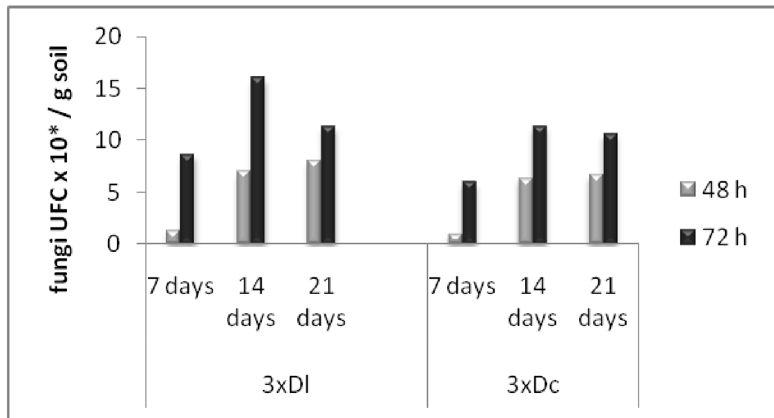


**FIG.8. The evolution of the fungi population in the DFC treated soil samples**

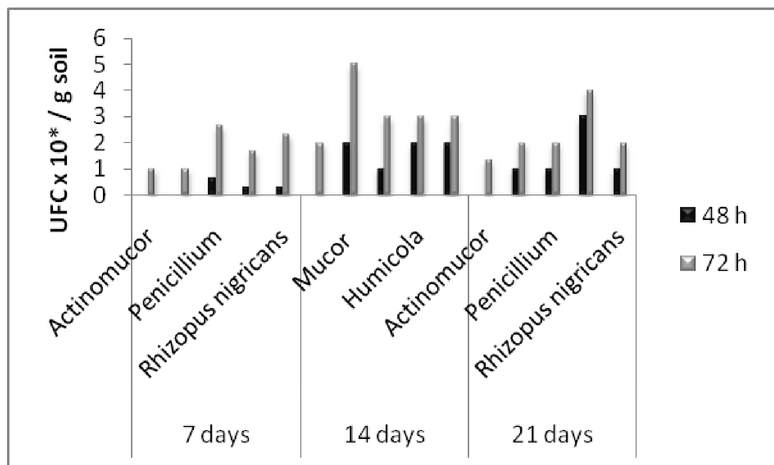


**FIG.9. The evolution of the fungi population in the DFC treated soil samples, maintained in laboratory**

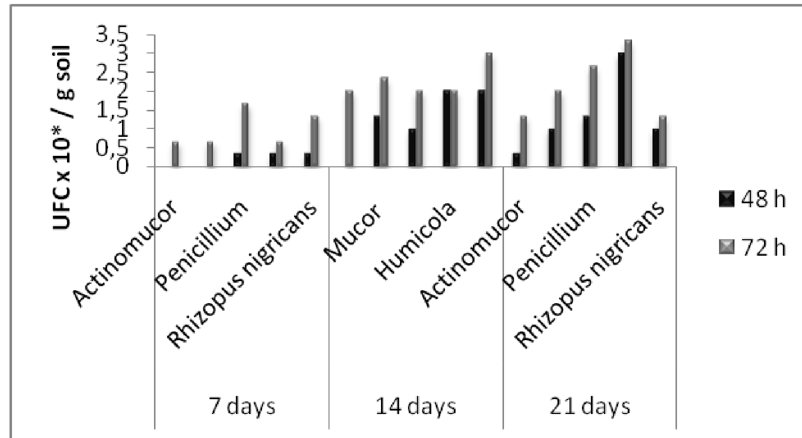


**FIG.10. The evolution of the fungi population in the DFC treated soil samples, maintained in field**

It produces a maximum number of colonies of *Actinomucor* of $2x10^3$ CFU / g soil (day 14 analysis), *Mucor* of $5x10^3$ CFU / g soil (day 14 analysis), *Penicillium* $3x10^3$ CFU / g soil (day 14 review), *Humicola* $4x10^3$ CFU / g soil (day 21 analysis), *Rhizopus nigricans* $3x10^3$ CFU / g soil (day 14 analysis). It may be noted the absence of genres from 48 h incubation (*Actinomucor*, *Mucor*) or extremely low values for *Penicillium*, *Humicola* and *Rhizopus nigricans*. Also *Actinomucor* sp. had a low development at 48 hours of incubation in all variants analyzed.

At 72 hours of incubation it is found that the dominant fungus is the genus *Penicillium* ($1,66x10^3$ CFU) at 7 days after application of the fungicide; *Rhizopus nigricans* ($3x10^3$ CFU) followed by *Mucor* ($2,33x10^3$ CFU) at 14 days after the application of the fungicide; *Humicola* ($3,33x10^3$ CFU) followed by *Penicillium* ($2,66x10^3$ CFU) at 21 days after the application of the fungicide (Figure 10). The fungi behavior outlines the negative effect of DFC application in high concentration. The balance of the soil is restored from day 14 of analysis, probably due to the involvement of microorganisms in the degradation of DFC.

## CONCLUSIONS

Difenoconazole administration in increased levels resulted in metabolic inhibition of microorganisms in the soil, reducing the synthesis and action of enzymes. Decreased number of microorganisms that are responsible for producing enzymes, causes reduction in decomposition and degradation processes if the organic waste and ultimately led to decreased soil quality. Increased concentration of DFC in soil has a negative impact on soil microbial communities and on soil quality.

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