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Growth kinetics, monitoring of biomarkers and respiratory metabolism to evaluate the combined effects of fludioxonil and difenoconazole in a freshwater ciliated protist: *Paramecium sp.*

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ABSTRACT

The intensification of agriculture and uncontrolled application of agrochemicals has resulted in an increased loading of ground water with pesticides, greatly impacting ecosystems by changing the biodiversity and threatening aquatic organisms. The aim of this study is to investigate the underlying effects of the mixture Fludioxonil – Difenoconazole, a newly introduced fungicide for the treatment of seeds, on an alternative cellular model: The ciliated protist Paramecium sp. Exposure experiments were conducted at 28°C and various endpoints were studied. We first interested to investigate the effects of sublethal exposure to Fludioxonil – Difenoconazole on population growth. Paramecium cells were exposed to four concentrations of (0.1, 0.5, 1, 1.5 mg/l) and the growth kinetics was followed for 96 hours. We also measured the oxygen consumption via a polarographic study. Under the same condition, total proteins rate, Glutathione content (GSH) and enzymatic activity of Catalase (CAT) were investigated to evaluate antioxidative response to fungicide effects. After 96h of treatment, the results showed a significant decrease in growth rate of about 70% in cells treated with 1.5 mg/l. Paramecia exposed to different concentrations of Fludioxonil - Difenoconazole showed a strong disruption in respiratory metabolism. On another hand, the monitoring of biomarkers has demonstrated a significant decrease in total proteins rate at the highest concentration. The present finding revealed a disruption of enzymatic activity of Catalase and a depletion of GSH content in dose dependent manner. By the obtained results, we may suggest the occurrence of oxidative stress generated by the studied xenobiotic on the ciliated protozoan Paramecium sp.

Keywords: *Paramecium sp.*, Fludioxonil, Difenoconazole, Toxicity, Growth kinetics, Respiratory metabolism, Biomarkers.

INTRODUCTION

If the beneficial effects of pesticides are well established, their use poses serious problems for the environment, human and animal health. The misuse and uncontrolled agrochemicals, has increased considerably over the last 35 years [1]. The Organization for Economic Co-operation and Development (OECD) has estimated that by the year 2020 global output will be 85% higher than it was in 1995 [2]. These products have a profound effect on the environment and contribute heavily to pollution when they are applied in a quantity exceeding what crops can absorb or when they are washed away by water or wind before their absorption. The excessive amounts of the constituents of pesticides (nitrogen, phosphate,..etc.) can be leached into groundwater or run off into surface waters thus affecting the “non-target” species of wildlife and flora. In addition, they reduce biodiversity and disrupt the ecosystems. Agrochemicals products received enormous attention for their toxic effects on organisms [3,4,5].

Triazole fungicides are one of the main classes of pesticides widely used for their properties, such as high chemical and photochemical stability, low biodegradability and easy transportation in the environment make them persistent in soil and water.

Difenoconazole (cis-trans-3-chloro-4-(4-methyl-2-(1H-1,2,4-triazol-yl methyl)-1,3-dioxolan-2-yl) phenyl 4-chlorophenyl ether) inhibits fungal ergosterol biosynthesis, thus resulting in the blocking of fungal cell wall chitin synthesis [6]. Unfortunately, recent research in southern China indicated that after 11.25 g of Difenoconazole per hectare was applied (lower than the recommended dosage), the drug dosage in paddy water reached 1.98 mg/l on spraying day, with a half-life of about 6 days [7] which may lead to an exposure to organisms. In addition to paddy field, previous study showed that the detected value of difenoconazole in surface water sample of Australia reached 0.15 mg/l [8]. According to the recent publication of the European Food Safety Authority (EFSA), it has been identified that difenoconazole is very toxic to aquatic organisms in view of its high toxicity to *Daphnia magna* (chronic NOEC = 0.0056 mg active substance/l) [9].

Fludioxonil (4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile) belonging to the family of Phenylpyrroles, its inhibitory effect appears in the inhibition of a protein kinase involved in the regulation of cellular metabolism thereby causing an alteration in protein phosphorylation process [10]. It was found that Fludioxonil has a long persistence in aquatic environment since residues of this fungicide were present up to 5 years from its use in grape vine-yards region [11], therefore, fludioxonil may also have additional effects on non-target plant species.

Usage of non-targeted organisms in environmental toxicology is needed to understand the wide range of toxic effects caused by the pesticides on different organisms. The common model systems used in the research of the environmental toxicity include bacteria, algae, daphnia, and zebrafish [3,12,13]. However, a variety of other organisms in the aquatic environment are important to maintain the balance of ecological systems. They have been introduced successfully in toxicological investigations. Protozoan cells are often used as bioindicators of chemical pollution, especially in aqueous environment. They are composed of a single cell, but unlike single-celled algae, they do not possess a protective cell wall. Thus, chemicals enter protozoan cells more easily than bacterial and algal cells and then interact directly with the cellular structures and organelles. Among protozoans, *Paramecium spp.*, widely distributed in freshwater, are one of the ciliate models, most commonly used for laboratory research. They have been widely used to evaluate the toxic effects of carcinogens, Carbamate pesticides, and pollutants [14,15,16].

In order to study the underlying effects of a fungicide newly introduced for treatment of seeds, the mixture Fludioxonil - Difenoconazole was tested at different concentrations on the ciliate protist, *Paramecium sp.* A growth monitoring and respiratory metabolism were performed. We sought to evaluate a possible oxidative stress. Enzymatic activity of Catalase was investigated to evaluate antioxidative response of *Paramecium sp.* We also measured the levels of reduced glutathione and total proteins.

MATERIALS AND METHODS

Chemicals

The mixture of Fludioxonil – Difenoconazole (Celest Extra, Syngenta) is a flowable concentrate containing 25 g/l (2.40% w/w) Fludioxonil and 25 g/l Difenoconazole (2.40% w/w). Stock solutions were prepared by dissolving the toxicant in distilled water.

Cell Culture

The biological material used in our work is a single-celled microorganism whose culture is obtained by simply soaking hay and lettuce in rainwater. The preparation is left in a warm place (20°C) dark and airy.

Acute toxicity test and growth kinetics

Preliminary toxicity test were conducted in our laboratory in order to determine sublethal concentrations. The experiments were performed according to [4]. Paramecium culture was exposed to a range of concentrations: 0.1, 0.5, 1 and 1.5 mg/l. Each test concentration was replicated 4 times. The culture was done at 28 ± 2° C in test tubes using 10 mL of the culture medium (about 60 cells/ tube). The growth kinetics study was realized by the daily cell counting, after fixation with formalin at under optic microscope using grooved blade. The count was repeated at least three times for each repetition.

Response rate, generation number and generation time

After 96h of exposure, changes in growth rates were examined. The number of living cells was counted and a percentage response was determinate according to the following formula:

$$Response\ percentage = \left[N_C - N_E / N_C \right] \times 100$$

Where N_C is the number control cells, N_E is the number of treated cells.

Based on the data, the number of generations and time required for each generation was calculated by the following formula:

$$n = LogNt - LogN0 / Log2$$

$$g = \frac{Generation\ time}{Number\ of\ generation}$$

Where n is number of generation, Nt is the number of cells at 72h, N0 is the number of cells at T0 and is the generation time.

Respiratory metabolism

The monitoring of the respiratory metabolism of the paramecium is measured through an oxygen electrode type Hansentech according to the method of [17] adapted at the Laboratory of Cellular Toxicology for monitoring the respiratory metabolism of the microorganisms. Oxygen consumption signals are visualized in spot shape on the computer screen.

Protein determination

The proteins content were quantified by colorimetry according to the method of [18], the reading of the absorbance is made at 595nm in spectrophotometer (Jenway 6300). The calibration range is made from a standard protein, Bovine Serum Albumin (BSA).

Catalase activity

Measuring the Catalase activity (CAT) is performed according to the method of [19] based on the hydrolysis of H_2O_2 to H_2O and O_2. For the assessment of enzymatic activity of Catalase, the samples were homogenized in phosphate buffer 1 mL (0.1M, pH 7.5) using an ultrasonic crusher (SONICS, Vibra cell). The homogenate thus obtained was centrifuged at 15 000 rpm/min for 10 minutes and the supernatant recovered serves as enzyme source. The reaction is initiated by the addition of hydrogen peroxide (500 mM, 30V). Reading is done against a blank prepared with hydrogen peroxide and phosphate buffer. The decrease in absorbance is measured every 15 seconds for one minute at 240 nm in a spectrophotometer (Jenway, 6300). Catalase activity is expressed in µmol H_2O_2 per minute per mg of protein.

Glutathione rate determination

The rate of glutathione (GSH) is quantified according to [20], whose principle is based on the colorimetric measurement of the 2-nitro-5 mercapturic acid at 412 nm. The samples are homogenized in 1 ml EDTA (0.02M, pH9.6). 0.2 mL of SSA is added to 0.8 mL of the homogenate prepared beforehand. The mixture was centrifuged at 1000 rpm / min for 5 min. An aliquot of 500 µl of the supernatant is added to 1 mL Tris / EDTA buffer. The amount of glutathione is expressed in µmol / mg protein.

Statistical analysis

All the experiments were run in triplicate. The obtained data were analyzed using Minitab16 student t-test and analysis of variance (ANOVA) to detect significant differences reported for the studied parameters.

RESULTS

Kinetics growth

In the acute toxicity experiment, we observed that the mixture Fludioxonil - Difenoconazole affected significantly the growth of paramecia ($P \leq 0.001$) in a concentration dependent manner (Fig. 1). The variation of paramecium cells number revealed the toxic effect of the fungicides. Indeed, the number of cells exposed to 1.5 mg/l is reduced by about 3600 cells compared to the control after 4 days of treatment.

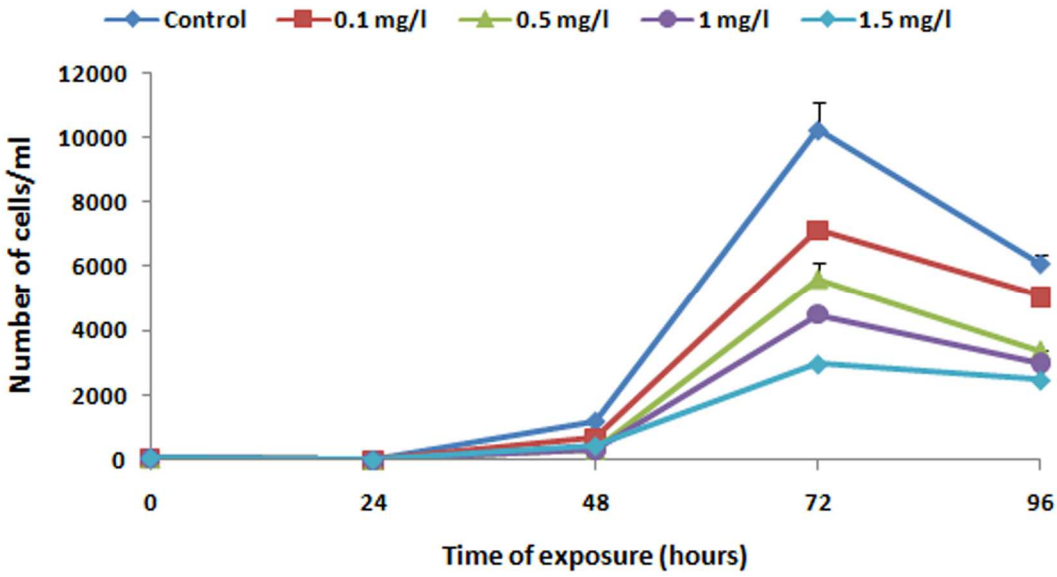

Fig. 1. Effects of increasing concentrations of the mixture Fludioxonil - Difenoconazole on the growth of *Paramecium sp.* at 28°C after 96h of treatment (P ≤ 0.001)

Response percentage

Based on the acute toxicity results, a percentage of inhibition was calculated for the tested fungicide. In the present experiments, when Paramecia were exposed to Fludioxonil - Difenoconazole mixture concentrations from 0.1 to 1.5 mg/l, a very important decrease in the growth rate was noticed after 96h of treatment (Fig. 2). The highest percentage of inhibition was obtained at the concentration of 1.5 mg/l where inhibition reached 70 %.

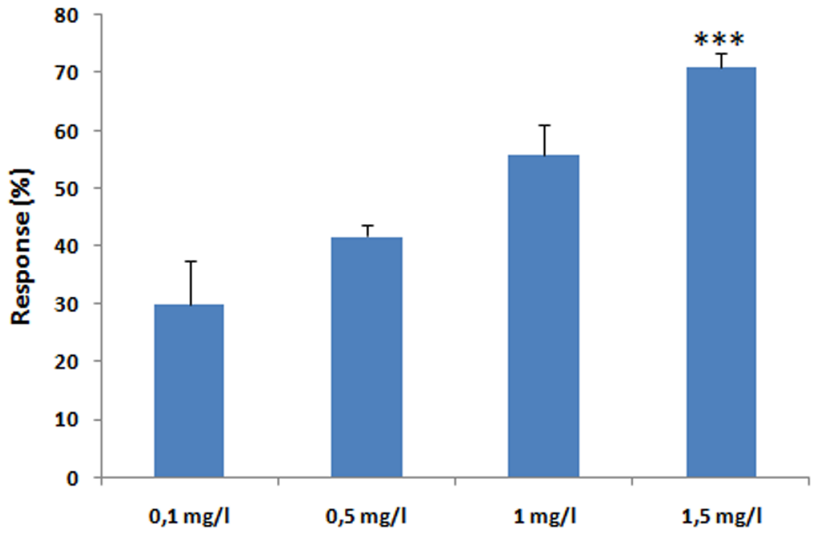

Fig. 2. Effects of increasing concentrations of the mixture Fludioxonil - Difenoconazole on the response percentage. Each value is average ± standard error of three replicates (* P< 0.001)**

In addition, we could calculate the number of generation and the generation time for this fungicide. The results are represented in Table 1. In exposed Paramecia, the results gave clear and gradual decrease in the number of generation with the increasing concentrations.

Table 1. Effect of the mixture Fludioxonil - Difenoconazole on *Paramecium sp.* generation number and generation time

Fludioxonil – Difenoconazole (mg/l)	Number of generations	Generation time (hours)
Control	7.41	12.95
0.1	6.89	13.93
0.5	6.55	14.65
1	6.22	15.43
1.5	5.62	17.08

Protein content

In exposed paramecia, protein content was significantly lower than that of the controls for the highest concentrations tested; the results are represented in Figure 3. The total protein rates of *Paramecium sp*. exposed to the mixture reached 0.36 mg/ml for the concentrations of 1.5 mg/l.

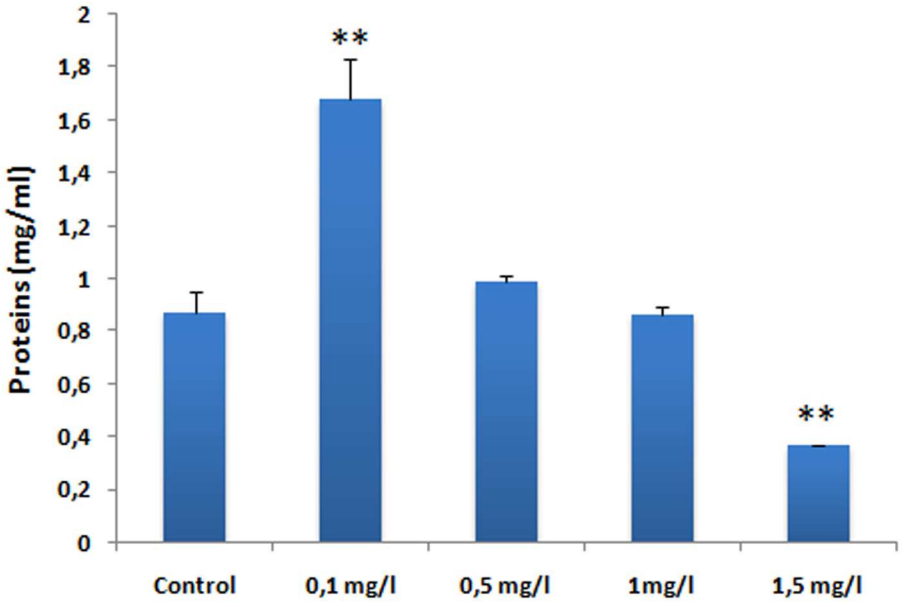

Fig. 3. Effects of Fludioxonil - Difenoconazole on the rate of total proteins in *Paramecium sp.* Each value is average ± standard error of three replicates (P< 0.01)**

Respiratory metabolism

The results reveal disturbances in the level of oxygen consumption as shown in figure 4. The amount of consumed O_2 increased at the highest concentrations (1.5 mg/l) with an oxygen consumption of the order of 100 nmol/ml at the 5th minute.

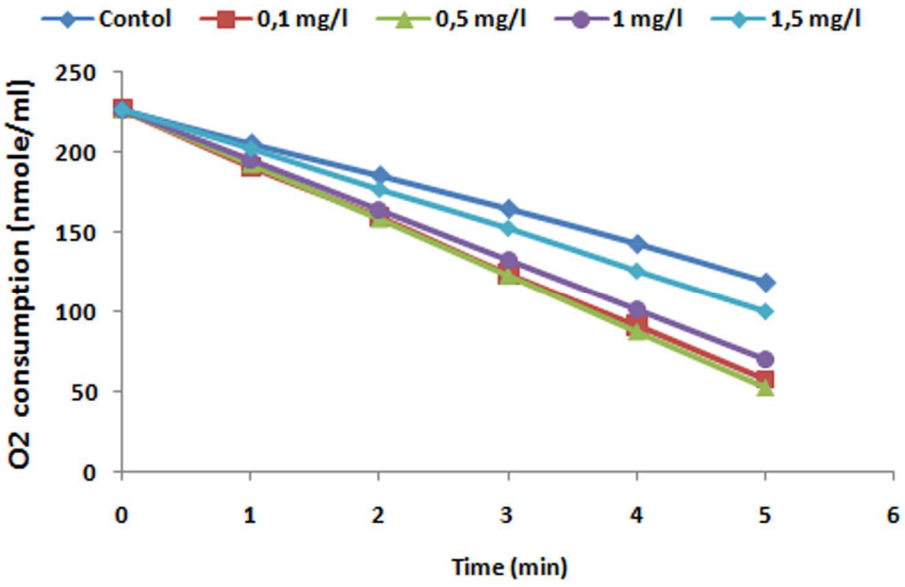

Fig. 4. Evolution of respiratory metabolism of *Paramecium sp.* exposed to increased concentrations of the mixture Fludioxonil – Difenoconazole

Glutathione (GSH) rate

The mixture Fludioxonil - Difenoconazole induces a decrease in the rate of glutathione from the concentration 0.5 mg/l (Fig. 5). Indeed, the rate of glutathione reached 0.124 µmol/mg pro compared to the control (0.182 µmol/mg pro). Therefore, the effect of the Fludioxonil – Difenoconazole on GSH content was concentration dependent ($P \leq 0.05$).

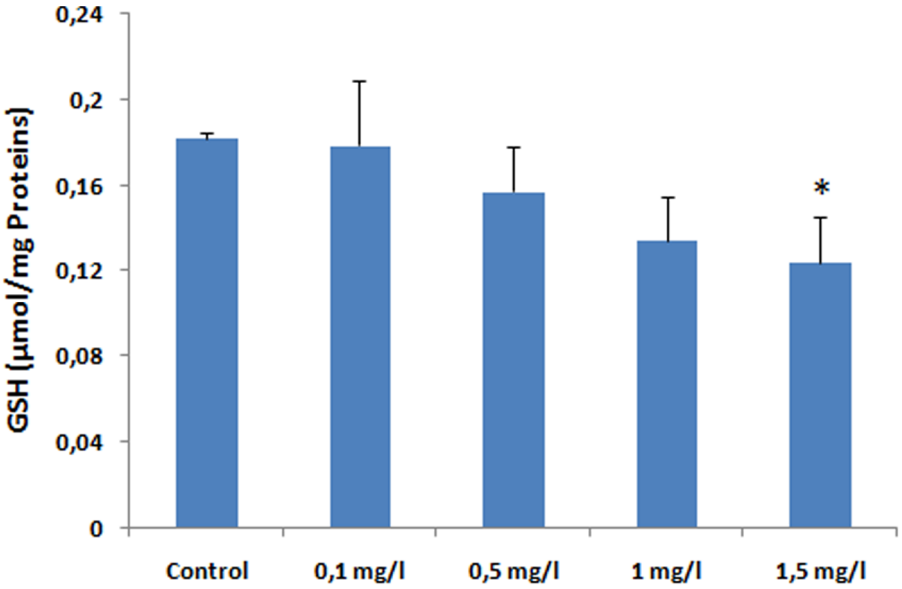

Fig. 5. Effects of the mixture Fludioxonil - Difenoconazole on the rate of GSH in *Paramecium sp.* Each value is average ± standard error of three replicates (* P< 0.05)

Catalase Activity

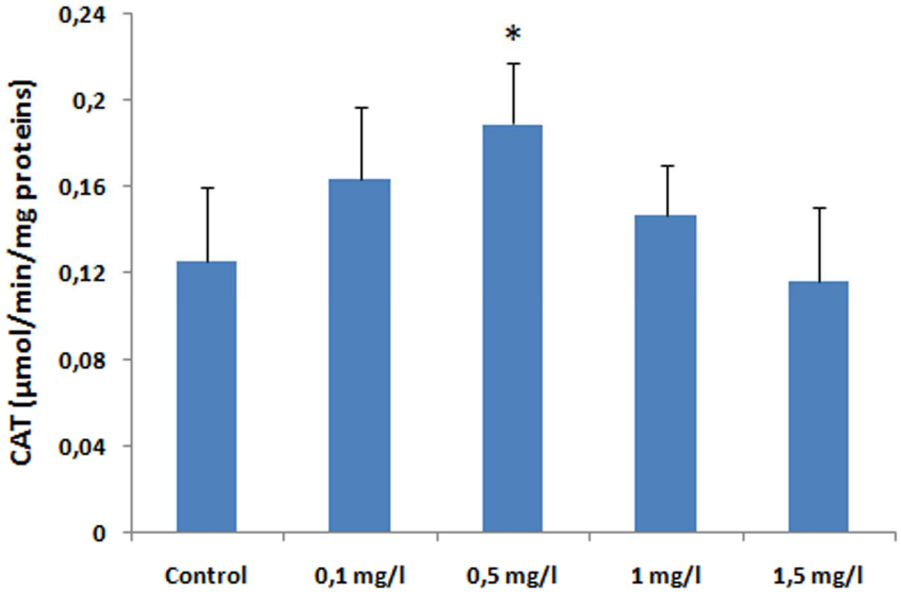

Fig. 6. Effects of the mixture Fludioxonil - Difenoconazole on the CAT activity in *Paramecium sp.* Each value is average ± standard error of three replicates (* P< 0.05)

Figure 6 illustrates the variations of CAT activity in *Paramecium sp.* exposed to increasing concentrations. Evident variations was noticed when cells were exposed to Fludioxonil – Difenoconazole. We note that the CAT activity reaches its maximum (0.182 µmol/min/mg pro) at the concentration 0.5 mg/l. However, the activity of the enzyme was less important at the concentrations 1 and 1.5 mg/l.

DISCUSSION

The intensification of agriculture has resulted in an increased loading of soil, surface and ground water with pesticides, greatly impacting ecosystems by changing the biodiversity and threatening aquatic organisms. Although the toxicity of pesticides has been extensively studied in many living microorganisms, very few results concern the action of this products on Protist. Due to their small size, they generally multiply through short cell cycles, making possible to study the effects of pollutants. On the other hand, it is known that microorganisms represent one of the links of which contaminants could be transmitted through the food chain leading to adverse effects on human health making them an excellent model for evaluating the toxicity of chemical compounds in aquatic life. Free living fresh water protozoan ciliates like Paramecia are the most commonly used ciliated and they

are considered as excellent bioindicators of toxicity stress and chemical pollution. Triazoles are the most important fungicides used in agriculture practice. Excessive amounts of such substances cause irreversible environmental damages and can indeed alter some physiological functions and biochemical parameters in organisms.

We therefore decided to evaluate the toxicity of the mixture Fludioxonil – Difenoconazole on the non-target aquatic organism, *Paramecium sp.*

In the present study, we first interested to investigate the effects of sublethal Fludioxonil – Difenoconazole exposure on population growth of *Paramecium sp.* We noticed that the selected concentrations affected the population growth in a dose-dependent manner inducing a strong inhibition. Furthermore, the obtained results can be compared with the previously published study who reported the effects of several groups of pesticides on the growth of *Paramecium sp.* [21,22]. This can be explained by the fact that the fungicides perturb the cellular divisions and certainly their metabolism systems and consequently cause their mortalities [23].

On the other hand, the response percentages confirm the toxic effects of the increasing concentrations. Indeed, for the higher concentrations for the mixture Fludioxonil – Difenoconazole, the response rate has reached a value of 71%. Moreover, the growth rate tests revealed that increasing concentrations provokes an important decrease in the number of generations by increasing the generation time.

Concerning the respiratory metabolism, our results showed that the cells exposed to Fludioxonil – Difenoconazole were affected; indeed we noticed a decrease in oxygen consumption for low concentrations which exhibited almost the same behavior. Regarding higher concentrations, a sleazy increase was detected. Other investigators reported the different changes in the respiratory activity in paramecia treated with other fungicides like Bifenazate, Phosphoramidate and Cypermethrin [22,24,5]. This can be explained by the fact that once the fungicide penetrates the cells, it will be responsible of triggering cell defense systems resulting in the induction of detoxification enzymes particularly Cytochrome P450 which participates in the metabolization of xenobiotics in rendering them hydrophilic and thus facilitating their elimination. However, the contribution of this monooxygenase could lead to the generation of reactive oxygen intermediates which are known as disruptive of respiratory metabolism.

The decreased dose – dependent of total protein rate was observed during exposure to sublethal fungicide concentrations. This is in agreement with [12] who showed a decrease in daphnids protein content when exposed to Tebuconazole (Triazoles group) and suggested that this decrease is an early defense reaction to the fungicide stress and to overcome this situation, living organisms require a high energy which lead to protein catabolism. Other authors [25,26] have reported that the decrease in protein content might be due to a lipoprotein formation, which will be used to repair damaged cells, tissues and organs.

In this work, we also investigate the effect of the fungicide on stress biomarkers. Low levels of reduced glutathione (GSH) indicate the activation of the detoxification system in cells treated with Fludioxonil – Difenoconazole. This tripeptide thiol is a vital protective antioxidant against oxidative stress, which plays a key role in the capture and sequestration of free radicals but also acts as a substrate for the regeneration of other essential antioxidants [27,28,29]. Several authors have reported a decrease in GSH content in paramecia exposed to pesticides [5, 24].

Concerning the Catalase activity, the exposure of *Paramecium sp.,* shows that the fungicide exerted effects depend on the concentration and the substance tested. Our results shows that low concentrations of Fludioxonil – Difenoconazole (0.1 and 0.5 mg/l) induced a significant increase on Catalase activity compared to the control. The activation was diminished when cells were exposed to 1 and 1.5 mg/l probably due to the deterioration of cellular system functions by the mixture. Our results corroborate those of [3] who reported disruption in the CAT activity of when algal cells of *Scenedesmus obliquus* were exposed to Fludioxonil.

It is known that under normal conditions, antioxidant defenses like Catalase (and other) usually prevent tissue damage caused by Reactive Oxygen Species (ROS). But when ROS overcome the defense systems of the cell, there is an alteration of the redox homeostasis, leading to oxidative stress.

CONCLUSION

Our results highlight the toxic effects of sublethal exposure to Fludioxonil – Difenoconazole on *Paramecium sp.* affecting its cellular growth and respiratory metabolism. Furthermore, the fungicide is responsible of radical species production, resulting in the induction of detoxification system in the used model (*Paramecium sp.*).

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REFERENCES

[1] FAO Agriculture mondiale: horizon, 2015/2030, Rapport abrégé. **2015.**
[2] OECD Environmental outlook for the chemicals industry. Environment Directorate, 2001. Paris.
[3] D. Dewez, L. Geoffroy, G. Vernet, R. Popovic. *Aquat. Toxicol* ., **2005**. 74, 150-159.
[4] Z. Azzouz, H. Berrebbah, M.R. Djebar. *African J. Microbiol. Res.*, **2011**. 5(20), 3243-3250.
[5] R. Amamra, M.R. Djebar, N. Grara, O. Moumeni, H. Otmani, A. Alayat, H. Berrebbah. *Ann. Res. & Rev. Biol.* **2015**. 5(5), 385-399.
[6] N.N. Ragsdale. **2**: 333-363. Inhibitors of lipid synthesis, Antifungal compounds, Siegel, M.R., Sisler, H.D. (Eds.), New York, **1977**, 2, 333-363.
[7] Z. Zhang, D. Wang, C. Zhang, C. Wu, X. Liu. *Chin. J. R. Sci.* **2011**. 25, 339-342.
[8] R.B. Schäfer, V. Pettigrove, G. Rose, G. Allinson, A. Wightwick, P.C. Ohe, J. Shimeta, R. Kühne, B.J. Kefford. *Environ. Sci. Tech.* **2011**. 45, 1665-1672.
[9] EFSA. Conclusion on the Peer Review of the Pesticide Risk Assessment of the Active Substance Difenoconazole. **2011**. Parma, Italy.
[10] C. Pillonel, T. Meyer. *Neurospora crassa*. *Pestic. Sci.* **1997**. 49, 229-236.
[11] NRAAVC. Evaluation of the new active Fludioxonil in the product Maxim 100 FS Fungicide Seed Treatment. **2000**. Common wealth of Australia, Canberra.
[12] E. Sancho, M.J. Villarroel, E. Andreu, M.D. Ferrando. *Chemosphere*. **2009.** 74, 1171–1178.
[13] S. Sunil, G. Rashi, S. Shilpi. *J. Hazard. Mater.* **2015**. 291, 102–110.
[14] S. Epstein, M. Burroughs, M. Small, *Cancer Res.* **1963**. 23, 35-44.
[15] C.E. Edmiston, M. Goheen, G.W. Maloney, W.L. Mills. *Environ. Res*. **1985.** 36, 338-350.
[16] J. Venkateswara Rao, S.K. Arepalli, V.G. Gunda, J. Bharat Kumar. *Pestic. Biochem. Phys.* **2008**. 91, 75-80.
[17] M.R. Djebar and H. Djebar. Revue des sciences et technologie, Synthèse, (Végarol Eds), Pu blication de l'Université de Annaba, **2000,** 103.
[18] M.M.A. Bradford. *Anal. Biochem.* **1976**. 72, 248-254.
[19] F. Regoli and G. Principato. *Aquat. Toxicol*. **1995**. 31, 143-164.
[20] Wechbeker, J.G.Cory. *Cancer let.* **1988**. 40**,** 257-264.
[21] R. Rouabhi, H. Berrebbah, M.R. Djebar. *Afr. J. Biotechnol*. **2006**. 5 (1), 45-48.
[22] I. Sbartai, H. Berrebbah, R. Rouabhi, H. Sbartai, S. Guy, and M.R. Djebar *Am-Euras. J. Toxicol. Sci.* **2009**. 1, 13-18.
[23] D. Mountassif, M. Kabine, R. Manar, N. Bourhim, Z. Zaroual, N. Latruffe, El M.S. Kebbaj. *Ecol. Ind*. **2007**. 7, 882–894.
[24] H. Benbouzid, H. Berrebbah, M.R. Djebar. *Adv. Environ. Biol.* **2015**. 9(3), 281-285.
[25] S. Ribeiro, J.P. Sousa, A.J.A. Nogueira, M.V.M .Soares, M.V.M. *Ecotoxol. Environ. Safe.* **2001.**49, 131-138.
[26] P.S. Bhavan, P. Geraldine.. *Pest Biochem. Physiol.* **2001**. 70, 27-41.
[27] R. Mittler, S. Vanderauwera, M. Gollery, F. Van Breusegem. *Trends Plant Sci*. **2004**. 9, 490-498.
[28] C. Foyer, G. Noctor. *Plant Cell. Environ.* **2005**. 28, 1056-1071.
[29] T. Bashandy, J. Guilleminot, T. Vernoux, D. Caparros-Ruiz, K. Ljung, Y. Meyer, J.P. Reichheld. *Plant Cell.* **2010**. 22, 376-391.