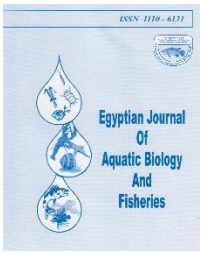
Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 25(1): 257 – 268 (2021)
www.ejabf.journals.ekb.eg



Antioxidant activities and lipid peroxidation in the freshwater bioindicator *Paramecium* sp. exposed to hydrazine carboxylate (Bifenazate)

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ARTICLE INFO

Article History:
Received: Oct. 20, 2020
Accepted: Dec. 30, 2020
Online: Feb. 27, 2021

Keywords:
Aquatic pollution,
Bifenazate,
Paramecia,
biomarkers,
lipid peroxidation.

ABSTRACT

Currently, in toxicology, the use of alternative models permits understanding the mechanisms of toxic actions at different levels of cell organization. In recent years, the fact that pesticides not only act against the target for which they are approved but on the whole ecosystem as well became unquestionable. Thus, the paramecium, as an alternative model, was chosen to test the effect of an acaricide (bifenazate) through measuring certain stress biomarkers. Four concentrations (10, 20, 40, and 80µM) were tested on aliquots of 50 ml of made-in-advance culture paramecia. The determination of enzyme biomarkers: catalase (CAT), ascorbate peroxidase (APX), and glutathione S-transferase (GST), revealed fluctuations in their activities, in conjunction with increasing both times and bifenazate concentration. In parallel, an induction of glutathione (GSH) after 24h of exposure and an increase in malondialdehyde (MDA) levels suggested a possible lipid peroxidation.

INTRODUCTION

Chemicals used in agriculture are mostly the radical cause of water pollution, the damage of which is numerous, diverse, and simaltaneously widespread. However, chemicals' presence leads to degradation and modification of aquatic ecosystems even when the concentration of substances remains below regulatory levels. It is worthmentioning that the overtime persistence of this pollution and the cocktail effects play a key role on both biodiversity disruption and the endocrine systems of aquatic organisms as well **(Capkin *et al.*, 2006; Schrack *et al.*, 2009)**. Nowadays, the integration of chemical data along with biological responses (biomarkers) are strongly recommended to characterize the effects of contaminants on organisms. Biomarkers are tools providing information of the effects of a given stress or a combination of stresses (physical, biological and chemical) on organisms at sub-individual and individual levels **(Lagadic *et al.*, 1997)**. At the biochemical level, studies of biomarkers examined the increased production of reactive oxygen species (ROS) as a systemic pathway of toxicity induced by many chemicals and other compounds that caused an oxidative stress condition, allowing the evaluation of the general damage induced on the metabolism **(Barata *et al*., 2005; Roed and Jones, 2010).**

Moreover, ROSs are derivatives of oxygen in wich certain electrons are found in an energized and highly reactive state. They represent the most important class of reactive species generated in living organisms and the major cause of oxidative stress in these organisms **(Bhagat *et al.*, 2016).** To minimize this damage, organisms developed antioxidant defence mechanism, whereas the failure to detoxify excess (ROS) production may lead to inactivation of enzymes, breakdown of both proteins and (DNA), and may also cause peroxidation of lipids **(Bandyopadhyay *et al*., 2007).** Several authors used protozoa, especially *Paramecia*, as an alternative model to demonstrate the harmful effects of certain pesticides on the locomotor behavior, biochemical metabolism and physiology of these organisms **(Sbartai *et al*., 2009, 2012; Azzouz *et al*., 2011, 2015; Saib *et al.*, 2014; Moumeni *et al*., 2016).** After many years of hard work, *Paramecium* has been determined to serve as a modern model organism that would make specific contributions to the cell, the molecular and the developmental biology **(Van Houten, 2019).** On the other hand, bifenazate, a molecule introduced on the market in December 2005 **(Dekeyser, 2005),** has been the subject of countless studies, though mostly limited to its acaricidal action against spider mites, mainly *Tetranychus urticae*, and its effect on resistance problems encountered in the field **(Tirello *et al*., 2012; Tang *et al*., 2014).** However, bifinazate has been used by other researchers to assess its toxicity in non-target species **(Sbartai *et al*., 2009),** using *Paramecium* sp., demonstrated the toxic effect of bifenazate on growth rate and respiratory metabolism. This in return, suggested another mode of acaricide action, initially known to be neurotoxic **(Dekeyser, 2005),** acting on the postsynaptic GABA receptors of the insect nervous system **(Grosscurt and Avella, 2005).** For their part, **Van Nieuwenhuyse et *al*., (2012)** confirmed this hypothesis by spotlighting the mitochondria as a potential target of bifenazate. In this context, the present study, which was conducted to better characterize the toxicity of bifenazate in a non-target cell model: *Paramecium* sp. through the use of a battery of stress bio-markers, which are considered relevant detection tools. Hence, an excellent approach to assess the effects of environmental contamination would be potential to highlight the capacity of the organism to defend itself. In this way, an excellent explanation of the phenomenon brought into play by the cell coping with the applied chemical stress would be attainable.

MATERIAL AND METHODS

2.1. Biologicol material

The culture of *Paramecium* was performed according to the method of **Beaumont and Cassier (1998).** Hay was infused in a vessel containing one litre of rainwater and placed in a warm (15 to 20 ° C), dark, and well-ventilated area. A few days later, the vessel was rained, and hence, the appearance of the first flagellates were observed. These organisms feed at the expense of the bacterial sailing.

2.2. Chemical material

The molecule used in this study is an acaricide belonging to the family of hydrazine carboxylate with a comercial name; bifenazate. The active molecule is: 1methylethyl 2-(4-methoxy[1,1'-biphenyl]-3-yl) hydrazine carboxylate. This substance has been authorized in the European union since December 1, 2005 **(Dekeyser, 2005)** and has been used against tetranyque (spider mites) since then. *Xenobiotic* was tested on 50 ml aliquots

of the culture, four concentrations were selected: 10, 20, 40 and 80µM (the tests were repeated thrice, and the results were expressed as the average). All the parameters were carried out after: 1, 24, 48 and 96 hours of exposure to the different concentrations of the pesticide in order to highlight the exact moment of the triggering of the antioxidant system.

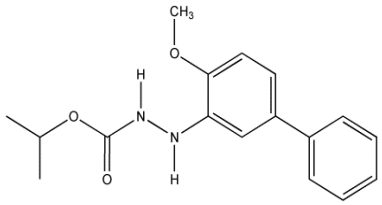

Figure.1. Plane structure of Bifenazate **(Van Leeuwen *et al.*, 2006)**

2.3. Studied parameters

2.3.1. Determination of biomarkers of stress

A series of biomarkers were assayed. Some were involved in the antioxidant system, in the present experiment, glutathione (GSH) was performed according to the method of **Weeckbeker *et al.*, (1988)** based on measuring the optical density of 2-nitro-5-mercapturic acid. The latter chemical results from the reduction of 5,5'-dithio-2-nitrobenzoic acid (Ellman's reagent or DTNB) by the groups (-SH) of glutathione. In addition, catalase (CAT) was carried out according to the method of **Cakmak and Horst (1991)** where the decrease in absorbance was recorded for three minutes for a wavelenght of 240nm. Ascorbate peroxidase (APX) was determined according to the method of **Nakano and Azada (1987)** where the reading was taken at 290nm for one minute. The monitoring of glutathione S-transferase (GST) activity, which is a detoxifying enzyme, was performed according to the method of **Habig *et al.*, (1974).** Optical density was measured at a wavelength of 340nm every minute for 5 minutes. Finally, the determination of malondialdehyde (MDA), a product of lipid peroxidation, was carried out by the technique of **Draper and Hadley (1990),** in order to demonstrate a possible radical attack on the various membranes. Based on the colorimetric measurement of the reaction between thiobarbituric acid (TBA) and MDA, a reddish-brown product whose intensity was measured at a wavelength of 532 nm, yielded.

2.3.2. Statistical study

For all parameters, a statistical analysis was performed. The results obtained were expressed by the average of three replicates (the standard deviation). The averages of the same series were compared using the statistical test, and an analysis of the variance (ANOVA) in accordance with the increasing concentrations of Bifenazate and time was performed, with a significance level (P). Calculations of this test were performed using statistical analysis software MINITAB version 16.0.

RESULTS

3.1. Effect of bifenazate on Glutathione S-transferase activity (GST)

Data obtained after determination of specific activity GST expressed in nmol / min / mg of protein measured in the cells of *paramecium* are grouped in Figure 2.

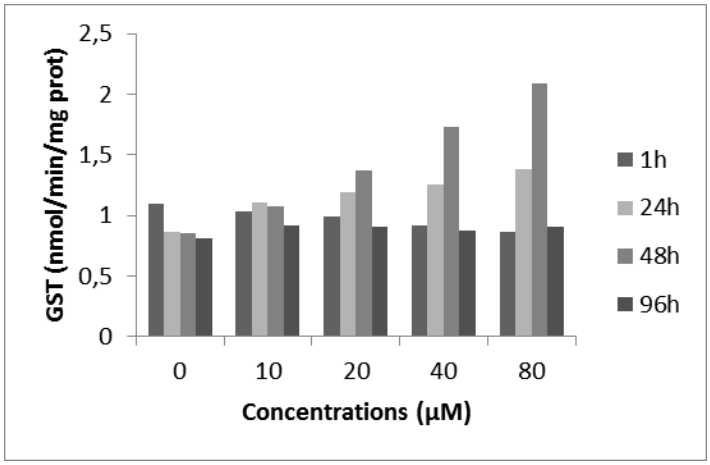

Figure.2. Effect of Bifenazate on GST activity of paramecia

In the figure above, it was noted that the GST increased insignificantly ($p > 0.05$) with the increasing concentrations of bifenazate and time, while peaked at 48 h when it was doubled compared to the control and the concentration of 80 µM. However, a decrease in GST was observed at the end of the treatment for all concentrations.

3.2. Effect of bifenazate on catalase activity (CAT)

The monitoring of the catalase activity in Figure(3) showed that it increased starting from the first hour of treatment compared to the control and the low concentrations (10 and 20 µM). At the same time, a peak was observed at 48 hours for high concentrations (40 and 80 µM) and gradually declined at 96 hours.

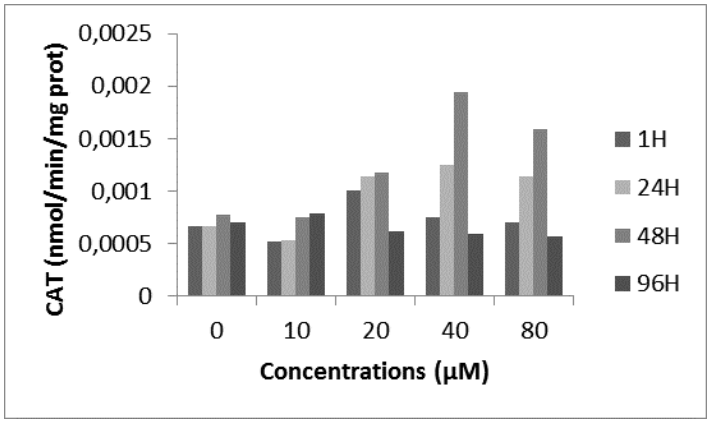

Figure.3. Effect of bifenazate on catalase activity of paramecia

However, a peak was observed at 48 h for concentrations 40 and 80 µM, where the rate was 0,0019 and 0, 0015 µM / min / mg of protein respectively. At the end of treatment (96h), there was a sharp non significant decrease in catalase activity($p > 0.05$) for all concentrations**.**

3.3. Effect of Bifenazate on ascorbate activity (APX)

In Figure (4), it was realized that the APX activity increased non significantly with time and concentration ($p > 0.05$) with a peak at 48 hours; about 4 times the control at the concentration of 80µM. However, the APX activity decreased at the end of treatment for all concentrations which was almost equivalent to that of the control.

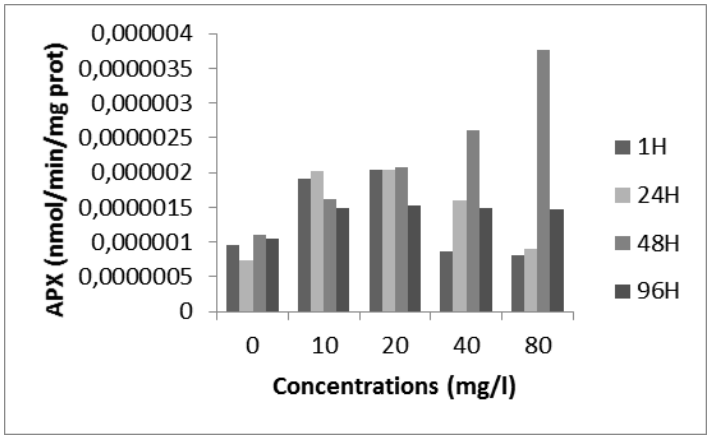

Figure.4. Effect of Bifenazate on APX activity of paramecia

3.4. Effect of bifenazate on the rate of glutathione (GSH)

After an hour of exposure, the rate of GSH in cells treated with 40µM concentration was two times higher than that in the control cells (Figure 5). However, after 24 hours, the induction of GSH was more apparent compared to the control with a peak observed at 40µM; about 3 times the control. Finally, the activity of GSH decreased at 48 hours with the high concentrations (40 and 80µM) marking a very highly significant decline ($p \leq 0.001$). It seems that the systems in question are outdated and the enzymes are completely inhibited.

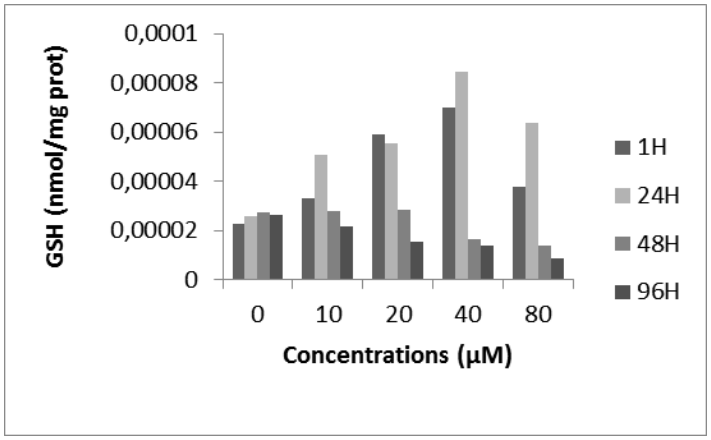

Figure.5. Effect of bifenazate on the rate of (GSH) of paramecia

3.5. Effect of bifenazate on the rate of malonedialdehyde (MDA)

Figure (6)showed that the MDA levels tended to increase in a dose-dependent manner which is very highly significant ($p \leq 0.001$). The level of MDA was 9.66µM / mg Prot to 10µM and reached up to 15.65µM / mg Prot to 80µM of bifenazate at 24 hours.

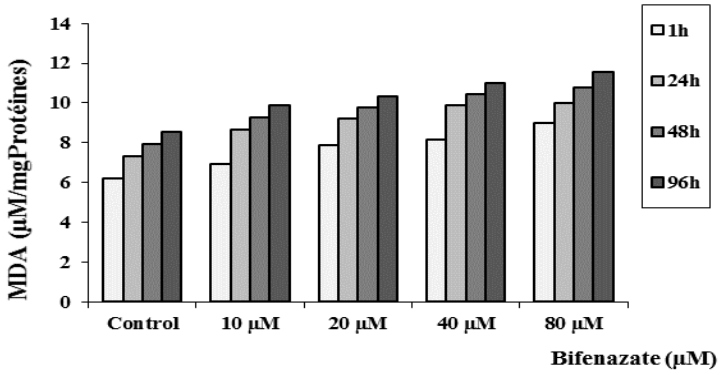

Figure.6. Effect of bifenazate on the rate of (MDA)

DISCUSSION

Whenever microorganisms are subjected to changes in their environment, they got stressed. This stress can be intense, and may cause the death of microorganisms if ever they could not react, particularly when their detoxification enzymes are inhibited. It can also be less intense, allowing them to deploy a battery of responses, through the activation of detoxification mechanisms, in order to fight, survive and in some cases, acclimatize to this new parameter **(Lagadic *et al.*, 1997)**. Among the studied biomarkers, glutathione is the main intracellular antioxidant which acts against oxidative stress and which exists mainly in the forms of reduced glutathione (GSH) and oxidized glutathione (GSSG) whose increase is indicative of a oxidative stress **(Jin *et al.*, 2010; Akerboom and Sies, 2017).** The processes of glutathione synthesis, transport, utilization and metabolism are tightly controlled to maintain intracellular glutathione homeostasis and

the redox balance of the cell **(Huanhuan *et al*., 2019).** On the other hand, GST is an enzyme that catalyzes the nucleophilic attack of GSH at the electrophilic center of many toxic compounds with very different chemical structures **(Bocedi *et al*., 2019)** and represents, due to its presence in the cytosole (5-8% of all soluble proteins), the most important line of defence (phase II) capable of transforming xenobiotics or sweeping away dangerous toxins by binding to them and promoting their extrusion from the cell **(Fabrini *et al*., 2010; Bocedi *et al*., 2016).**

Thus, during the current study, it was noticed that when paramecia were subjected to chemical stress, GSH and GST activity appeared to be sensitive and responded quickly to the presence of the pollutant. In fact, the increase in GST activity indicated both a high concentration of xenobiotics present in the environment, and the induction of oxidative stress following the increasing production of ROS **(Bhagat *et al*., 2016),** and that may explain the depletion of GSH in detoxification processes **(Lee *et al*., 2012)**. Moreover, at low concentrations, detoxification systems were triggered which, among other things, resulted in the induction of antioxidant enzymes such as CAT and APX. The latter play a crucial role in the decomposition of hydrogen peroxide (H_2O_2) and the maintenance of cellular redox homeostasis ,and together with superoxide dismutase (SOD) constitute the first line of defence (**Ribera *et al*., 2001)** against oxidative stress damage, thus, allowing the cell to tolerate and then, adapt to the xenobiotic.

Meanwhile, the decrease in the level of GSH could be attributed to its spontaneous bond with the acaricide; thanks to its thiol group (SH) ,or to its use in the scavenging of free radicals (ROL) produced during the reaction of its oxidation in GSSG **(Kadry *et al*., 2012)** or its use as a cofactor for GST. However, this decrease can alter the antioxidant system and lead to an increased generation of ROS which accelerates mitochondrial damage thus, inducing apoptosis **(Akhtar *et al*., 2017; Huanhuan *et al*., 2019)**. It is known that the depletion of GSH by cystine deprivation or the degradation of GSH leads to oxidative stress which leads to necroptosis and ferroptosis by directly oxidizing lipids **(Chen *et al*., 2017)**. This, in return, justifies the increase in the level of MDA recorded in the present work, thus suggesting a probable lipid peroxidation and a much greater disintegration of cell membranes at high concentrations. The systems in question are overwhelmed and the detoxifying enzymes are completely inhibited due to the susceptibility of the systems to wear the massive influx of free radicals resulting from exposure of paramecia to high concentrations of this xenobiotic **(Huang *et al*., 1993)**. These results are in complete agreement with the studies of several authors who reported fluctuations in GSH levels, increased MDA levels and induction of certain defence enzymes in paramecia exposed to different pesticides (**Sbartai *et al*., 2012; Saib *et al*., 2014; Moumeni *et al*., 2016**) as well as an intensification of GST in *Paramecium tetraurelia* after exposure to pyrethroids (cypermethrin and deltamethrin) **(Amamra *et al*., (2015a, 2015b).**

5. CONCLUSION

Several studies have reported the toxicity of bifenazate as an acaricide action against mites, emphasizing its neurotoxicity as well as the phenomenon of insect resistance to this molecule. However, little work has focused on the effect of this pesticide on non-

target species, particularly, *Paramecium*, using biomarkers as an early means of detecting chemical pollution in aquatic environments. In this context, the present experiment was conducted to demonstrate the severity of the toxicity of bifenazate in a freshwater ciliated protest (*Paramecium* sp.) that is known as a pollution bio-indicator. In conclusion, bifenazate proved to be toxic for the paramecia that led to an over production of free radicals, a condition that was studied through several detoxification systems among which was the induction of free radicals to the low concentrations. However, at high concentrations, these systems were overwhelmed, and the massive influx of ROS led to lipid peroxidation (increased AMDA) and membrane alteration causing cell death. The aforementioned results considering the behavior of this cellular model are very close to that of the higher organisms, in condition that the installation of oxidative stress is concerned.

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