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APPLICATION EFFECTS OF CHLORPYRIFOS-ETHYL AND MALATHION ON THE LICHEN *Xanthoria parietina* AND THE MOSS *Funaria hygrometrica*

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Abstract

Insecticides used in agriculture are important for pest's management. However, due to their known environmental damage, the study of their effect on non-target organisms is crucial. The literature on this topic deals with the effect of insecticide on plants, animals, fungus, algae, and cyanobacteria. So far, no ecotoxicological studies could be found about their effect on lichen or mosses. Our study aimed to evaluate the effect of Chlorpyrifos-ethyl and Malathion on Xanthoria parietina and Funaria hygrometrica respectively a lichen and moss widely used in biomonitoring. Laboratory biotests were conducted applying insecticides by pulverization in controlled conditions. The results have shown no significant effect of the two pesticides in the two species within three days as F. hygrometrica treated with malathion within seven days. After seven days CPE is adversely affecting the lichen and the mosses resulting in a decrease of the photosynthetic pigment, in addition to, an increase in the concentrations of the products of oxidations. From this study, it was observed that that X. parietina and F. hygrometrica may be proposed to be used in biomonitoring of some insecticides risk.

Key words*: insecticides, lichens, mosses, ecotoxicity.*

INTRODUCTION

Agriculture plays an important role in many economies (Toop et al., 2017). The improvement of its productivity is fundamental to achieve food security and sustainable economic development (Godfray and Garnett, 2014).

Insecticide and other kinds of pesticides associated with modern inputs have certainly led to an increase in agricultural outputs. During decades, they have induced an increase in agricultural productivity, but more recently there has been a growing concern about the risks associated with their use (Pretty and Bharucha, 2015). They have been considered as one of the major threats to ecosystems leading to environmental pollution, pest resistance and biodiversity loss (Geiger et al., 2010). Insecticides can have widespread dissemination and may accumulate in different habitats water, air, soil and leaving organisms (Kumari et al., 2008). It is proven that they have been implicated in many population regressions (Davidson et al., 2002).

The Evaluation of their impact on non-target organisms is important to prove their safety. Although many papers have been published on ecotoxicological risk assessment of insecticides, the majority of the studies were about insecticides toxicity on superior plants, animals, fungus, algae and cyanobacteria. So far, no ecotoxicological studies could be found about insecticide effect on the mosses (bryophyte) or lichens. In this context, we have decided to study in the laboratory the insecticide toxicity on lichens and mosses.

The ecotoxicological assessment of insecticide on species such as fungi, algae, and cyanobacteria is complex due to the variety of species and the absence of specific toxicity

tests. Moreover, the existing results in the literature are conflicting; some reported a negative effect (Jha and Mishra, 2005; Sheridan and Simms, 1975). Others did not find any significant effects of insecticide on algae; macrophytes and cyanobacteria (Stratton and Corke, 1982; Wendt-Rasch et al., 2004); even in one study, they noted a positive effect (Leganés et al., 2001).

Among the insecticide largely used in Algeria and all over the world we find Chlorpyrifos-ethyl (CPE: 0.0-Diethyl-0-3,5,6-trichloro-2-pyridyl Phosphorothioate) and Malathion (MAL: 2-[(dimethoxy phosphorothioyl) sulfanyl] butanedioate, Diethyl) which despite its prohibition, it is still used by many farmers. Both malathion and Chlorpyrifos-ethyl are organophosphorus pesticides used in agriculture, residential areas, public recreation areas and pest control programs. They are toxic to humans (Meng et al., 2010). Currently, they are used by the USEPA as case studies to evaluate their risks to endangered or threatened species (Moore et al., 2018).

Their effect on fungi, cyanobacteria, and algae has been well studied. At low dose CPE has a stimulatory effect on the growth of cyanobacteria and negative impact at a high dose (Jha and Mishra, 2005), it has a negative impact on several algae species (Van Donk et al., 1992), it causes a transitory shift on fungi (Elgueta et al., 2017) and it has a stimulatory effect on the fungal population at low concentration and the inhibiting effect at a high one (Bisht et al., 2016). At a low dose, MAL does not have a direct inhibitory effect on algae, but it reduces algal biomass at a high dose (Steven et al., 1999). it affects total carbohydrate and protein contents of algal strains (Ibrahim et al., 2014).

One study, indicate that MAL has a negative impact on fungal growth with a dose-effect correlation (Ma and Ghany Tm, 2016). The current paper aims to present the results of the study that has been done in the laboratory under controlled conditions about the effects of exposure of the lichen *X. parietina* and the moss *Funaria hygrometrica* to the insecticide CPE and MAL.

MATERIALS AND METHODS

Biological materiel

X. parietina, a foliose epiphytic lichen widely distributed in urban and rural areas (Money, 2016) and *F. hygrometrica* known as bonfire moss were chosen for this study because they have been used for a long time in biomonitoring surveys (Ebong, 2015; Kumar and Tewary, 1999; Paoli et al., 2013, 2014; Scerbo et al., 2002; Vannini et al., 2016) as well as for their availability with big amount in the study area. The samples of both species were randomly collected from a remote urban area away from any agricultural pollution in a small forest situated in 35.70˚ N and -0°577 ˚ at 150 m above sea level in Oran city, western Algeria, which has Mediterranean climate. The lichens were taken from pine trees (*Pinus halepensis*). The mosses were taken from the soil of the same sites. The samples were transferred in paper bags to the laboratory at room temperature. They have been carefully cleaned, the thalli were separated from the twigs and the soil to avoid any contaminations of particles of the wood or soil. The experiment has been done after the sampling day to avoid any stress.

Agriculture contaminant

The insecticides CPE and MAL applied in the experiment are branded respectively PYRICAL 480® EC (formulated by ACI SPA) and Sif® Malathion 50 (formulated by cheminova). They were obtained from the market.

Treatment

The exposure system was inspired from the work of (Carrera and Carreras, 2011). The samples were placed in a glass box (15 g of plant material per box) under temperature (20°C at day and 10°C at night), light intensity (10 µmol photons m^{-2} s^{-1} at day). The tests have been conducted by using the pulverization of two different concentrations of the used insecticides in addition to the control which consist only water. Dilutions were made to match the concentration of the formula used in the field (1.5 l/ha for the CPE and 2 l/ha for MAL), considering the surface

of the exposure box. The second concentration is the double of the first one (3 l/ha for the CPE and 4 l/ha for MAL), considering the surface of the exposure box. In a control box, the lichens and the moss were sprayed only with water (control of possible effects produced only by an increase in the humidity of the chamber). The physiological effects were measured after 3 and 7 days. The treated samples were set in a factorial design with five samples per treatment.

Biomarkers response

Photosynthetic pigment

Chlorophyll and carotenoid extraction were done using the Bajpai et al. (2012) method which consists of the grounding of 1 g of the lichen sample in 10 ml acetone 80% and 25 mg of $CaCO_3$ then we have processed the centrifugation. The supernatant was analyzed by spectrometry Optima SP-300. The Chlorophyll content was determined using the formula of Arnon (1949) and carotenoid using the formula of Perron and Juneau (2011).

Proline

Proline dosage was done using the technique of Dreier and Goring (1974) which consists of isolating the proline from 1 g the fresh matter with methanol, then containing it in the ninhydrin reagent in an acid medium and finally extracting it with toluene. The Proline contents were determined by spectroscopy at 528 nm wavelength than by comparing the measured optic density with those of the calibration curve.

Total Protein

The total protein concentration was determined by a spectrophotometric technique using the Bradford method Bradford (1976). We used bovine serum albumin as a standard. The samples were rinsed in deionized water and homogenized in a mortar with sand and 2 ml of 50 mM phosphate buffer solution (pH = 6.8) then centrifuged at 12,000 rpm at 4°C for 20 minutes; 1.5 ml of Bradford solution was added to the100 ml supernatant. The resulting mixture was shaken and left to react for 10 minutes. The Protein contents were deducted by comparing the measured optic density with those of the calibration curve.

Soluble sugar

The soluble sugar concentration was determined using the methods of Dubois et al. (1956). To extract the sugar, 100 mg of fresh material was placed in test tubes with 3 ml of ethanol 80%, the tubes were left for 48 hours in darkness at ambient temperature. 2 ml of the resulting solution was put in a tube with 1 ml of phenol 5% and 5 ml of sulfuric acid. The tubes were let for 10 minutes, then agitated and placed for 20 minutes at a temperature of 30°C. The absorbance measurements were made in length of 485 nm.

Statistics

To show the significance of the differences between groups of data, the ANOVA statistical analysis, and posthoc test of Tukey were executed using the xlstat program (version 2018.7) P-value < 0.05 was considered as significant.

RESULTS AND DISCUSSIONS

The data that show the effect of different concentrations of CPE and MAL on the moss and the lichen after 3 and 7 days of exposure are presented in Table 1, Figure 1 and Figure 2. The study revealed that the used insecticides causes a reduction of the photosynthetic performance and an increase in the concentration of protein, proline, and soluble sugar. Among all samples, the adverse effect was more accentuated in the sample treated with a double dose compared to the single dose. However, no statistically significant ($P > 0.05$) effect of the two pesticides was seen in the two species within the period of three days (Table 1). The physiochemical parameters of both species were unaffected by the exposure to an insecticides that the literature describes as being highly phytotoxic to many species; in one study they noticed that the inhibitory effect of the insecticides on the algae was apparent within two days (Megharaj et al.,

1989). We assume that it may not be excluded that the duration of exposure was not enough to allow the pesticide to exert effects on the moss or lichen. Furthermore, we assume also, the fact that *X. parietina* was unaffected possibly due to 1) the particular structure of the lichen thalli, where the algae are surrounded and protected by a fungus (Pisani et al., 2011); 2) The capacity of fungi to degrade insecticide such as MAL (Mostafa et al., 1972) and 3) their negligible effect on some algae species (Tandon et al., 1988). In the case of *F. hygrometrica*, the biomarkers were unaffected maybe du to according to its cell wall and vascular compartmentation that might be considered as tolerant to the exposition to a certain xenobiotic (Basile et al., 2008).

After 7 days in *F. hygrometrica* treated with Malathion it was noticed: a non-significant decrease in the concentration of chlorophyll 'a', chlorophyll 'b', chlorophyll a + b and carotenoid (Figure 1).

Table 1. Concentration of photosynthetic pigments (µg/mg), Soluble protein (mg/g), Proline (µg/g), and total sugar (µg/g) in treated samples of *X. parietina* and *F. hygrometrica*. Data are reported as mean ±standard deviation. Means (n = 5) with the same lower-case letter are not significantly different at $P < 0.05$ according to Tukey's test

Carotenoids			Chlorophyll a			Chlorophyll b			Chlorophyll a+b		
Control	1 dose	2 doses	Control	1 dose	2 doses	Control	1 dose	2 doses	Control	1 dose	2 doses
Effects of CPE concentrations on *X. parietina* After 3 days											
0,65± 0.08a	0.66± 0.09a	0.60± 0.12a	3.13± 0.9a	2.97± 0.55a	2.72± 0.72a	0.90± 0.29a	0.85± 0.18a	0.62± 0.16a	4.15± 0.42a	3.85± 0.42a	3.65± 0.70a
Effects of MAL concentrations on *X. parietina* After 3 days											
0.68± 0.14a	0.69±0. 17a	0.68± 0.14a	3.66± 1.57a	3.67± 1.66a	3.67± 1.47a	1.07±0. 47a	1.06± 0.53a	0.82± 0.32a	5.14± 1.56a	4.49± 2.19a	4.52± 1.70a
Effects of CPE concentrations on *F. hygrometrica* After 3 days											
0.84± 0.21a	0.78±0. 19a	0.73± 0.11a	4.72± 1.001a	4.46± 1.80a	3.98± 0.95a	3.98±1. 22a	3.75± 1.27a	3.80±1. 38a	8.07± 1.32a	8.19± 1.82a	7.50± 1.69a
Effects of MAL concentrations on *F. hygrometrica* After 3 days											
0.83± 0.12a	0.79± 0.1a	0.72± 0.12a	3.81± 0.26a	3.32±0 .40a	3.35± 0.26a	2.82±0. 55a	2.73± 0.48a	2.84±0. 28a	6.43± 0.96a	6.22± 0.52a	5.85± 0.57a

Total soluble protein			Proline			Total sugar		
Control	1 dose	2 doses	Control	1 dose	2 doses	Control	1 dose	2 doses
Effects of CPE concentrations on *X. parietina* After 3 days								
10,87± 0.46a	10.82± 0.43a	10.96± 0.87a	22.434± 3.10a	23.04± 1.85a	25.86± 1.61a	115.49± 4.38a	122.43± 10.27a	130.36± 21.39a
Effects of MAL concentrations on *X. parietina* After 3 days								
10.89± 1.14a	10.92± 0.97a	10.53± 0.54a	23.33± 2.55a	24.03± 3.28a	26.1± 3.60a	110.82± 5.14a	121.25± 18.63a	132.28± 18.44a
Effects of CPE concentrations on *F. hygrometrica* After 3 days								
10.52± 0.41a	11.49± 1.02ab	11.53± 0.39b	38.58± 2.65a	39.30± 2.87a	42.73± 2.99a	122.14± 2.98a	124.60± 4.32a	126.92± 6.76a
Effects of MAL concentrations on *F. hygrometrica* After 3 days								
10.71± 0.5a	10.75± 0.46a	11.36± 0.14a	41.81± 2.98a	41.77± 6.20a	46.00± 3.97a	125.28± 4.77a	126.73± 5.97a	132.20± 6.28a

A non-significant decrease the concentration of carotenoids and a non-significant increase in the concentration of protein, proline and sugar (Figure 2). Thus, it can be assumed that this moss is tolerant to MAL exposition or the used concentrations were not able to provoke any stress in *F. hygrometrica*. treated with CPE it was noticed a significant decrease in the concentration of chlorophyll 'a', chlorophyll 'b', chlorophyll a + b and a non-significant decrease the concentration of carotenoids (Figure 1) and a significant increase in the concentration of protein, proline and sugar. Moreover, after 7 days In *X. parietina* treated with both insecticide it was noticed: a significant decrease in the concentration of chlorophyll 'a', chlorophyll 'b', chlorophyll a+b. A non-significant

decrease the concentration of carotenoids. A non-significant increase in the concentration of protein and a significant increase in proline and sugar (Figure 2). Photosynthetic pigment content is a valuable indicator of the physiological state of lichen and Mosses (Augusto et al., 2013; Garty et al., 2001; Tyler, 1990).

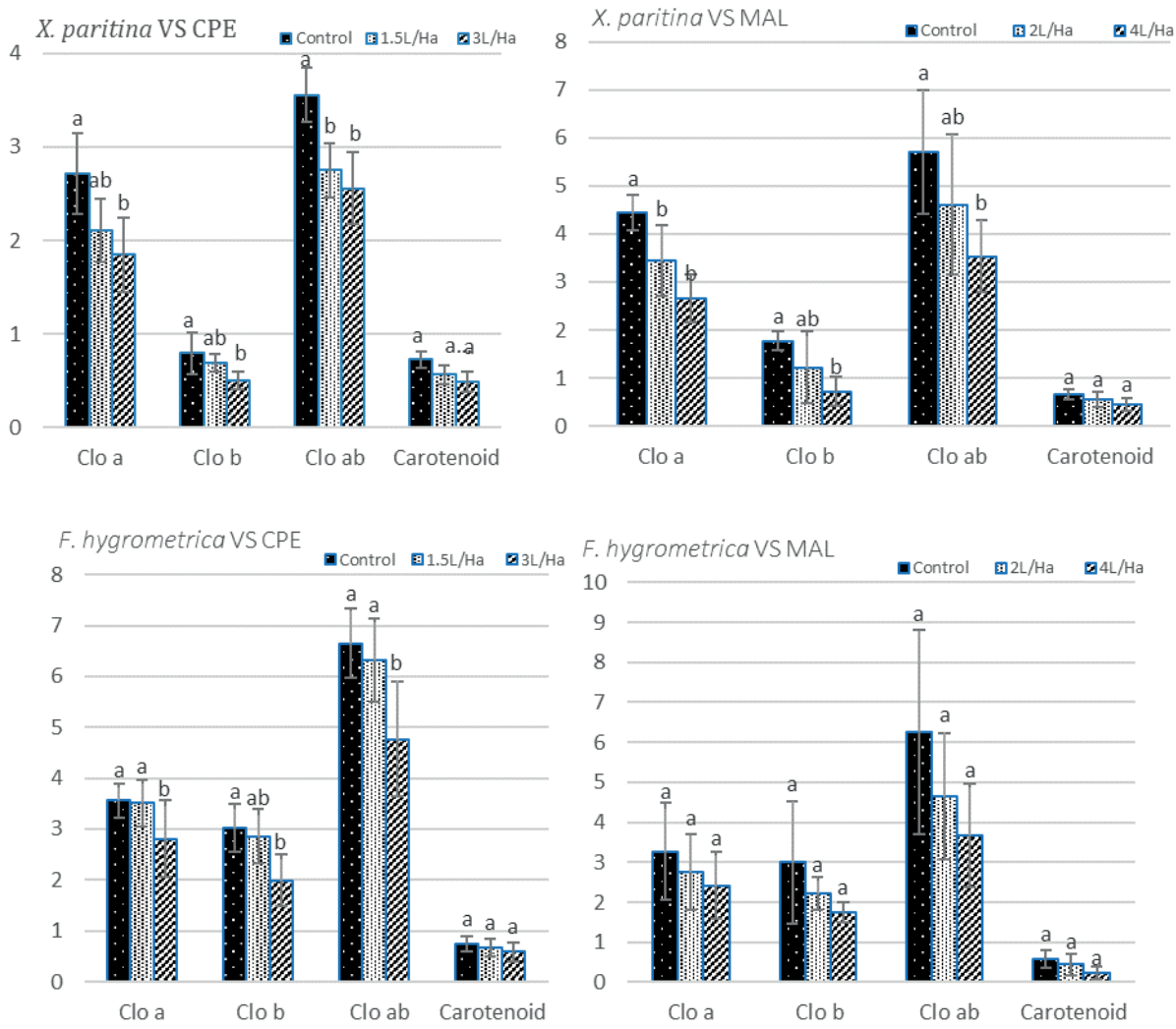

Figure 1. Concentration of photosynthetic pigments (µg/mg), after 7 days, in treated samples of *X. parietina* and *F. hygrometrica*. Vertical bars indicate standard deviation. Means (n = 5) with the same lower-case letter are not significantly different at $P < 0.05$ according to Tukey's test

Several studies about different types of pollutants from agricultural sources found a positive correlation between chlorophyll in lichens and the amounts of pollutants in the air (Gaio-Oliveira et al., 2005; Munzi et al., 2009; Sanchez-Hoyos and Manrique, 1995). Found a positive correlation between chlorophyll of different species of lichens and the amounts of pollutants in the air. However (Boonpragob and Nash III, 1991; Gombert et al., 2003; Schmull et al., 2002) found a negative correlation. (Steven et al., 1999; Ware and Roan, 1970) reported that MAL reduced phytoplankton photosynthesis.

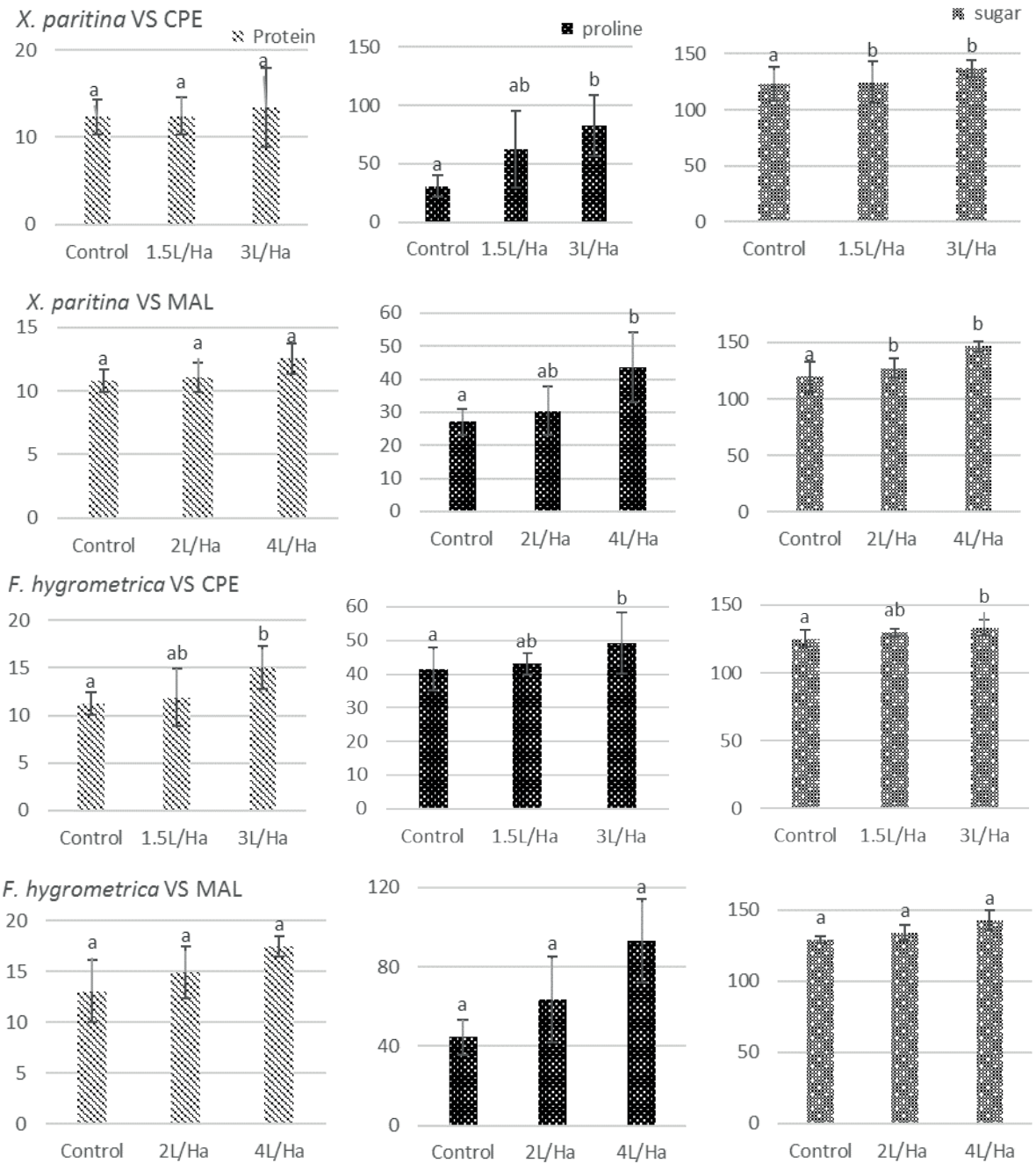

Figure 2. Concentration of Soluble protein (mg/g), Proline (µg/g), and total sugar (µg/g) after 7 days in treated samples of *X. parietina* and *F. hygrometrica*. Data are reported as mean ± standard deviation. Vertical bars indicate standard deviation. Means (n=5) with the same lower-case letter are not significantly different at $P < 0.05$ according to Tukey's test

In the affected samples of our study; photosynthetic activity appears to be sensitive to pesticide concentrations in both lichens and mosses. Indeed, the treated samples have significantly lower levels of chlorophyll a, chlorophyll b, chlorophyll a+b compared to the control sample. These results are in agreement with those found in the literature, which show that the degradation of the pigments depends on the concentrations of the pollutants. Ebenezer and Ki (2014) noticed the reduction of Chla content of marine microalgae caused by toxicity of a pesticide. Carrera and Carreras (2011); González and Pignata (1997) reported a correlation between changes in photosynthetic pigment content of lichens and air pollution. In the case of *X. parietina*, the

measurement of the average levels of Chla, Chlb, Chla+b and carotenoid obtained indicate the existence of a state of stress due to the presence of the insecticide; chlorophyll is sensitive to oxidative processes initiated by stress, such as photochemical oxidation (Chettri et al., 1998). Mascher et al. (2002) concluded that the decline in chlorophyll content and carotenoid is an indication of poisoning. Silberstein and Galun (1988) report that chlorophyll degradation is one of the most obvious indications of damage caused by air pollutants. The production and accumulation of carotenoids in plants are a highly regulated process (Cunningham Jr and Gantt, 1998). According to our results; CPE causes a decrease of this pigment in *X. parietina*. No decrease of later pigment was noticed in all other cases; thus, we suggest further analysis to understand the mechanism of interaction.

Proline is considered as a biomarker of stress; its accumulation is one of the most remarkable manifestations of stress (Nash III and Gries, 1995). The proline content in our affected samples allowed us to detect a stress phenomenon, both mosses and lichens experiencing a metabolic disturbance caused by the treatment. Prolines accumulate in the majority of living organisms that are subject to abiotic stress (Saradhi and Vani, 1993). Our results are in an agreement with the work on the behavior of mosses and lichens vis-a-vis atmospheric pollutants. *X. parietina* and *F. hygrometrica* had a metabolism disrupted by pesticide treatment resulting in the accumulation of proline. According to Carceller (1995), the accumulation of proline is due to the inhibition of the oxidation caused by mitochondrial dysfunction.

To evaluate the effect of the insecticides on the soluble protein level in the two selected biological models, we have used the method of Bradford (1976). According to many studies the organophosphorus pesticides reduce the growth rate and prevent the protein biosynthesis (Jena et al., 2012; Mohapatra and Mohanty, 1992; Mohapatra et al., 1997; Mohapatra et al., 2010). In our study, the insecticides provoked a non-significant increase in soluble protein content except in the case in *F. hygrometrica* treated with CPE where the increase was significant.

The increase in soluble protein content may be explained by the improvement of nitrogen uptake and by biosynthesis of the detoxification enzyme (Chaillou et al., 1986; Chaillou et al., 1991). In consistent with our results, the study of Ahmed (2009); Alonge (2000) who worked on the effects of insecticides on higher plants; they noticed a significant improvement in protein levels in treated species. However, these results are inconsistent with those of Bačkor and Fahselt (2005); Bačkor and Loppi (2009); Pisani et al. (2011) who found a decrease in protein levels following exposure of *X. parietina* to the pollutant and this is also the result of (Paoli et al., 2013) who explain this decrease by the membrane lipid peroxidation, which causes the inactivation of proteins.

The sugars occupy a central position in the physiological stress response through their close relationships with photosynthesis, mitochondrial respiration (Couée et al., 2006). The regulation of sugar levels is complex (F., 2013). The ability of plants to respond to changes in soluble sugar levels can serve as a control mechanism that integrates external environmental conditions (Xiao et al., 2000). The treated samples showed an increase trends which was statistically significant only at the highest dose and after 7 days of incubation. This augmentation may be due to the fact that soluble sugars accumulate during different abiotic stress conditions related to oxidative stress(Couée et al., 2006). According to De Raïssac (1992), the process of accumulation of soluble sugars and/or proline in the leaf tissues of stressed plants is known as an adaptation characteristic. These results are in consistent with ours and with the one of Ramel (2009) that studied the involvement of soluble sugars in the responses to xenobiotic and oxidative stress in Arabidopsis thaliana in interaction with molecules of a pesticide. Our results disagree with what has been reported by Jena et al. (2012) that pesticides prevent the biosynthesis of sugars.

CONCLUSIONS

The results of the present study showed that experimental exposure of the lichen species *X. parietina* and the moss *Funaria hygrometrica* to the insecticides CPE and MAL does not cause a toxicity within three days. The damage appears within seven days. Their response appears to be insecticide specific; they may be relevant as biomarker species for pesticide research. However, further studies are suggested to understand the process of insecticides bioaccumulation and their toxicity on lower plants.

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