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Molybdenum: Antioxidant or Pro-oxidant Role in Lichens?

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Abstract Most of the trace elements toxicity data on ecophysiological status of lichen species were the result of laboratory experiments where thalli were totally immersed in solutions at different concentrations of heavy metals to test their relative stressing effect. This made it possible, when using same/comparable concentrations and time of exposure, to rank their toxicity and, at the same time, the sensitivity of lichens versus the same element. However, such data hardly reproduce the environmental toxicity due to wet depositions where the mode of exposure (drops) and the contact time (seconds – minutes) are much shorter. In addition, many works studied the effect of a restricted group of elements often ignoring unreasonably others. Molybdenum is one of the least studied elements in lichens concerning its effect on ecophysiological status and interaction with other micronutrients. Based on its involvement in several metabolic pathways, and multiplicity in valency values, we carried out exposure of thalli of the lichen *Pseudevernia furfuracea* (L.) Zopf to sprays at different concentrations of molybdenum (0.5, 1, 2, 4 ppm), copper (6, 12 mM) and mixed treatments of the two elements to evaluate the response in cell peroxidation, photopigment amounts, chlorophyll a – chlorophyll b ratio and chlorophyll degradation. Results showed that copper promoted a strong increase in thiobarbituric acid reactive species levels, whereas molybdenum reduced them below the limits of detection of the analytical procedure. No significant variations in photobiont parameters were detected versus control. Interestingly, when the lichens were co-exposed to the combinations of the four molybdenum concentrations with the highest one of copper oxidative stress dropped, on average, 20% compared to only copper exposure, whereas in case of the lower Cu^{2+} concentrations malondialdehyde levels increased 80%, most time showing a statistically significant variation compared to controls. Algal physiology was not affected by metals exposure. Together these data suggest that molybdenum in itself plays, in *Pseudevernia furfuracea*, a potential antioxidant role. It can be overwhelmed or, paradoxically, translated in a pro-oxidant one by the presence of elements like copper, affecting the formation of oxidative damage. This is the first work where such an outcome is showed in lichens.

Keywords Molybdenum, Copper, Lichens, Antioxidant Effect

1. Introduction

The circulation of trace elements (TEs) through the ecosphere depends on many natural sources like weathering of rocks, volcanoes eruptions, forest fires, geothermal systems including undersea smokers [1]. Moreover, microbial communities drive a large part of their fluxes between and within environmental compartments [2, 3]. Nevertheless, mining exploitation, industrial manufacturing, combustion process for heating and power generation, agricultural practices and degradation of man-made products represent nowadays the main contributors to TEs cycling.

Atmosphere is the subsystem most heavily impacted [4], and following dry and wet depositions can seriously pollute terrestrial and aquatic ecosystems. The use of biomonitors such as lichens and mosses is one of the most effective

methods to detect local/widespread contamination process of TEs evaluating relative spatial and temporal variation [5, 6]. In particular, lichens depend for their growth and metabolism on nutrients obtained from wet and dry atmospheric deposition [7].

However, excessive amounts of TEs in the environment can cause several damages to the lichen thallus, such as alterations in cell membrane permeability, degradation of photosynthetic pigments, oxidative stress, alteration of metabolism [8]. Considering the significant risks posed by certain TE concentrations to lichen species, great importance has been attached to the implementation of studies focusing on TEs toxicity. Many lab investigations have been carried out to test their toxicity [9-12], but less is the workload focused onto elucidating the physiological role of most of the TEs. One of the TEs lacking sufficient study in controlled conditions experiments is molybdenum. This is a quite surprising finding due to its multiple valency that makes it a potential electron exchanger as well as its location in active sites of numerous enzymes involved in nitrogen metabolism such as nitrogenase and nitrate reductase, aldehyde oxidase, xanthine dehydrogenase, sulfite oxidase [13]. Very few data exist on the response of ecophysiological status of lichens and mosses due to changing concentrations of this element alone [14], and in association with other TEs [15].

Conversely, studies carried out on higher plants suggest a potential antioxidant role of molybdenum in case of exposure to environmental stress such as very low temperatures and metal toxicity [16, 17]. Among TEs, copper is an element quite well studied about its harmful effects both in field and in laboratory trials showing to be a very strong promoter of biological damage, especially that based on triggering reactive oxygen species (ROS) formation [18, 19]. Interestingly, several anthropogenic activities such as agriculture [20, 21], traffic [22, 23], metallurgy [24, 25] and mining [26, 27] are significant sources of atmospheric emissions of molybdenum and copper, as well contributing to an alteration of their biogeochemical cycles [28, 29].

1.1. Experimental Set Up and Goal of Work

Based on the aforementioned scenario, preliminary laboratory trials were set up to evaluate the response of mycobiont and photobiont parameters in the lichen *Pseudevernia furfuracea* (one of the most used organisms in air quality biomonitoring) following spraying of thalli with: a) 4 different solutions of molybdenum, b) 2 different solutions of copper, c) combinations of the 4 molybdenum x 2 copper solutions for a total of 8 treatments.

Such a combination of treatments was aimed at checking: 1) the toxicity of copper at the tested concentrations, 2) the contribution of molybdenum to the physiological status of thalli, 3) the interactive effect of the two TEs versus mycobiont and photobiont as well as the potential protective role of molybdenum versus the damage promoted by copper.

2. Materials and Methods

Thalli of the lichen *Pseudevernia furfuracea* were collected from an area with a low level of anthropization (Sila National Park, location La Fossiata, Calabria Region, Southern Italy, Figure 1) and transported to the Laboratory of Ecology and Ecotoxicology of Department of Biology, Ecology and Earth Sciences (University of Calabria), where the experimental trials were carried out. Twigs and pieces of bark as well as microfauna inhabiting thalli were carefully removed by plastic tweezers.

Before the start of the experiment, lichens were pre-adapted for 4 days in a climatic chamber (temperature: 18 ℃, humidity: 60%, illumination: 9000 lx with a dark-light cycle of 12 hours) whose parameters set up were the same of the following copper/molybdenum exposure trials (Figure 2).

In total 15 treatments were planned. Each consisted in 1 g of lichen located on plastic nets suspended in a box of 15 cm x 15 cm, replicated 6 times, and sprayed with a volume of 5 mL respectively of: double distilled and demineralised water (Controls), 6 mM and 12 mM $CuSO_4$ (Copper Treatments), and 0.5 ppm, 1 ppm, 2 ppm and 4 ppm Na_2MoO_4 (Molybdenum Treatments).

The complete experimental scheme is showed in Table 1. At the end of the trials all replicates were sprayed with the same amount of water because lichens are highly responsive to hydration, and dissimilar levels may result in a different metabolic activation.

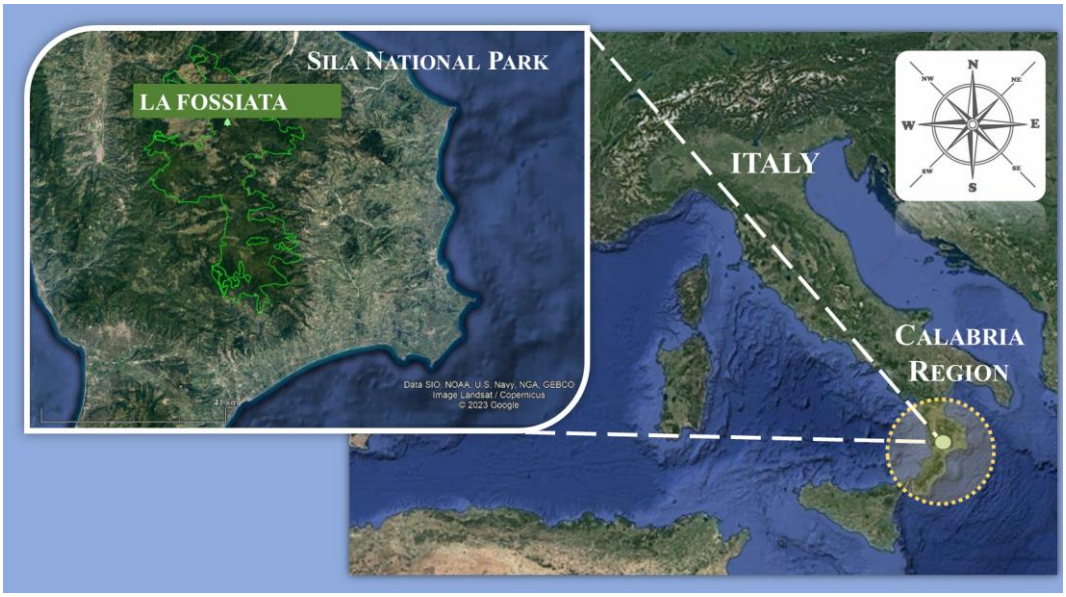

Figure 1. Area of origin of lichen thalli (figures taken from Google Earth and edited by the authors)

Figure 2. Lichens exposure in the climatic chamber

Table 1. Experimental set up of exposure of thalli of the lichen *Pseudevernia furfuracea* at equal amount (5 mL) of 2 solutions of copper sulfate (6 mM and 12 mM), 4 solutions of sodium molybdate (0.5, 1.0, 2.0 and 4.0 ppm) and 8 treatments resulting from combination of the copper (Cu) and molybdenum (Mo) solutions. X = application of treatment, water = application of a volume of double distilled and demineralised water equal to that used to spray $CuSO_4$ and Na_2MoO_4 solutions. Thalli were sprayed only with molybdenum at day "1" to allow lichen metabolize it before copper exposure (i.e. for a better detection of a potential antioxidant role of molybdenum). Total number of replicates across treatments = 90

Treatments (n = 6)			**Time**		
			Day "1" Spraying 5 mL	**Day "4" Spraying 5 mL**	**Day "7" Retrieval of thalli**
Control (water)			X	X	Evaluation of ecophysiological parameters
$CuSO_4$	6 mM		water	X	
	12 mM		water	X	
Na_2MoO_4	0.5 ppm		X	water	
	1 ppm		X	water	
	2 ppm		X	water	
	4 ppm		X	water	
$CuSO_4$ 6 mM	Na_2MoO_4	0.5 ppm	X (only Mo solution)	X (only Cu solution)	
		1 ppm	X (only Mo solution)	X (only Cu solution)	
		2 ppm	X (only Mo solution)	X (only Cu solution)	
		4 ppm	X (only Mo solution)	X (only Cu solution)	
$CuSO_4$ 12 mM	Na_2MoO_4	0.5 ppm	X (only Mo solution)	X (only Cu solution)	
		1 ppm	X (only Mo solution)	X (only Cu solution)	
		2 ppm	X (only Mo solution)	X (only Cu solution)	
		4 ppm	X (only Mo solution)	X (only Cu solution)	

2.1. Ecophysiological Parameters

2.2.1. Thiobarbituric Acid-reactive Substances

Cellular peroxidation levels were evaluated by the detection of malondialdehyde (MDA) [30], a keto-enol tautomer resulting from the rearrangement of peroxides following the increase in temperature and low pH values. 50 mg of lichen thalli were rinsed in distilled water, homogenized by an Ultraturrax (T25, IKA, Germany) in 2.5 mL of 0.1% trichloracetic acid (TCA) solution, and then 1.5 mL of the homogenate was centrifuged at 12,000 g for 20 min. An aliquot of 0.5 mL of the supernatant was transferred into glass tubes, mixed to 1.5 mL of 0.6% of thiobarbituric acid (TBA) in 10% TCA and held at a temperature of 95 ℃ for 30 min. After cooling them in an ice bath, the reaction mixtures were centrifuged again at 12,000 g. Absorption of the supernatant was measured at 532 nm (Spectrophotometer Lambda 40, PerkinElmer) and MDA levels were quantified using the extinction coefficient of the TBA-MDA complex (155 mM cm^{-1}) and expressed as μMol of MDA g^{-1} of lichen dry weight.

2.2.2. Photopigments

The whole extraction procedure was performed under green light to avoid pigment degradation. Lichens were preliminary washed with $CaCO_3$ saturated acetone to remove lichenic substances that may denature pigments. 60 mg of thalli were desiccated for 24 h, added to glass tubes containing 3 mg of polyvinylpolypyrrolidone and homogenized in 3 mL of dimethyl sulfoxide (DMSO) for 1.5 min using an Ultraturrax (T25, IKA, Germany). Mixture was transferred to new glass tubes adding 4 mL of DMSO. Extraction (in the dark) lasted 18 h followed by centrifugation of the suspension at 4000 rpm for 10 min and addition of 3 mL of DMSO to the precipitate. After 6 h the two aliquots of DMSO were mixed, again centrifuged and absorbance was measured at 665, 649 and 480 nm by means of a Lambda 40 Spectrophotometer (PerkinElmer). Pigments concentrations were evaluated using Wellburn equations [31]. Phaeophytization coefficient was calculated as the ratio of absorbance at 415 nm and 435 nm [32].

2.2.3. Statistical Analyses

Comparisons between treatments were made by performing Kruskal-Wallis test with post hoc Nemenyi test using Minitab 19 Statistical Software.

3. Results and Discussion

3.1. Mycobiont Response

Evaluation of the levels of TBArs in thalli of *P. furfuracea* showed that the two TEs, when sprayed separately, promoted an opposite effect, whereas the combined treatments resulted in a complex outcome not easily interpretable (Figures 3-5).

The concentrations of copper tested in this work correspond to those frequently used in organic agriculture for treatments of stone/pome fruits (6 mM) and olive/vegetable crops (12 mM) to reproduce a scenario of environmental contamination [33].

The highest one concentration strongly promoted cellular peroxidation (+2370% vs control), while the lowest one, despite the relevant increase in TBArs levels (+792% vs control) resulted in an effect not statistically different from both treatment "A" of copper and Control (Figure 3, Table 2). This suggests that *P. furfuracea* thalli are relatively resistant to the toxic effects of copper. Indeed, the 12 mM solution corresponds to the average maximum yearly allowable rate of copper (28 kg per ha over a period of 7 years) [34].

Moreover, when the present data are compared with those of copper exposure of other lichen species [35-38], all of those studied (*Parmelia caperata*, *Parmelia perlata*, *Parmelia subrudecta*, *Parmelia sulcata*, *Parmelia tiliacea*, *Ramalina farinacea*, *Usnea amblyoclada*, *Peltigera rufescens*, *Cladina arbuscula*) showed a very different and remarkable sensitivity to copper, with concentrations ranging from 50 μM to about 8 mM i.e. from 2400 to 1.5 times lower than that resulting in a harmful effect in case of *P. furfuracea*.

However, it should be emphasized that, in the aforementioned studies, the exposure of lichens to copper consisted in total immersion (soaking) in different $CuSO_4$ solutions for changing time periods (from 30 min to 24 h), probably resulting in a more uptake of the metal, whereas our trials aimed at reproducing a more realistic environmental exposure simulating a short term event of wet deposition.

We observed a quite unexpected result of molybdenum exposure. All the tested concentrations (8-fold range) gave systematically the same result (n = 6) i.e. levels of MDA below the detection limit of the method, a value registered per each replicate (Figure 4).

Control MDA amounts are essentially low and they are the consequence of the basal production of ROS due to an aerobic metabolism. In fact, mitochondrial and photosynthetic transport chains show an electron leaking that amounts 1-4% of their total fluxes [39]. To quantify the amount of molybdenum complexed and not complexed was out of the aim of the present work, however no concentrations promoted an increase of basal oxidative stress. Quite the opposite, all of them showed a protective effect against ROS induced damage. Studies carried out on higher plants systems clearly suggested an antioxidant role of molybdenum (supplied at the same oxidation state of the present work (6+)), when exposed to aluminum [16] or different N sources [40] by enhancing synthesis of antioxidant enzymes (superoxide dismutase, peroxidase, catalase and ascorbate peroxidase) and, at the same time, reducing MDA levels.

Moreover, toxicant treatments with cadmium [41] showed that protective response following molybdenum supply consisted also in stimulating synthesis of non-enzymatic defense (glutathione). The molecular mechanism underlying molybdenum-induced protection remains unexplored. Recent literature [42] indicates that levels of metals in lichens are correlated to allantoin concentrations, a ureide that is known to exert an antioxidant role [42-44] in higher plants.

Conversely, molybdenum is an essential element for the functioning of enzymes like xanthine dehydrogenase/oxidase involved in the synthesis of ureides [45]. The interpretation of our results is that molybdenum may have stimulated, in *P. furfuracea* thalli, the synthesis of allantoin or other ureides, whichever was the amount of molybdenum supplied, enhancing amounts/activity of antioxidant molecules which reduced ROS damage to very low levels in the absence of external factors promoting peroxidation processes. Figure 5 shows the effect of the 8 treatments resulting from the combination of 2 copper solutions and 4 molybdenum solutions. When 12 mM copper treatment is associated to the whole range of molybdenum concentrations an average reduction of 20% of TBArs levels was detected. Although there is a not statistically significant difference vs only 12 mM copper solution effect, however it may suggest a moderate molybdenum antioxidant capacity.

Copper seems to promote oxidative stress mainly via alike Fenton reaction where O^{2-} anion plays a crucial role in reducing cupric ions to cuprous ions [46, 47] which in turn decompose hydrogen peroxide (H_2O_2) to produce hydroxyl radical. However, interaction between different copper oxidation states and H_2O_2 is a complex mechanism, indeed H_2O_2 can also reduce Cu^{2+} resulting in the formation of superoxide [48]. This increases O^{2-} intracellular pool and its reduction of Cu^{2+} to Cu^{+}. When cupric form is supplied in high quantity (like the case of 12 mM solution) most of superoxide reacts with Cu^{2+} generating $^{\cdot}OH$. This means that, probably, not complexed molybdenum (6+) (net of the fraction used to activate metabolism, included antioxidant systems) hardly interacts with superoxide. All the combinations of 6 mM copper treatment with the 4 molybdenum solutions are shown to strongly promote TBArs levels (an average 83% increase statistically significantly different from both 6 mM copper treatment and control) that are still 32% lower than those associated to 12 mM copper treatment (although not significantly different). Our explanation of this not predictable outcome is that when Cu^{2+} concentration decreased not all

superoxide generated within Fenton reaction cycles (and possibly some of that resulting from basal metabolism) reacts with cupric ion increasing the probability of reacting with not complexed molybdenum (6+). A study carried out to improve efficiency of oxidation of organic compounds in sewage treatment facility [49] showed that molybdenum co-catalytic Fenton system may effectively contribute to the abatement of organic load due to the production of singlet oxygen ($^1O_2$), a very strong oxidative form of O_2 [50] following interaction of molybdenum (6+) with $O_2^{\cdot-}$ anion. The same result was obtained in another work where a Cu^{2+}/Mo system, in presence of oxygen, generated $^1O_2$ to degrade several organic compounds [51]. Reduced molybdenum (4+) can be re-oxidized by cellular oxygen, present in high concentrations in aerobic organisms, so that the element acts as a catalyst, promoting singlet formation also at low concentrations [52]. This means that the paradoxical result showed by combining the lower concentration of copper solution with the 4 molybdenum treatments may be a consequence of the extra-formation of singlet oxygen (due to molybdenum recapture of the superoxide aliquot not reacted with copper) that takes place also at the lowest tested molybdenum concentration (0.5 ppm).

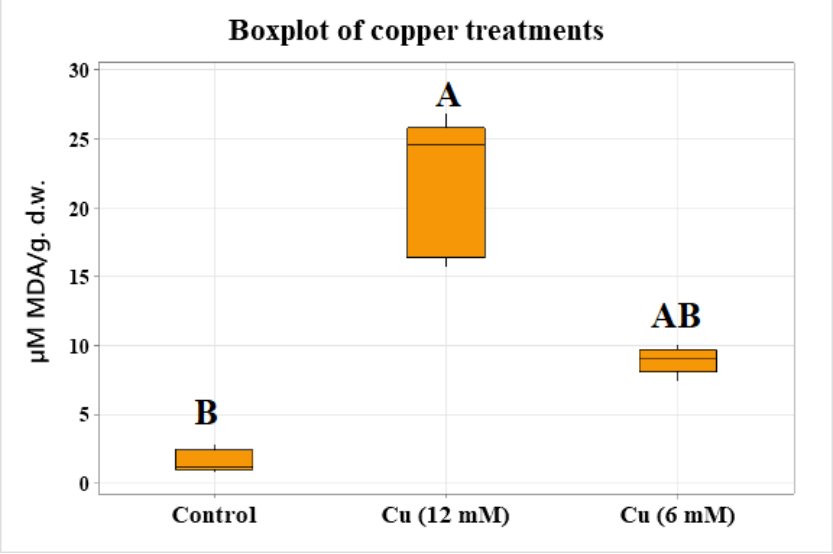

Figure 3. Box-plot of MDA levels (medians) measured in thalli of the lichen *Pseudevernia furfuracea* sprayed with equal amounts (5 mL) of 2 solutions of $CuSO_4$ (12 mM, 6 mM). Different letters mean statistical significant differences in post hoc Nemenyi test

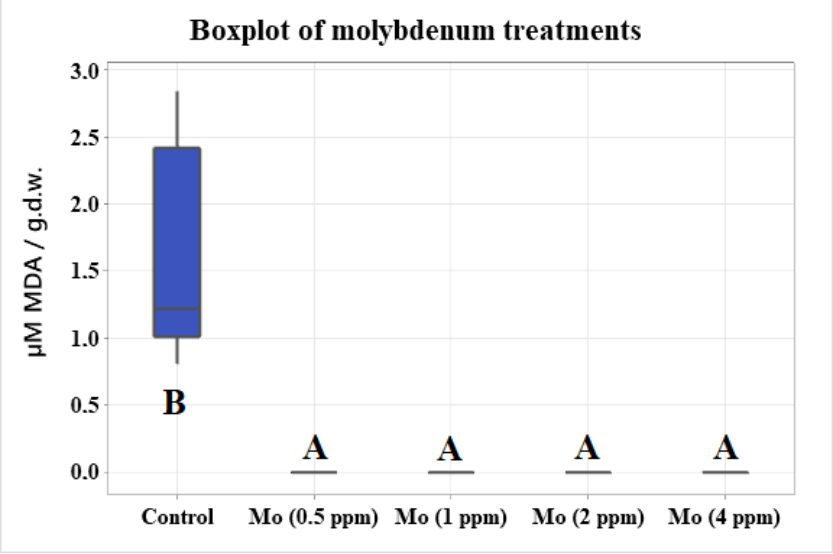

Figure 4. Box plot of MDA levels (medians) measured in thalli of the lichen *Pseudevernia furfuracea* sprayed with equal amounts (5 mL) of 4 solutions of Na_2MoO_4 (0.5 ppm, 1 ppm, 2 ppm and 4 ppm). Different letters mean statistical significant differences in post hoc Nemenyi test

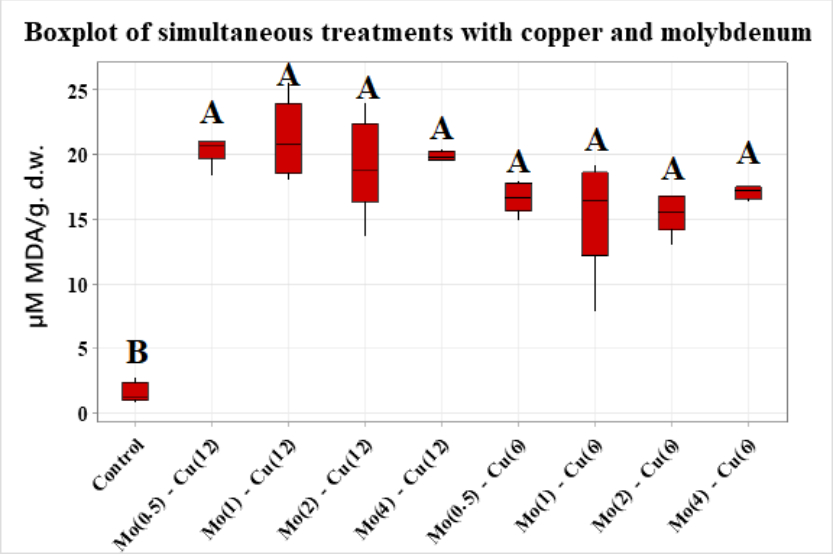

Figure 5. Box plot of MDA levels (medians) measured in thalli of the lichen *Pseudevernia furfuracea* sprayed (5 mL) first with 4 solutions of Na_2MoO_4 (0.5 ppm, 1 ppm, 2 ppm and 4 ppm), and then 2 solutions of $CuSO_4$ (12 mM, 6 mM) for a total of 8 treatments. Different letters mean statistical significant differences in post hoc Nemenyi test

Table 2. Results of Non-Parametric 1 Way ANOVA (Kruskal-Wallis) relative to the comparisons of Control with copper treatments, molybdenum treatments and copper x molybdenum treatments as well as comparisons between treatment levels. Chl a = chlorophyll a, Chl b = chlorophyll b

Comparisons		Ecophysiological Parameters	H	p
Control Cu (6 mM) Cu (12 mM)		TBArs	15.16	0.001
		Chl a	1.27	0.529
		Chl b	0.95	0.623
		Xanthophylls+Carotenoids	1.37	0.504
		A435/A415/g d.w.	1.77	0.414
		Chl a/Chl b	1.30	0.523
Control Mo (0.5 ppm) Mo (1 ppm) Mo (2 ppm) Mo (4 ppm)		TBArs	28.54	<0.00005
		Chl a	1.82	0.769
		Chl b	6.98	0.137
		Xanthophylls+Carotenoids	5.57	0.234
		A435/A415/g d.w.	9.6	0.11
		Chl a/Chl b	10.88	0.06
Control		TBArs	15.16	0.004
		Chl a	6.2	0.27
Cu (12 mM)	Mo (0.5 ppm) Mo (1 ppm) Mo (2 ppm) Mo (4 ppm)	Chl b	6.9	0.24
		Xanthophylls+Carotenoids	7.7	0.20
		A435/A415/g d.w.	7.8	0.20
		Chl a/Chl b	8.56	0.73
Control		TBArs	16.39	0.003
		Chl a	8.34	0.80
Cu (6 mM)	Mo (0.5 ppm) Mo (1 ppm) Mo (2 ppm) Mo (4 ppm)	Chl b	5.28	0.26
		Xanthophylls+Carotenoids	5.57	0.234
		A435/A415/g d.w.	10.1	0.09
		Chl a/Chl b	4.06	0.398

Table 3. Variation of ecophysiological parameters of the photobiont of *P. furfuracea* following exposure to copper (Cu) and molybdenum (Mo) solutions and their combined treatments. Values are expressed as medians, Chl a = Chlorophyll a, Chl b = Chlorophyll b, Xan+Car = Xanthophylls and Carotenoids, Pigments (Chl a, Chl b, Xan+Car) = µg g^{-1} d.w., Q1 = first quartile, Q3 = third quartile

Treatments	Chl a	Chl b	Xan+Car	A435/A415/g d.w.	Chl a/Chl b
Control	1923 Q1 = 1582, Q3 = 2035	538 Q1 = 460, Q3 = 595	791 Q1 = 673, Q3 = 853	240 Q1 = 237, Q3 = 243	3.5 Q1 = 3.36, Q3 = 3.59
Cu 12 mM	1684 Q1 = 1616, Q3 = 1950	486 Q1 = 467, Q3 = 579	710 Q1 = 692, Q3 = 817	238 Q1 = 235, Q3 = 239	3.46 Q1 = 3.39, Q3 = 3.46
Cu 6 mM	1860 Q1 = 1793, Q3 = 2028	537 Q1 = 518, Q3 = 584	780 Q1 = 741, Q3 = 827	238 Q1 = 234, Q3 = 240	3.46 Q1 = 3.39, Q3 = 3.54
Mo 0.5 ppm	1740 Q1 = 1689, Q3 = 1820	480 Q1 = 454, Q3 = 494	763 Q1 = 728, Q3 = 815	242 Q1 = 242, Q3 = 244	3.83 Q1 = 3.61, Q3 = 3.97
Mo 0.5 ppm – Cu 12 mM	2051 Q1 = 1982, Q3 = 2116	592 Q1 = 578, Q3 = 607	843 Q1 = 822, Q3 = 864	234 Q1 = 231, Q3 = 235	3.44 Q1 = 3.42, Q3 = 3.50
Mo 0.5 ppm – Cu 6 mM	1754 Q1 = 1717, Q3 = 1946	483 Q1 = 435, Q3 = 532	742 Q1 = 716, Q3 = 789	237 Q1 = 237, Q3 = 238	3.83 Q1 = 3.61, Q3 = 3.97
Mo 1 ppm	1769 Q1 = 1577, Q3 = 1959	549 Q1 = 498, Q3 = 589	763 Q1 = 728, Q3 = 815	236 Q1 = 234, Q3 = 237	3.3 Q1 = 3.14, Q3 = 3.36
Mo 1 ppm – Cu 12 mM	1923 Q1 = 1742, Q3 = 2061	521 Q1 = 459, Q3 = 556	797 Q1 = 702, Q3 = 834	237 Q1 = 228, Q3 = 238	3.7 Q1 = 3.39, Q3 = 4.09
Mo 1 ppm – Cu 6 mM	1859 Q1 = 1658, Q3 = 2098	497 Q1 = 443, Q3 =544	764 Q1 = 698, Q3 =842	239 Q1 = 231, Q3 = 240	3.73 Q1 = 3.41, Q3 = 4.07
Mo 2 ppm	1800 Q1 = 1604, Q3 = 1894	518 Q1 = 473, Q3 = 547	771 Q1 = 710, Q3 = 813	238 Q1 = 236, Q3 = 239	3.43 Q1 = 3.35, Q3 = 3.56
Mo 2 ppm – Cu 12 mM	1756 Q1 = 1613, Q3 = 2005	477 Q1 = 408, Q3 = 584	730 Q1 = 674, Q3 = 836	235 Q1 = 232, Q3 = 241	3.7 Q1 = 3.36, Q3 = 3.98
Mo 2 ppm – Cu 6 mM	1708 Q1 = 1647, Q3 = 2100	475 Q1 = 415, Q3 = 614	723 Q1 = 683, Q3 = 867	233 Q1 = 229, Q3 = 237	3.68 Q1 = 3.33, Q3 = 3.96
Mo 4 ppm	1707 Q1 = 1632, Q3 = 1820	479 Q1 = 458, Q3 = 512	714 Q1 = 688, Q3 = 753	242 Q1 = 241, Q3 = 242	3.55 Q1 = 3.55, Q3 = 3.56
Mo 4 ppm – Cu 12 mM	1606 Q1 = 1424, Q3 = 1720	444 Q1 = 360, Q3 = 444	652 Q1 = 604, Q3 = 705	230 Q1 = 227, Q3 = 233	3.93 Q1 = 3.86, Q3 = 3.99
Mo 4 ppm – Cu 6 mM	1594 Q1 = 1571, Q3 = 1614	440 Q1 = 430, Q3 = 463	685 Q1 = 673, Q3 = 711	242 Q1 = 240, Q3 = 245	3.52 Q1 = 3.47, Q3 = 3.70

3.2. Photobiont Response

Table 3 illustrates the response of photobiont (lichenized green alga, genus *Trebouxia*) to the exposure of copper and molybdenum solutions. No statistically significant differences were detected between comparisons of 14 treatments vs control with a coefficient of variation ranging from +18% to –12% around central tendency values. Moreover, both the phaeophytization coefficient and the ratio chlorophyll a-chlorophyll b (Chl a/Chl b) show to approach values typical of healthy lichens [53-55] supporting the lack of effect of copper and molybdenum at the tested concentrations, also when supplied in sequence.

When the previous mentioned works related to copper response of lichens are taken into consideration, a damaging effect on photobiont results to be evident. The parameter most often affected is the Chl a-Chl b ratio, showing systematically to be increased by copper exposure, followed by the reduction of Chl a concentration. This result is evident in the case of photobiont like the genus *Trentepohlia* and *Cyanobacteria* but it is contradictory when algal partner is the genus *Trebouxia* [36, 39] that is present in the fruticose lichen (*P. furfuracea*) we used in this experimental trial. A partial explanation of these results may arise from the observation that in lichens showing *Trebouxia* as photobiont and stressed with TEs (copper included) the synthesis of chelating agents (phytochelatins and glutathione) takes place in photobiont (and not in mycobiont) attenuating the harmful effect of metals on the algal cells [56, 57]. Possibly not all *Trebouxia* species show this attitude explaining the differential response of lichens with a *Trebouxioidae* partner. However, we hypothesize that the different way of exposure to TEs may have, at least partially, conditioned our results also in the case of the photobiont, particularly considering that it represents a very small percentage of lichen biomass, located within mycobiont structures that, as consequence, is the partner mostly affected by "external" environmental stress.

4. Conclusions

In the present work thalli of the epiphytic lichen *Pseudevernia furfuracea* were sprayed with solutions of copper and molybdenum separately and in combination. This represents a method of exposure different from most of the past toxicological tests where lichens were immersed (soaked) in metal solutions for several hours or fractions of hours. These tests lacked similarity with true environmental exposures and were used to compare the relative toxicity of metals (if used at comparable concentrations and times).

Our approach may be viewed as the opposite of the setup of these works and, according to our opinion, may better simulate an environmental exposure (as wet depositions) to TEs and, as a consequence, the potential stress response of lichen thalli. Under these experimental conditions copper resulted clearly toxic only at the highest tested concentration (12 mM), while the 6 mM solution showed an "intermediate" damage (oxidative) effect (not different from both control and 12 mM solution). The most interesting result is the strong systemic reduction of MDA levels following the spraying of all 4 molybdenum solutions significantly below the values measured in not treated thalli. This outcome suggests an antioxidant role of molybdenum for *P. furfuracea*. However, being preliminary trials, further tests are necessary to confirm this datum as well as to associate it to an increase in ureides concentrations or other antioxidant molecules. When molybdenum is supplied with copper, the antioxidant role is preserved only with the highest copper exposure, resulting in a relatively weak protection (TBArs levels 20% less than 12 mM solution) against ROS induced damage. Molybdenum exposure with the lower copper concentration solution shows to promote strongly MDA production. It may be due to an oxidative co-catalytic role of molybdenum that causes the formation of singlet oxygen following reaction with superoxide ion mainly generated within Fenton reaction cycles. Overall, these data support an antioxidant activity of molybdenum in *P. furfuracea* that, depending on its oxidation number, may be switched into a damage promoting activity based on the concentration of co-exposed metals affecting ROS formation.

Caution is needed in extrapolating environmental projection from the outcomes of this work. A rough indication is that oxidative stress in lichens may be reduced when total molybdenum exposure ranges from 0.5 to 4 ppm.

However, our activity did not analyze the effect of environmental factors causing free-radicals induced damage different from TEs (i.e. thermal peaks, excess of salts, organic contaminants) so nothing can be said about a potential protective role of molybdenum vs their exposure.

Nevertheless, based on our results, the chemical patterns and interactions between molybdenum and other TEs, involved in like Fenton reactions, seem to be quite complex. These organisms uptake, as much simultaneously as separately, a remarkable variety of TEs, originating from natural or anthropogenic sources, capable of promoting ROS formation such as Fe, Cu, Co, Mn, Ce, Ru, Cr, Ti [58, 59]. As a consequence, a simple interpretation of measured levels of oxidative stress, possibly induced by these, and the protective role of molybdenum could not be always appropriate especially in absence of molybdenum speciation. It is better to provide further laboratory tests where to reduce concentrations combinations and increase the number of TEs co-exposed with molybdenum to improve their environmental predictability.

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