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Original Article

Biomarkers of pollution by glyphosate in the lichens, *Parmotrema tinctorium* and *Usnea barbata*

Biomarcadores da poluição por glifosato nos líquens *Parmotrema tinctorium* e *Usnea barbata*

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Abstract

Glyphosate is a herbicide commonly used in agriculture for weed control. Current agricultural production demands vast amounts of this product, which are applied by ground or aerial spraying. The concomitant aerial currents promote glyphosate drift to vegetated or urban areas. In this context, we hypothesized that the lichens, *Parmotrema tinctorum* and *Usnea barbata*, could be sensitive to the action of glyphosate and therefore be used to bio-indicate the presence of this herbicide in areas affected by drift. Since living organisms respond in different ways to the action of herbicides, our interest was also to indicate biological markers responsive to the action of glyphosate, through concentrations and exposure times of the thallus, besides identifying the most sensitive species. We evaluated the effect of different concentrations (0.0, 4.8, 9.6, and 19.2 mg L^{-1}) and exposure times (24, 48, and 72 hours) to glyphosate on the morphoanatomy, photobiont vitality, photosynthetic efficiency, and oxidative metabolism of the thalli. We found that the lichens, *P. tinctorum* and *U. barbata*, respond to glyphosate stress, with prospects for use in the biomonitoring of pollutant dispersal from plantation areas. When using *P. tinctorum* as a bioindicator, lichen morphoanatomy, photobiont vitality, and photosynthetic pigment concentration were efficient biomarkers for the effect of concentration and exposure time. For *U. barbata*, the lichenic morphoanatomy and the activity of SOD and APX enzymes were essential tools to indicate the herbicide action. *Parmotrema tinctotum*, however, was characterized as more sensitive in bio-indicating the presence of this herbicide to diagnose the air quality in urban areas or vegetation sectors adjacent to agricultural environments.

Keywords: biomonitoring, photobionts, herbicides, mycobionts, Parmeliaceae.

Resumo

O glifosato é um herbicida comumente utilizado na agricultura para o controle de ervas daninhas, contudo, a produção agrícola atual demanda quantidades gigantescas deste produto, que são aplicadas por pulverizações terrestres ou aéreas, que acompanhadas de correntes aéreas, promovem a deriva do glifosato para áreas vegetacionais ou urbanas. Neste contexto, nós levantamos a hipótese de que os líquens *Parmotrema tinctorum* e *Usnea barbata* pudessem ser sensíveis à ação do glifosato e, portanto, bioindicar a presença deste herbicida em áreas afetadas por deriva. Como os organismos vivos respondem de diferentes formas à ação dos herbicidas, nosso interesse foi também de indicar marcadores biológicos responsivos à ação do glifosato, por meio de concentrações e tempos de exposição dos talos, além de identificar a espécie mais sensível. Para isso, nós avaliamos o efeito de diferentes concentrações (0.0, 4.8, 9.6 e 19.2 mg L^{-1}) e tempos de aexposição (24, 48 e 72 horas) ao glifosato, sobre a morfoanatomia, vitalidade do fotobionte, eficiência fotossintética e metabolismo oxidativo dos talos. Nós verificamos que os liquens *P. tinctorum* e *U. barbata* respondem ao estresse por glifosato, com perspectivas para serem utilizados no biomonitoramento da dispersão de poluentes a partir de áreas de plantio. Contudo, para o uso de *P. tinctorum* como bioindicador, a morfoanatomia liquênica, a vitalidade do fotobionte e a concentração de pigmentos fotossintéticos consistiram em biomarcadores eficientes para o efeito de concentração e tempo de exposição. Para *U. barbata*, a morfoanatomia liquênica, bem como a atividade das enzimas SOD e APX constituíram ferramentas importantes para evidenciar a ação do herbicida. A espécie *P. tinctotum*, contudo, foi caracterizada como mais sensível, sendo indicada para bioindicar a presença deste herbicida e para diagnosticar a qualidade do ar em áreas urbanas ou fragmentos vegetacionais imersos em matrizes agrícolas.

Palavras-chave: biomonitoramento, fotobiontes, herbicidas, micobiontes, Parmeliaceae.

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1. Introduction

Glyphosate [N-(phosphonomethyl)glycine] is a post-emergent, systemic, non-selective, broad-spectrum herbicide commonly employed in agriculture for weed control and to promote soil protection by plant residues obtained from natural vegetation or a cover crop grown during intercropping using a no-till practice (Zimmer et al., 2018; Costa et al., 2021). Global use of the glyphosate active ingredient has exceeded 8.6 billion kilograms (Benbrook, 2016), and production without this herbicide today seems a major challenge (Brookes et al., 2017; Beckie et al., 2020).

Currently, the application of glyphosate and other herbicides in agricultural areas occurs by ground or aerial spraying, the latter being carried out by aircraft (Martin et al., 2020). These practices may be accompanied by air currents that promote the drift of glyphosate to vegetation or urban areas near the planting areas. Cordova et al. (2020) demonstrated that a 5% drift of glyphosate reached up to 400 m away from the application area. This herbicide is the only one capable of ensuring high-level inhibition of the enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), which is involved in the biosynthesis of crucial aromatic amino acids for plants. Glyphosate has been shown to be toxic to non-target organisms (Batista et al., 2017; Abraham et al., 2018; Brito Rodrigues et al., 2019; Fernandez et al., 2021; Strandberg et al., 2021; Vieira et al., 2022), such as lichen photobionts, as the shikimate pathway is also present in these algae (Vannini et al., 2016).

Glyphosate drift has been commonly associated with losses or changes in biodiversity in vegetated areas, especially in peripheral areas (Palharini et al., 2020; Silva Borges et al., 2021), as well as severe impacts on human health, including environmental accidents and increased incidence of some diseases. Laboratory evidence has shown that glyphosate can affect human cells even at concentrations below regulatory limits (Benachour et al., 2007, Benachour and Séralini, 2009), inducing cases of cancer (van Straalen and Legler, 2018). Thus, legal proceedings have been conducted in the USA, and Europe threatens to ban the use of this herbicide (Mesnage et al., 2015; The Economist, 2016; Hakim, 2017; Peng et al., 2020), demonstrating the dire need for bioindicators to account for the presence of this herbicide in environments. As in Brazil, agricultural production is concentrated mainly in areas of the Cerrado biome, demanding significant amounts of glyphosate (Ribeiro Bizuti et al., 2020). We hypothesized that lichens commonly found in this biome, such as *Parmotrema tinctorum* (Despr. Ex Nyl.) Hale and *Usnea barbata* (L.) Weber ex FH Wigg, could be sensitive to the action of glyphosate, and therefore bio-indicate the presence of this herbicide in forest and urban areas subject to drift.

We suggest this hypothesis since lichens are sessile and cannot avoid pollutants by migration or other means. Mycobiont and photobiont components have relatively short life cycles and respond rapidly to changing environmental conditions (Koch et al., 2019; Hurtado et al., 2020). In general, we know that glyphosate affects most monocotyledons and dicotyledons by inhibiting protein synthesis and thus, growth (Senseman, 2007), but research results on the effects of this herbicide on lichens are still scarce.

Fungi and algae have been shown to be sensitive to glyphosate individually (Salman et al., 2016; Vázquez et al., 2021) and in combination. Mallik et al. (2002) found that all species of the lichen *Cladonia* investigated in a forested area died within three years after a single application at standard glyphosate operational rates. However, McMullin et al. (2012) demonstrated that lichen species exhibit different glyphosate tolerance classes with high population mortality for *Bryoria furcellata*, *Cladonia uncialis,* and *T. granulosa.* Since we know that these organisms can respond in different ways to the action of herbicides, our interest was also to indicate biological markers responsive to the action of glyphosate through concentrations and exposure times of the thallus of *P. tinctorum* and *U. barbata.* To this end, we evaluated the effect of different glyphosate concentrations and exposure times on the morphoanatomy, photobiont vitality, photosynthetic efficiency, and oxidative metabolism of the thallus. We chose to evaluate classical techniques, routinely implementable in laboratories, or metrics that can be easily obtained using portable equipment (Rascher et al., 2008; Ruiz-Espinoza et al., 2010; Xavier et al., 2021). Furthermore, as the choice of a bioindicator as an environmental tool should take into account biological sensitivity (Ferreira and Olivati, 2014), our goal was also to indicate a potentially sensitive species possible to be used in the biomonitoring of glyphosate dispersion to forest fragments and urban areas adjacent to agricultural environments.

2. Material and methods

2.1. Lichen material and experimental conditions

Two species of lichens widely distributed and commonly found in areas of the Cerrado biome were used: one species of the foliose morphotype [*Parmotrema tinctorium* (Despr. Ex Nyl.) Hale] and the other of the fruit morphotype [*Usnea barbata* (L.) Weber ex F.H. Wigg]. The collection of material was done in an area of permanent preservation, characterized by vegetation formation of Cerrado *sensu stricto*, situated in the region of the green plateau, municipality of Caiapônia, GO, Brazil (17°19'27.5" S and 51°33'25.3" W). The samples were carefully removed from the tree trunk with the aid of a spatula and stored separately in trays covered with moist paper. After collection, the material was taken to the Laboratory of Metabolism and Biodiversity Genetics of the Instituto Federal Goiano, Rio Verde campus, for immediate processing.

In the experiment, the lichens were exposed to an abiotic stress model based on overexposure. For this, the stalks were immersed in different glyphosate (N-(phosphonomethyl) glycine) solutions for 30 min. Glyphosate was provided by using a commercial herbicide, Nortox 480 SL® (active ingredient offered at a concentration of 480 g L^{-1}), at four increasing concentrations of the active ingredient: 0.0 mg L^{-1} (distilled water); 4.8 mg L^{-1}; 9.6 mg L^{-1}, and 19.2 mg L^{-1}). After exposure, the stalks were dried on paper

towels and sampled for further analyses, which occurred 24, 48, and 72 hours after the exposure event.

2.1.1. Evaluation of algal cell viability, tolerance index, and lichen anatomy

The photobiont cells were stained using neutral red dye. A total of 100 cells were counted per sample, and classified as living, dead, or plasmolyzed cells (Zetsche and Meysman, 2012). The counting occurred under an Olympus microscope (BX61, Tokyo, Japan), using magnifications of 40–100×. To express the tolerance of each lichen species to glyphosate as a function of the doses and exposure times evaluated, revealing the lichen species more sensitive to the action of glyphosate, the tolerance index was calculated. For this, the percentage of live cells observed in the thallus submitted to the highest dose of glyphosate (19.2 mg L^{-1}) and the longest exposure time (72 hours) was divided respectively by the percentage of live cells observed in the thallus submitted to the 0.0 mg L^{-1} dose of herbicide and the shortest exposure time (24 hours) (adapted from Souza et al., 2013).

The lichen anatomy was evaluated to identify tissue damage caused by glyphosate exposure. Thus, samples of the stalks were embedded in HistoResin (Leica Microsystem, Monsheim, Germany), went through the stages of fixation (FAA50), dehydration in increasing ethyl series, pre-infiltration, and infiltration, according to the manufacturer's recommendations. Subsequently, the samples were cross-sectioned on a rotary microtome (Model 1508R, Logen Scientific, China), with 5 µm thick sections. The sections were stained with toluidine blue polychromatic stain at 0.05% in a 0.1 M phosphate buffer, pH 6.8 (O'Brien and Mccully, 1981), and permeabilized with Canada balsam. Three slides were mounted for each treatment, and each slide contained ten histological sections from which the integrity of the anatomical layers was evaluated.

2.1.2. Evaluation of photosynthetic pigments

The concentration of photosynthetic pigments (chlorophyll *a*, *b*, total, the ratio between Cl*a*/Cl*b*, and carotenoids) in the thallus was evaluated. In lichens, the high concentration of acidic substances can increase the phaeophytization quotient of chlorophyll. In order to avoid this effect, the thalli were washed in 100% acetone saturated with $CaCO_3$. The chloroplast pigments were extracted in an extraction solution consisting of DMSO and polyvinylpolypyrrolidone (PVPP) at 2.5 mg mL^{-1}. The stalks were covered with 5 mL of the extraction solution; the vials sealed and covered with aluminum foil, and kept at 65 °C in the dark for 40 min. The absorption spectrum was measured in a UV-VIS spectrophotometer (UV-1800, Shimadzu Corp., 115 VAC, Tokyo, Japan) for wavelengths 665, 648, and 480 nm, with calibration against the blank containing only extraction solution. Turbidity was checked at 750 nm, and in cases where the value was greater than 0.01 optical density, the extract was centrifuged (2000 × *g*) for 90 seconds, and the supernatant was re-evaluated. The pigments were quantified based on the work and methodology of Wellburn (1994). The phaeophytization quotient was expressed as the ratio of the absorbances at 435 and 415 nm (and indicated as OD435/OD415) (Ronen and Galun, 1984).

2.2. Chlorophyll a fluorescence parameters

The OJIP transient fluorescence of chlorophyll *a* was determined using a portable fluorometer, FluorPen FP 100 (Photon Systems Instruments; Drasov, Czech Republic). The stalk of all sample units was previously adapted to the dark for 30 min for complete oxidation of the photosynthetic electron transport system. Subsequently, they were subjected to a pulse of 3000 µmol m^{-2} s^{-1} of blue light, measuring the minimum fluorescence (F0) at 50 µs when all Photosystem II (PSII) reaction centers were open and defined as the O step, followed by the J step (at 2 ms), the I step (at 30 ms), and the maximum fluorescence (Fm) when all PSII reaction centers were closed, known as the P step. These values were used for the estimation of various bioenergetic indices of PSII, according to Strasser et al. (2000). We estimated values for the specific light absorption flux per reaction center (ABS/RC); the captured energy flux per reaction center at t = 0 (TR_0 /RC); the electron transport flux per reaction center (ET_0 /RC); the specific energy dissipation flux at the level of the chlorophylls of the antenna complex (DI_0 /RC); the photosynthetic performance index (Pi_{Abs}) that incorporates the processes of the energy cascade from the first uptake events to the reduction of PQ; the maximum quantum yield of primary photochemistry (PHI_{P0}); probability of an exciton moving an electron through the electron transport chain after the Quinone (PHI_0), and the quantum yield of electron transport (PHI_{E0}), after adaptation of the stalks to the dark (30 min).

Images of chlorophyll *a* fluorescence were obtained using an Imaging-PAM modulated fluorometer (Imaging-PAM M Series, Wals) and analyzed using ImagingWin v2.41a software. Initially, initial fluorescence (F0) and maximum fluorescence (Fm) were determined in stalks pre-adapted to the dark for 30 min. From this, it was possible to calculate the potential quantum yield of photosystem II (FSII) (Equation 1):

$$\left(F_V / F_M\right) = \left(Fm - F0\right) / Fm) \quad (1)$$

The variables of the slow phase of fluorescence induction were obtained sequentially: fluorescence in a light-adapted sample before the saturation pulse (F) and Fm in a light-adapted sample (Fm').

The effective quantum yield of photochemical energy conversion in FSII, Φ_{II} = (Fm'-F)/Fm'; and the quantum yields of regulated energy dissipation, Φ_{NPQ} = (F/Fm') - (F/Fm) and unregulated, Φ_{NO} = F/Fm, were calculated. The Φ_{II} was further used to estimate the apparent electron transport rate employing the adapted equation (Kromkamp et al., 1998) (Equation 2),

$$ETR = \Phi II.RFA.Thalli\ ABS\ .\ 0.5 \quad (2)$$

2.2.1. The activity of antioxidant metabolism enzymes

The samples, consisting of 1g of lichen thallus, were collected, conditioned in liquid nitrogen to quantify the

antioxidant system enzyme activity, and stored in an ultra-freezer at -80 °C. The enzymes were extracted by maceration of 200 mg of lichen tissue in liquid nitrogen with 50% PVPP according to the extraction protocol proposed by Biemelt et al. (1998), with an extraction buffer composed of 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, and 10 mM ascorbic acid. Then, the extract was centrifuged at 13000 × *g* for 10 min at 4 °C, and the supernatants were used to evaluate the activity of catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD).

The CAT activity was evaluated according to the methodology proposed by Havir and McHale (1987). For this, an aliquot of the enzyme extract was added to an incubation medium containing 100 mM potassium phosphate (pH 7.0) and 12.5 mM hydrogen peroxide. Enzyme activity was determined based on the consumption of H_2O_2 every 15 s for 3 min at 240 nm in a spectrophotometer. The molar extinction coefficient used was 36 mM^{-1} cm^{-1}. The CAT activity was quantified in µmol H_2O_2 min^{-1} mg^{-1} protein.

The activity of APX was evaluated based on the methodology of Nakano and Asada (1981), in which the oxidation rate of ascorbate at 290 nm was monitored every 15 s for 3 min. An aliquot of the enzymatic extract was added to a medium containing a 100 mM potassium phosphate buffer at pH 7.0, 0.5 mM ascorbic acid, and 0.1 mM H_2O_2. The molar extinction coefficient used was 2.8 mM^{-1} cm^{-1}. APX activity was determined as µmol AsA min^{-1} mg^{-1} protein.

The activity of SOD was determined based on the methodology of Giannopolitis and Ries (1977), in which the enzyme's capacity to inhibit the photoreduction of nitrotetrazolium blue (NBT) was evaluated. For this, an aliquot of the enzyme extract was incubated in a medium containing 50 mM potassium phosphate at pH 7.8, 14 mM methionine, 0.1 µM EDTA, 75 µM NBT, and 2 µM riboflavin. The samples and incubation medium were illuminated with a 20 W fluorescent lamp for 7 min. Readings were performed in a spectrophotometer at 560 nm. The activity of SOD was determined in U mg^{-1} protein, where 1U corresponds to the amount of enzyme necessary to inhibit the photoreduction of NBT by 50%.

2.2.2. Quantification of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and total soluble sugars (AST)

The concentration of H_2O_2 and MDA in the stalks was determined by macerating 200 mg of lichen tissue in liquid nitrogen and PVPP, followed by homogenization in 0.1% (w/v) trichloroacetic acid (TCA) and centrifugation at 10000 × g for 15 min at 4 °C. The concentration of H_2O_2 was obtained by spectrophotometry according to the method of Velikova et al. (2000). The concentration of MDA was determined using the methodology proposed by Buege and Aust (1978).

Total soluble sugars (TSS) was determined using the phenol-sulfuric acid method (DuBois et al., 1956) and spectrophotometry at 490 nm wavelength. The values were expressed as soluble sugar content (% sugars per gram of fresh mass), with D-glucose as the standard (standard curve: y = 0.0143x - 0.0041, R^2 = 0.9908).

2.2.3. Experimental design and statistical analyses

The experiments were conducted in an entirely randomized design, in a double factorial scheme: four glyphosate concentrations (0.0, 4.8, 9.6, and 19.2 mg L^{-1}) and three sampling times (24, 48, and 72 hours after exposure). The lichen species (*P. tinctorium* and *U. barbata*) were analyzed separately, and all analyses were conducted in triplicate. For the analyses of enzymatic activity, H_2O_2, MDA, and AST were considered as triplicates of the triplicates.

The data obtained from each response variable for each lichen were submitted to a two-way ANOVA to verify the effect of the doses and time on the anatomy, photochemistry, physiology, enzymes of oxidative metabolism, and synthesis of H_2O_2, MDA, and AST in the thallus of the lichens evaluated. The data were also subjected to regression analysis, and the effect of the predictor variables was evaluated through the adjustment of the linear models. The model fit was evaluated based on the coefficient of determination, the significance of the regression coefficient and using the *t*-test at the 5% probability level. The tolerance index was used to compare the lichen species used, and the means were analyzed using a Student's *t*-test at 0.05% ($p < 0.05^{**}$) significance level.

Subsequently, all variables were jointly evaluated for doses and exposure times for each lichen. This evaluation aimed to analyze the behavior of each variable in order to define potential biomarkers. These variables were analyzed using correlation environments and combined in principal component analyses (PCA). As the variables had different measurement units, correlation PCAs were performed, constructed using data standardized to have a mean = 0 and standard deviation - 1. The number of components was chosen according to the eigenvalues (>1.0) and the explained variance (above 80%). All statistical tests were performed in R 4.2.1 (R Core Team, 2021).

3. Results

3.1. Algal cell viability and lichen anatomy

We observed a linear decrease in the percentage of live algal cells as the glyphosate concentration increased in the two lichens (Figure 1a). However, the opposite behavior was evident for the percentage of dead cells, which increased linearly with the increase in the herbicide concentration (Figure 1b). Regarding the presence of plasmolyzed cells, the increase in glyphosate concentration increased the percentage of these cells only in *U. barbata* samples (Figure 1c). Concerning the vitality of the photobiont cells, therefore, the species *P. tinctotum*, seems to be more sensitive than *U. barbata* to the effect of dosage, which was evident in the adjustment of a linear model of greater slope for the decrease in the percentage of live cells (β = -4.180) and an increase in the percentage of dead cells (β = 4.128).

We also observed an effect of the exposure time to glyphosate on the percentage of live and dead cells of the photobiont of *P. tinctorum* (Figure 2a, b). In *U. barbata*, however, these percentages were not affected by this explanatory variable, exposing a higher sensitivity

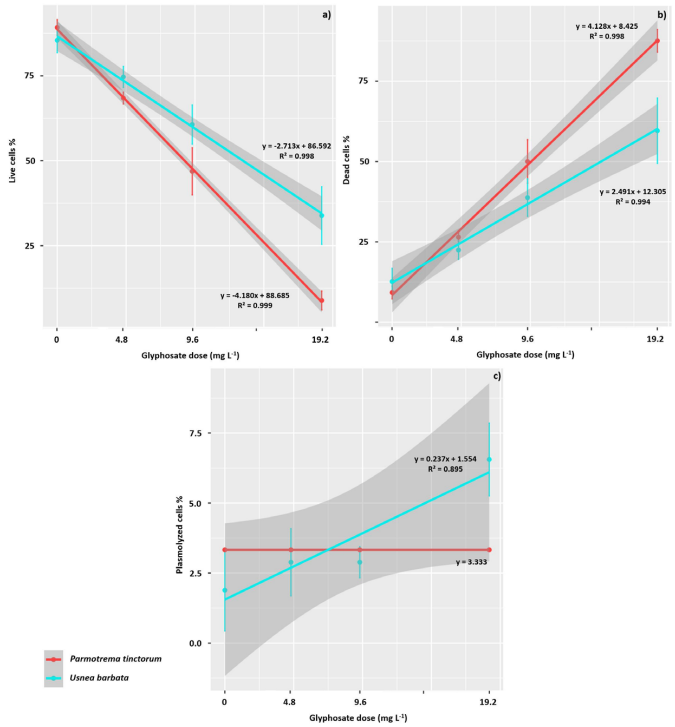

Figure 1. Percentage of live (a), dead (b), and plasmolyzed (c) cells observed for the photobiont of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, submitted to different concentrations of glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}). The straight lines represent the fitted model and the prediction intervals (95%) are in grey.

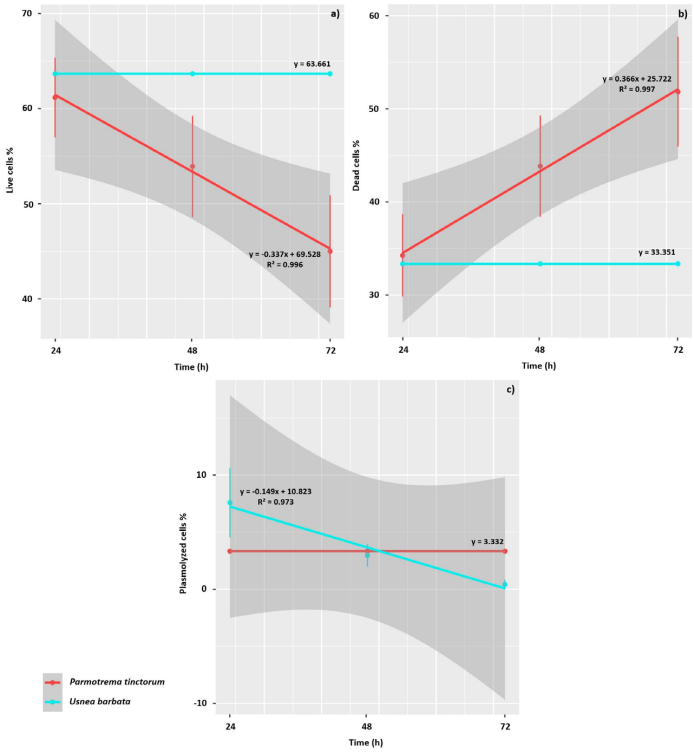

Figure 2. Percentage of live (a), dead (b), and plasmolyzed (c) cells observed for the photobionts of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, submitted to the action of glyphosate herbicide for three exposure times (24, 48, and 72 hours). The straight lines represent the adjusted model and the prediction intervals (95%) are in grey.

of *P. tinctorum algae* not only to the increase of the dose but also to the increase in contact time with the herbicide. The percentage of plasmolyzed cells decreased over the exposure time of *U. barbata* lichen (β = -0.149), suggesting the activation of some resistance system (Figure 2c).

The increase in glyphosate concentration also affected the integrity of the anatomical layers of the thallus of *P. tinctorum* and *U. barbata*. In *P. tinctorum*, there was a reduction in the number of cells composing the algal layer, as well as an increase in the disintegration of the hyphae that make up the upper and lower cortex as the concentration of the herbicide increased (Figure 3a–d). In *U. barbata*, the reduction of cells composing the algal layer also followed the increase in herbicide concentration; the cortex region was greatly affected by glyphosate exposure and disaggregation of hyphae and asci was observed (Figure 3e–h). In the control treatment, the spore-producing structures appear integrated and were deconfigured as they were exposed to increasing concentrations of glyphosate. In *U. barbata* stalks submitted to the highest herbicide concentration, it was impossible to distinguish the presence of asci and ascospores in the cortex region, given the degradation of the anatomical structures.

3.1.1. Photosynthetic pigment concentration and chlorophyll a fluorescence in the photobiont

Exposure to different doses of glyphosate affected the concentration of photosynthetic pigments only in

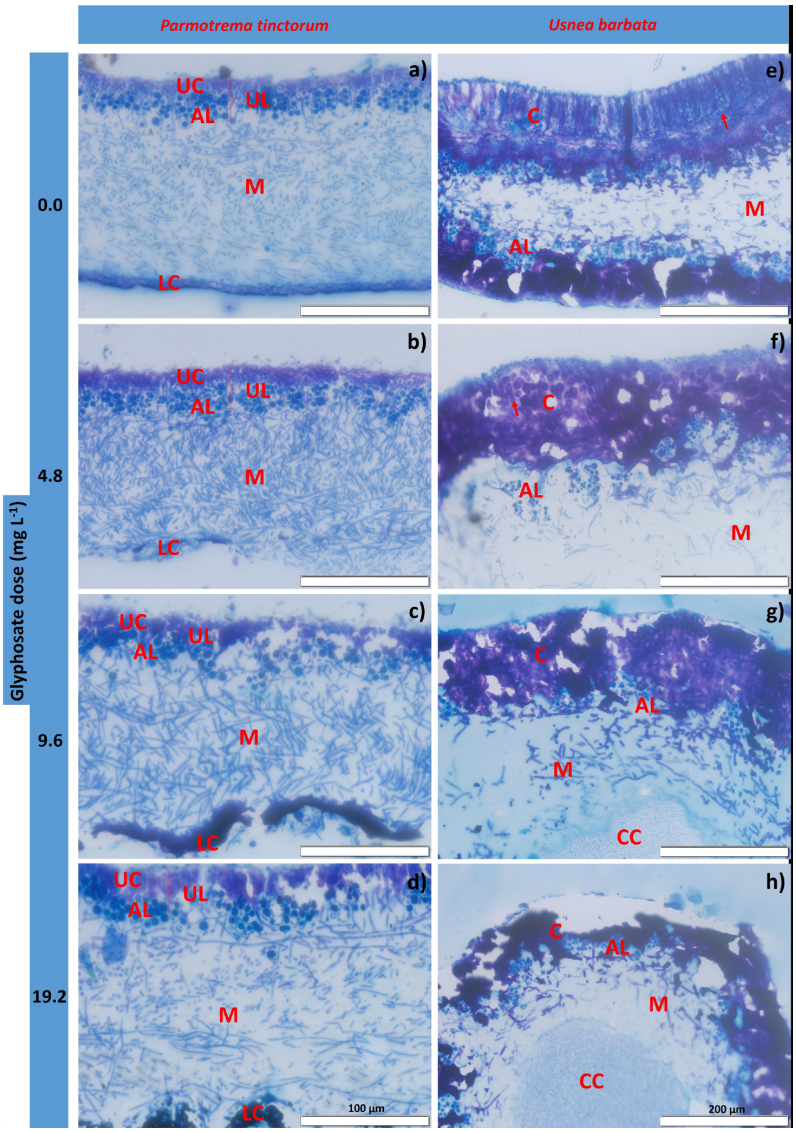

Figure 3. Anatomical sections showing the tissue organization of thalli of the lichens, *Parmotrema tinctorum* (a–d), and *Usnea barbata* (e–h) submitted to different concentrations of the herbicide, glyphosate (0.0, 4.8, 9.6, and 19.2 mg L^{-1}). C = cortex, UC = upper cortex, AL = algal layer, UL = upper layer (upper cortex + photobiont layer), M = medulla, LC = lower cortex, CC = central cylinder. In e and f, arrows indicate the presence of ascospores stored in asci.

the photobionts of *U. barbata*. Thus, there was a linear reduction in the concentration of chlorophyll *a*, total chlorophyll, and carotenoids in the thallus of this lichen as a function of the dose of herbicide used (Figure 4a–c).

A contrary behavior to that observed for the effect of glyphosate dosage was evaluated in the effect of exposure time on photosynthetic pigments. This is because the exposure time affected only the photobiont of *P. tinctorum*, with a linear decrease in the content of chlorophyll *a*, chlorophyll *b*, and total chlorophyll (Figure 5a–c). There was an increase in the chlorophyll *a/b* ratio as a function of time (Figure 5d), which is compatible with a sharper decline in the tissue concentration of chlorophyll *b* (β = -0.428) than of chlorophyll *a* (β = -0.162) as a function of time. The concentration of carotenoids followed the same pattern as chlorophylls, with a decrease in concentration over the time of exposure of *P. tinctorum* stalks to glyphosate herbicide (Figure 5e). The absence of an effect of exposure time on *U. barbata* again indicates the activation of defense mechanisms that become more effective over time.

The increasing doses of glyphosate also affected the algal photochemistry in *P. tinctorum* and *U. barbata* thalli; a linear increase in the parameters ABS/RC, TR_0 /RC, and DI_0 /RC was observed. However, for these variables, the *U. barbata* stalks seemed to have been more affected, which is indicated by the more positive slopes observed in the curves obtained for this lichen in ABS/RC (β = 0.113), TR_0 /RC (β = 0.034), and DI_0 /RC (β = 0.094) (Figure 6a–c).

Other photochemistry parameters were also negatively affected by increasing doses of glyphosate in the lichen algae that were tested, with a linear reduction in Pi_{Abs}, PHI_{E0}, PHI_0, and PHI_{P0} of the stalks (Figure 7a–d). The fitting of linear models of higher slope for the decrease in the values observed in the parameters Pi_{Abs} (β = -0.020), PHI_{E0} (β = -0.006), and PHI_{P0} (β = -0.009) also indicates a higher photochemical sensitivity of *U. barbata* algae to increasing doses of herbicide.

Image fluorescence analysis visually proved the effect of herbicide dosage on the primary photochemistry of *P. tinctorum* and *U. barbata*. The values of F_V/F_M were reduced as a function of increasing glyphosate dose (Figures 8 and 9). ETR and Φ_{II} presented the same response pattern observed for F_V/F_M in both lichens. The tolerance mechanism, Φ_{NPQ}, was profoundly affected, especially in *U. barbata* (Figure 9). A diffusion of the energy intended for Φ_{II} and Φ_{NO} was observed in both lichens.

When we evaluated the effect of the exposure time to glyphosate on the chlorophyll *a* fluorescence of the photobionts of *P. tinctorum* and *U. barbata*, the effect on ABS/RC, TR_0 /RC, ET_0 /RC, and DI_0 /RC was verified only for *P. tinctorum*. For this species, there was a linear reduction over time in the values of ABS/RC and

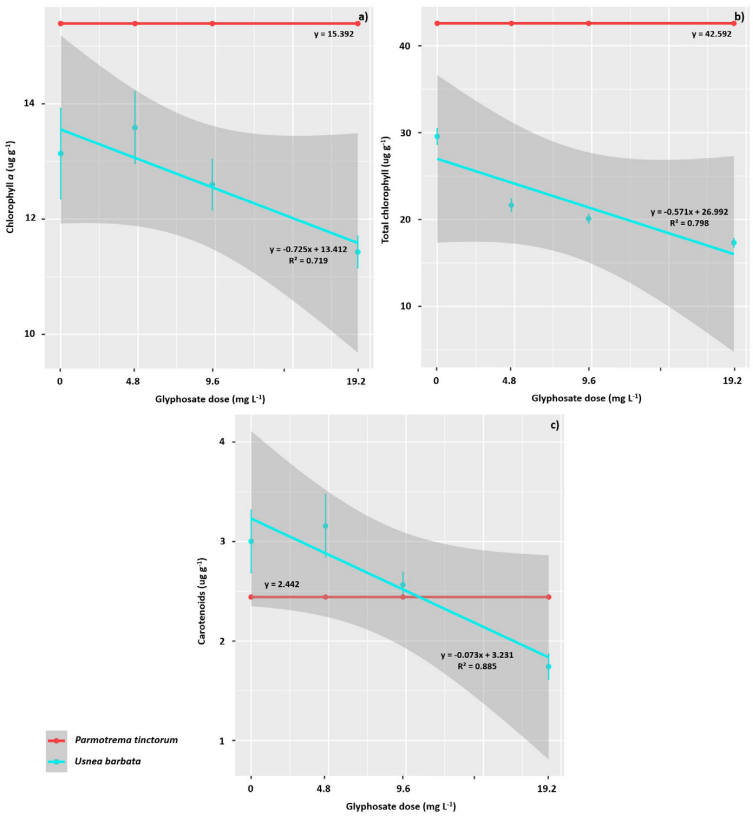

Figure 4. Concentration of photosynthetic pigments chlorophyll *a* (a) and total (b) and carotenoids (c) observed for the photobiont of lichen species, *Parmotrema tinctorum* and *Usnea barbata*, submitted to different concentrations of glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}). The straight lines represent the fitted model and the prediction intervals (95%) are in grey.

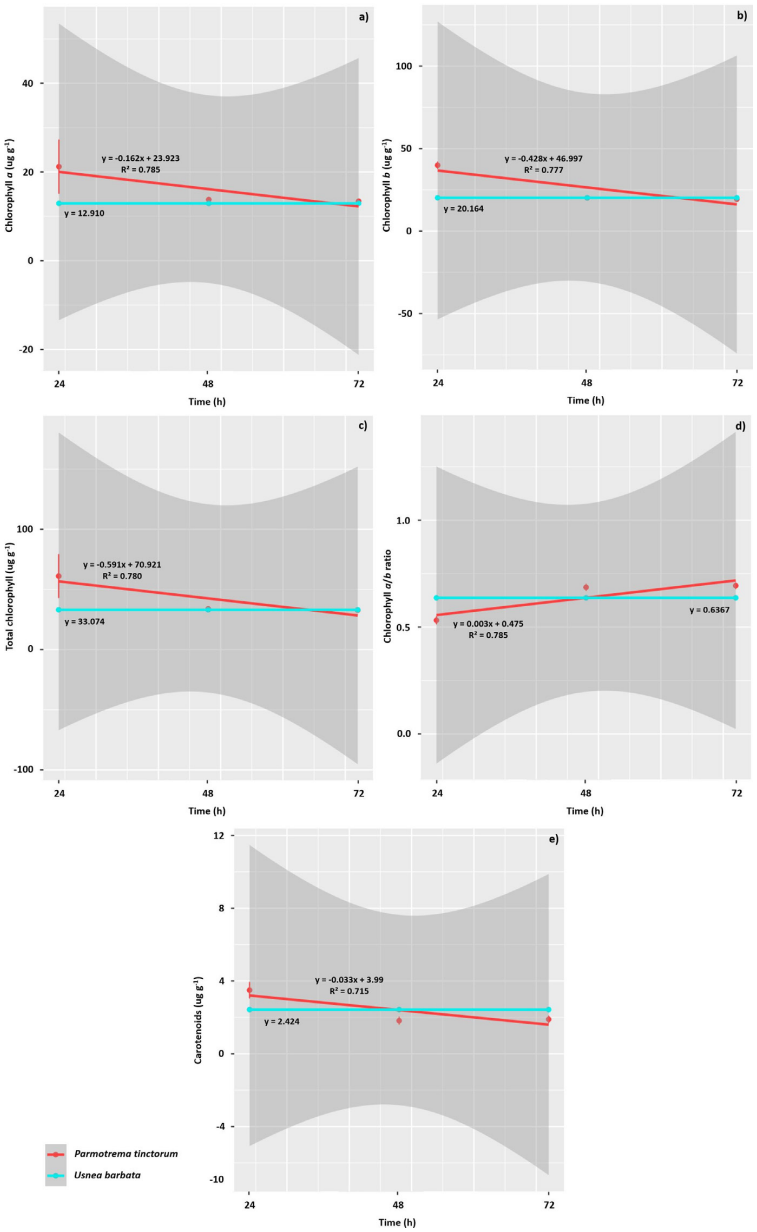

Figure 5. The concentrations of the photosynthetic pigments, chlorophyll *a* (a), chlorophyll *b* (b), and total chlorophyll (c), chlorophyll *a*/*b* ratio (d), and carotenoids (e) were observed for the photobionts of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, submitted to the action of glyphosate herbicide for three exposure times (24, 48, and 72 hours). The straight lines represent the adjusted model and the prediction intervals (95%) are in grey.

TR_0 /RC (Figures 10a and b), an increase in the values of ET_0 /RC (Figure 10c), and a reduction in the levels of DI_0/RC (Figure 10d), indicating a slight photochemical recovery of the thalli over the time of exposure.

Indeed, the fluorescence parameters Pi_{Abs}, PHI_{E0}, and PHI_0 were unaffected by herbicide exposure time in the lichen, *U. barbata*. However, for *P. tinctorum*, the fit of linear models of higher slope to the increase in the values observed in the parameters Pi_{Abs} ($\beta = 0.003$), PHI_{E0} ($\beta = 0.001$), and PHI_0 ($\beta = 0.001$) (Figure 11a–c), indicated a trend of photochemical recovery in this lichen over time.

3.1.2. The activity of the enzymes of antioxidant metabolism: H_2O_2, MDA, and TSS

We observed a linear increase in SOD activity as a function of glyphosate dosage in the thallus of both lichens (Figure 12a). However, *P. tinctorum* seemed to respond more expressively through the action of this enzyme, which was demonstrated by fitting a model with a higher slope ($\beta = 0.021$). For the CAT enzyme, dosage effects were observed only in the thallus of *P. tinctorum*, with a linear increase in the activity of this enzyme as the exposure

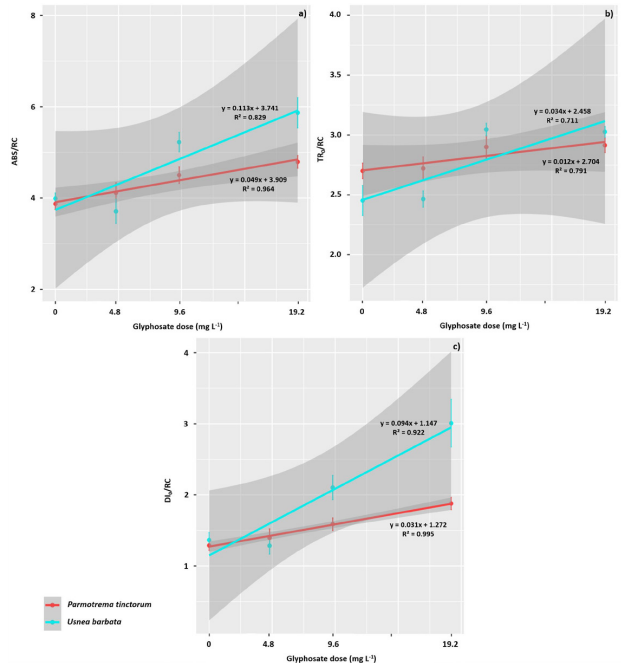

Figure 6. Chlorophyll *a* fluorescence parameters: Specific light absorption flux per reaction center - ABS/RC (a), captured energy flux per reaction center at t = 0 - TR_0/RC (b), and specific energy dissipation flux at the level of the chlorophylls of the antenna complex - DI_0 /RC (c) were observed for the photobiont of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, subjected to different concentrations of the glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}). The straight lines represent the fitted model and the prediction intervals (95%) are in grey.

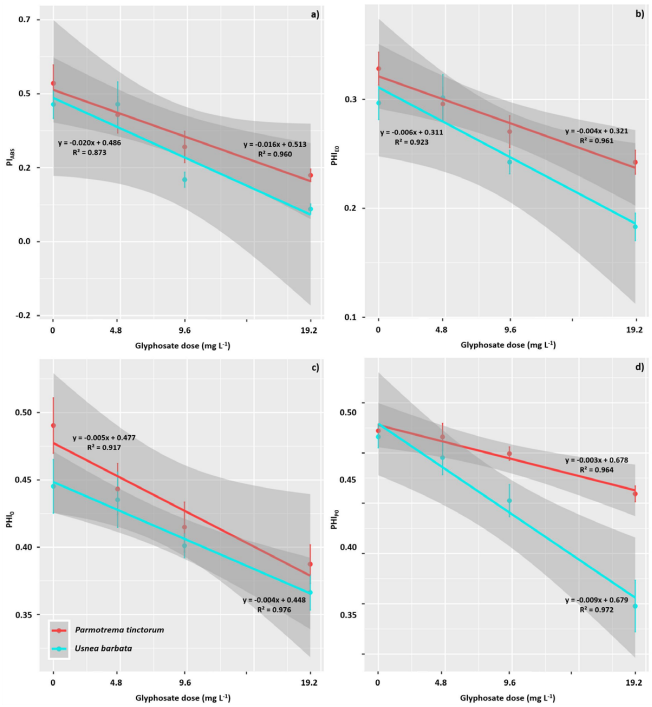

Figure 7. Chlorophyll *a* fluorescence parameters: Photosynthetic performance index - Pi_{Abs} (a), quantum yield of electron transport - PHI_{E0} (b), electron transport chain after the Quinone - PHI_0 (c), and maximum quantum yield of primary photochemistry - PHI_{P0} (d) observed for the photobiont of lichen species, *Parmotrema tinctorum* and *Usnea barbata*, subjected to different concentrations of glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}). The straight lines represent the fitted model and the prediction intervals (95%) are in grey.

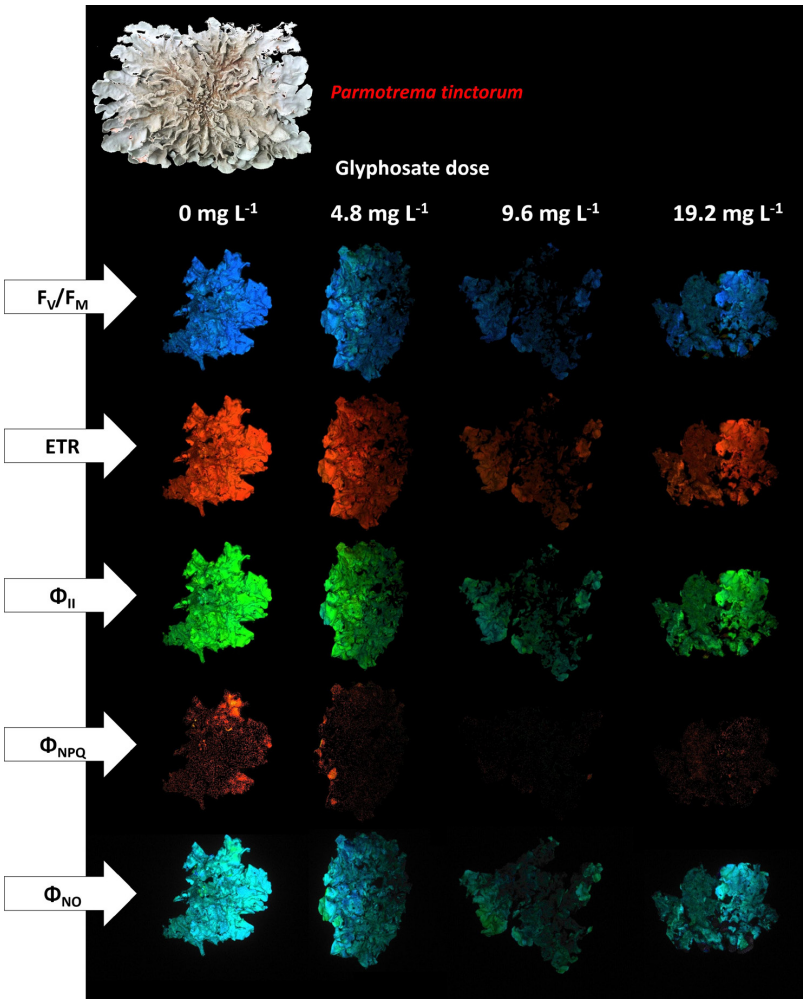

Figure 8. Chlorophyll *a* fluorescence parameters: F_V/F_M - maximum quantum efficiency of PSII photochemistry; ETR - apparent electron transport rate; Φ_{II} - effective quantum yield of photochemical energy conversion in PSII; Φ_{NPQ} - quantum yield of regulated energy dissipation, and Φ_{NO} - quantum yield of unregulated energy dissipation, observed for the lichen photobiont, *Parmotrema tinctorum*, subjected to different concentrations of glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}).

dose increased (Figure 12b). Only the lichen, *U. barbata*, responded by linearly increasing APX enzyme activity in response to increasing the applied dose of herbicide (Figure 12c).

The enzymatic antioxidant system was also affected by the time of exposure to glyphosate in both lichens. The lichens responded by increasing the activity of the SOD enzyme over time. The model adjustment showed an identical slope pattern for both lichens (β = 0.004), indicating a similar pattern of activity (Figure 13a). The lichens responded by decreasing activity of CAT enzyme in the thallus over the exposure time; the model with the highest slope for the reduction of values was observed for *U. barbata* (β = -0.059) (Figure 13b).

APX enzyme activity increased in response to exposure time in the two lichens. the species, *U. barbata*, seemed to be more responsive to dose, by increasing the concentration of this enzyme over the exposure time, which was indicated by the increase in the slope of the model (β = 0.236) (Figure 13c).

The concentration of H_2O_2 was affected by the concentrations of glyphosate only in the thallus of *U. barbata*. Thus, there was a reduction in the concentration of this oxidant as a function of increasing dosage (Figure 14a). The concentration of TSS was also affected only in *U. barbata* but followed an opposite behavior to that of peroxide, linearly increasing as the exposure dose increased (Figure 14b). MDA increased in *U. barbata* tissues as a function of the dose, with a positive linear slope (β = 58.559). For *P. tinctorum*, however, an opposite behavior pattern was observed, with a negative slope (β = -12.724) for MDA (Figure 14c).

The exposure time also affected the H_2O_2 and MDA concentrations in the thallus of the two lichens. As a function of dose, *U. barbata* responded by decreasing peroxide concentrations over time (β = -0.268), while *P. tinctorum*

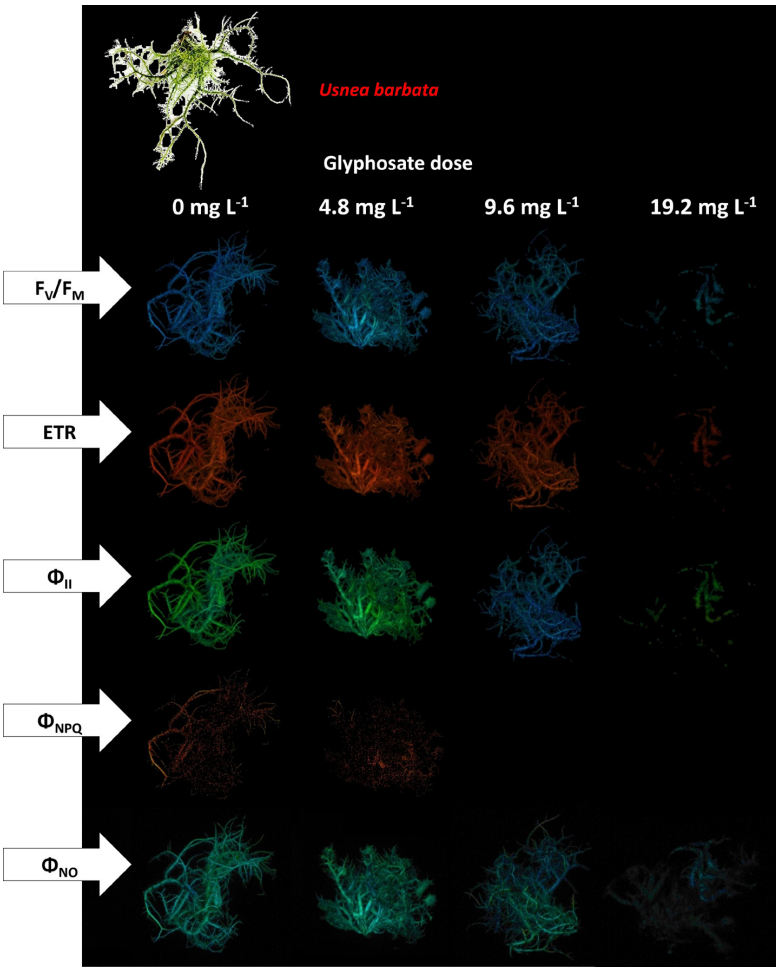

Figure 9. Chlorophyll *a* fluorescence parameters: F_V/F_M - maximum quantum efficiency of PSII photochemistry; ETR - apparent electron transport rate; Φ_{II} - effective quantum yield of photochemical energy conversion in PSII; Φ_{NPQ} - quantum yield of regulated energy dissipation, and Φ_{NO} - quantum yield of unregulated energy dissipation, observed for the lichen photobiont, *Usnea barbata*, subjected to different concentrations of glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}).

responded by slightly increasing the concentration of this oxidant over exposure time (β = 0.164) (Figure 15a). Exposure time affected the concentration of MDA only in *U. barbata* stalks; MDA concentrations reduced over time (β = -62.460) (Figure 15 b).

3.1.3. Analysis of variables as biomarkers of the effect of glyphosate

In general, when we plotted the observed data for *P. tinctorum* together, we found that for the dose-effect, cell viability was an excellent biomarker for damage since the percentage of live cells was essential in defining the differences between the control and the highest dose of herbicide applied (Figure 16a). Likewise, the percentage of dead and plasmolyzed cells were essential to defining the differences observed between the highest dose and the control dose. We also found that chlorophyll *a* fluorescence was a good marker of effect since stress indicators such as ABS/RC and DI_0 /RC were associated with the highest dose of glyphosate, while indicators of photochemical efficiency, such as Pi_{Abs}, PHI_{P0}, PHI_0, and PHI_{E0}, were associated more with the control treatment. The concentration of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids were also associated more with the control plants. The enzymes, CAT and SOD, as well as the content of H_2O_2, were associated more with the plants treated with the highest concentration of herbicide, thus also constituting good biomarkers of stress for the dose effect of glyphosate herbicide in *P. tinctorum*. Contrary to expectation, MDA content, TSS, and phaeophytization quotient were not crucial in defining the dose-effect (Figure 16a).

When we evaluated the effect of exposure time, cell viability (in particular the percentage of live and dead cells) was an important biomarkers to define this effect (Figure 16b); similarly for the contents of pigments such as chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids, which were associated with a shorter exposure time. The activities of the enzymes, SOD and APX, can also be

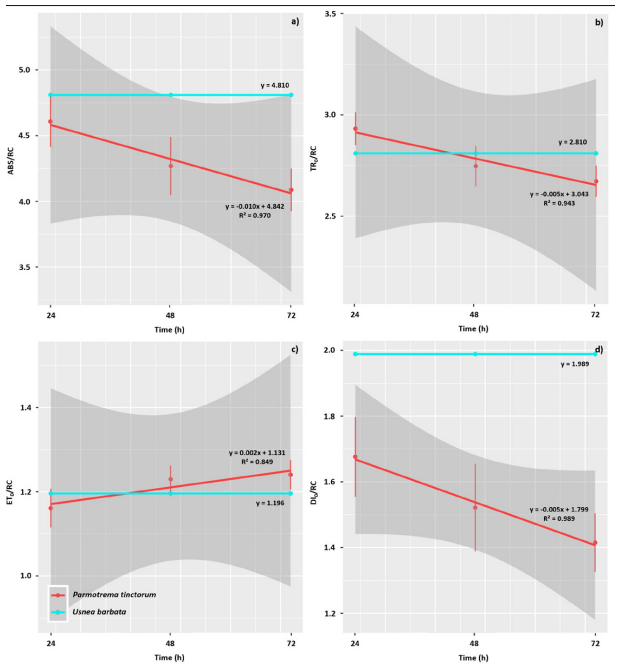

Figure 10. Chlorophyll *a* fluorescence parameters: Specific light absorption flux per reaction center - ABS/RC (a), captured energy flux per reaction center at t = 0 - TR_0 /RC (b), electron transport flux per reaction center - ET_0 /RC (c), and specific energy dissipation flux at the level of the chlorophylls of the antenna complex - DI_0 /RC (d) observed for the photobiont of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, subjected to the action of glyphosate herbicide over three exposure times (24, 48, and 72 hours). The straight lines represent the adjusted model and the prediction intervals (95%) are in grey.

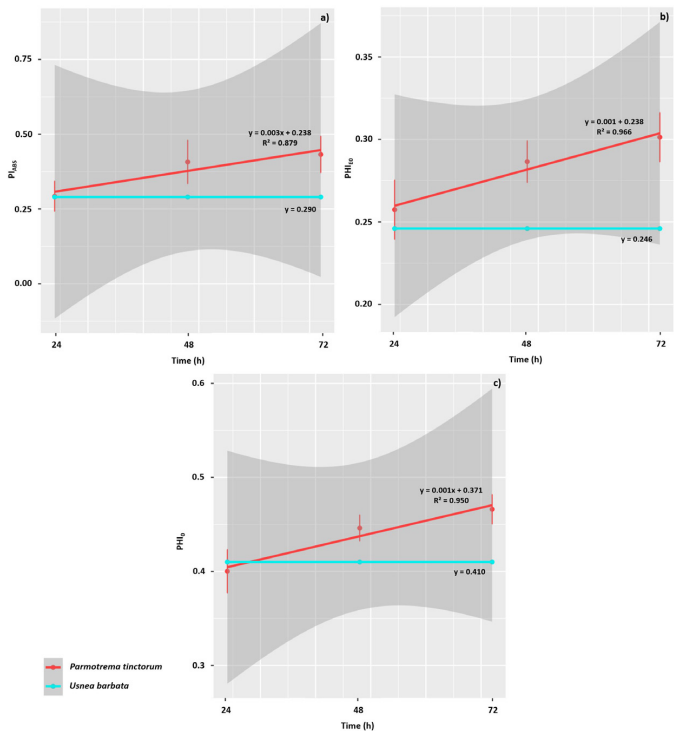

Figure 11. Chlorophyll *a* fluorescence parameters: Photosynthetic performance index - Pi_{Abs} (a), quantum yield of electron transport - PHI_{E0} (b), and electron transport chain after the Quinone - PHI_0 (c) observed for the photobiont of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, subjected to the action of glyphosate herbicide over three exposure times (24, 48, and 72 hours). The straight lines represent the adjusted model and the prediction intervals (95%) are in grey.

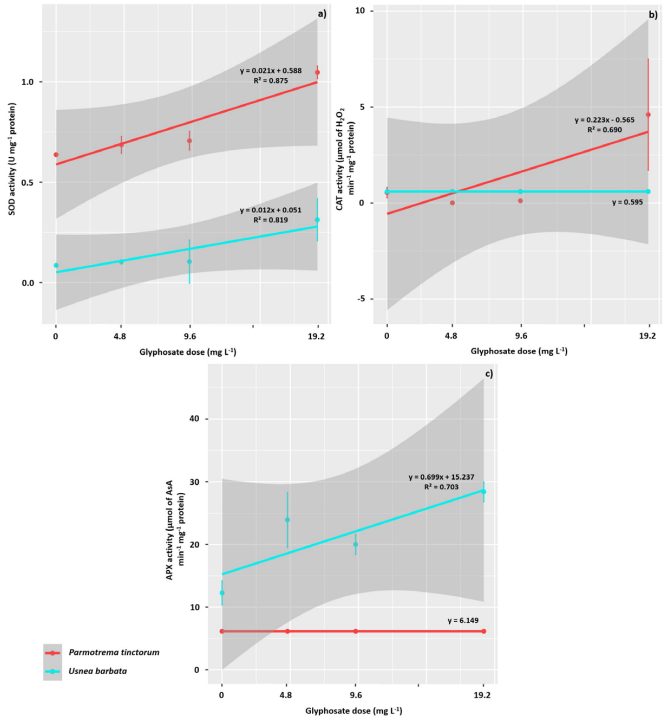

Figure 12. Activities of the enzymes SOD - superoxide dismutase (a), CAT - catalase (b), and APX - ascorbate peroxidase (c) observed in the thalli of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, submitted to different concentrations of glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}). The straight lines represent the fitted model and the prediction intervals (95%) are in grey.

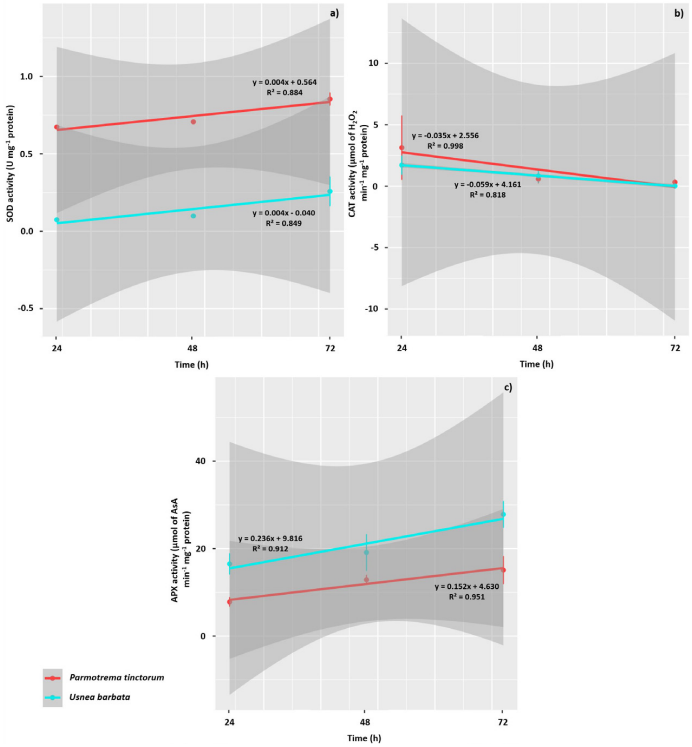

Figure 13. Activities of the enzymes SOD - superoxide desmutase (a), CAT - catalase (b), and APX - ascorbate peroxidase (c) observed in the thalli of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, submitted to the action of glyphosate herbicide over three exposure times (24, 48, and 72 hours). The straight lines represent the adjusted model and the prediction intervals (95%) are in grey.

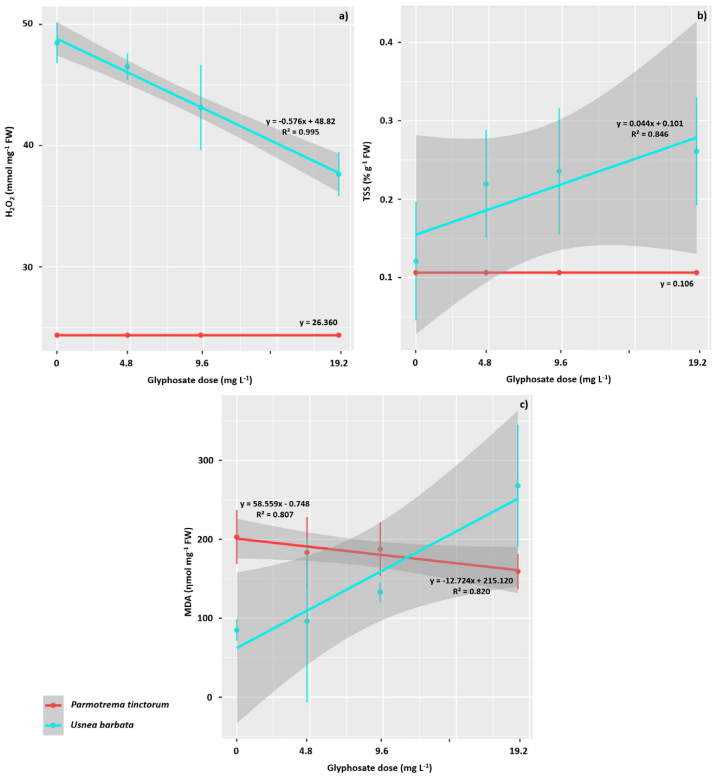

Figure 14. Concentrations of H_2O_2 - peroxide (a), TSS - total soluble sugars (b), and MDA - malondialdehyde (c) observed in the thalli of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, submitted to different concentrations of glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}). The straight lines represent the fitted model and the prediction intervals (95%) are in grey.

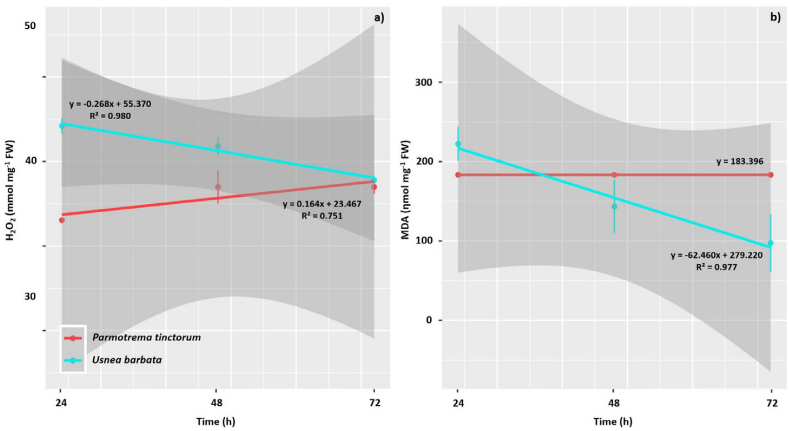

Figure 15. Concentrations of H_2O_2 - peroxide (a) and MDA - malondialdehyde (b) observed in thalli of the lichen species, *Parmotrema tinctorum,* and *Usnea barbata,* submitted to the action of glyphosate herbicide for three exposure times (24, 48, and 72 hours). The straight lines represent the adjusted model and the prediction intervals (95%) are in grey.

used as biomarkers for the time of exposure of *P. tinctorum* to glyphosate. However, the fluorescence of chlorophyll *a* cannot be considered an interesting biomarker for this question since *P. tinctorum* showed recovery of the algal photosynthetic apparatus over time. Thus, the parameters associated with photochemical stress were associated with shorter exposure time (ABS/RC, DI_0 /RC, and TR_0 /RC), while the photochemical efficiency parameters (such as PHI_{P0}, PHI_0, and PHI_{E0}) were associated with longer algal exposure time. MDA content, TSS, and phaeophytization quotient were also not efficient in defining the stress induced by the longer herbicide exposure time (Figure 16b).

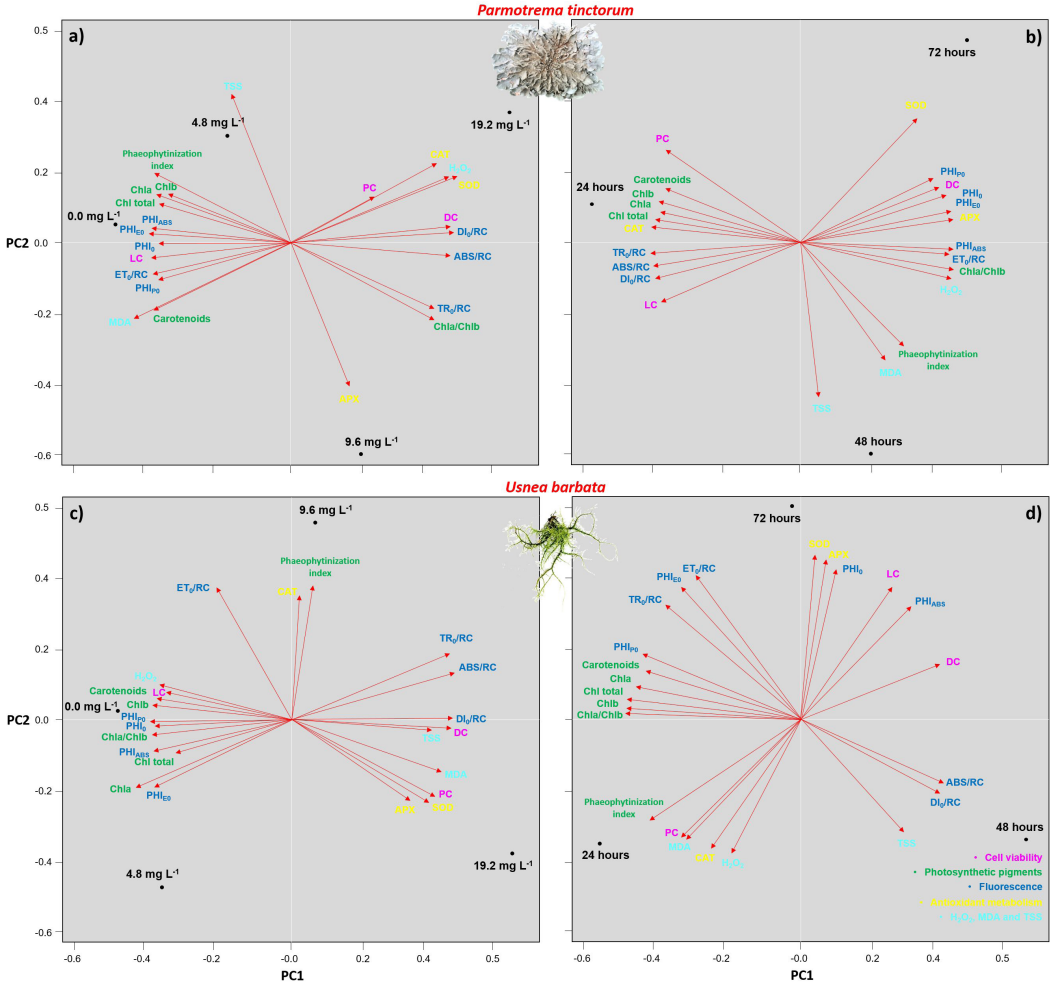

Figure 16. Principal component analysis of the concentrations of cell viability, photosynthetic pigments concentration, chlorophyll *a* fluorescence, enzymes of antioxidant metabolism, peroxide (H_2O_2), malondialdehyde (MDA), and total soluble sugars (TSS) in the thallus of the lichen. *Parmotrema tinctorum*, analyzed as a function of four doses of glyphosate herbicide (0.0, 4.8, 9.6, and 19.2 mg L^{-1}) (a) and as a function of three exposure times (24, 48, and 72 hours) (b); the thallus of the lichen *Usnea barbata*, analyzed as a function of four doses of the herbicide (c) and as a function of the three exposure times (d). LC = living cells, PC = plasmolyzed cells, DC = dead cells, ABS/RC = the specific flux of light absorption per reaction center, TR_0 /RC = the energy flux captured per reaction center at t = 0, ET_0 /RC = the electron transport flux per reaction center, DI_0 /RC = the specific energy dissipation flux at the level of the chlorophylls of the antenna complex, Pi_{Abs} = the photosynthetic performance index, PHI_{P0} = maximum quantum yield of primary photochemistry, PHI_0 = probability that an exciton moves an electron down the electron transport chain after the Quinone, PHI_{E0} = quantum yield of electron transport, Chl*a* = chlorophyll *a*, Chl*b* = chlorophyll *b,* Chl total = total chlorophyll, CAT = catalase, APX = ascorbate peroxidase and SOD = superoxide dismutase.

When we plotted the data for *U. barbata*, the cell viability, especially the percentage index of alive cells, behaved as an essential biomarker associated with the control treatment (Figure 16c). In contrast, the percentage of plasmolyzed and dead cells were primarily associated with the highest concentration of herbicide tested. As biomarkers, the photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids) and the Chl*a*/Chl*b* ratio, also reflected the effect of the dose, with the highest values associated with the control treatment. Similar behavior was verified for photochemical efficiency (PHI_{P0}, $PHI_{0,}$ and PHI_{ABS}), although the stress parameters were not effectively associated with the highest dose of herbicide. APX and SOD enzymes and MDA and TSS concentrations were efficient in indicating the stressful effect of the highest dose of glyphosate on stalks. Phaeophytization quotient, CAT, and H_2O_2 were not good markers for the effects evaluated (Figure 16d).

The vitality indices did not behave as good markers for the effect of exposure time to herbicide on *U. barbata* thallus. The enzymes, SOD and APX, indicated increased stress with exposure time, but the photochemical performance indices, PHI_0 and PHI_{ABS}, indicated recovery of the photosynthetic apparatus with exposure time (Figure 16d). The phaeophytization quotient, CAT activity, MDA, and TSS concentration are therefore not suitable biomarkers for this effect.

3.1.4. Tolerance index

Parmotrema tinctorum was more sensitive to the herbicide in terms of tolerance to the highest dose of glyphosate and the longest exposure time (Figures 17a and b). The tolerance index of *P. tinctorum* to the highest dose of herbicide tested was 0.099 and that of *U. barbata* was 2.521. The tolerance index to the longest exposure time were 0.735 in *P. tinctorum* and 0.939 in *U. barbata*.

4. Discussion

4.1. Glyphosate herbicide affects photobiont viability and promotes morphoanatomical damage in P. tinctorum and U. barbata

The lichenized fungi that constitute *P. tinctorum* and *U. barbata* are associated with unicellular green algae of the division, Chlorophyta. Fernandez et al. (2021) demonstrated that glyphosate can affect the population growth of algae of this division, causing significant damage to the ultrastructure of exposed cells, including disruption of thylakoids and mitochondria, formation of electrodense bodies, accumulation of lipids, and increased size and number of starch granules. El-Sheekh (2000) found that glyphosate concentrations above 20 mM could inhibit the growth parameters of Chlorophyta species by ~50%. Decreases in the abundance of Chlorophyta as a result of the action of this herbicide were also reported by Gonzalez et al. (2019). In the work of Iummato et al. (2019), glyphosate affected not only the growth of crops of the Chlorophyta, *Scenedesmus* vacuolatus, but morphological and ultrastructural damage were also detected.

Morphoanatomical damage has already been observed in *P. tinctorum* in peripheral areas of vegetation fragments adjacent to agricultural environments, indicating that the dispersion of agricultural pollutants such as glyphosate can negatively impact lichen populations (Palharini et al., 2021). This same herbicide induced ultrastructural changes in algal cells and hyphae observed in samples of the lichen, Xanthoria parietina (Vannini et al., 2016). Moreover, micrographs showed that the toxic effects were dose- and time-dependent. We found a dose-dependent toxic effect of glyphosate on the morphoanatomy of *P. tinctorum* and *U. barbata;* these changes make for efficient biomarkers for toxicity analysis.

4.1.1. Glyphosate herbicide affects the concentration of photosynthetic pigments in the photobionts of P. tinctorum and U. barbata

The pigment concentration of the Chlorophyta algae that make up the photobiont layer of *U. barbata* was reduced as a function of glyphosate dose, while *Trebouxia cortícola,* in *P. tinctorum,* was affected by exposure time. Indeed, work has shown that herbicides can affect the chlorophyll content in lichen thallus and increase chlorophyll degradation (Sujetovienė et al., 2019). Wong (2000) found that glyphosate, at a concentration of 2 mg L^{-1} or more, reduced growth, photosynthesis, and chlorophyll synthesis in the Chlorophyta, *Scenedesmus quadricauda*. This is because this herbicide inhibits the shikimate pathway in algae.

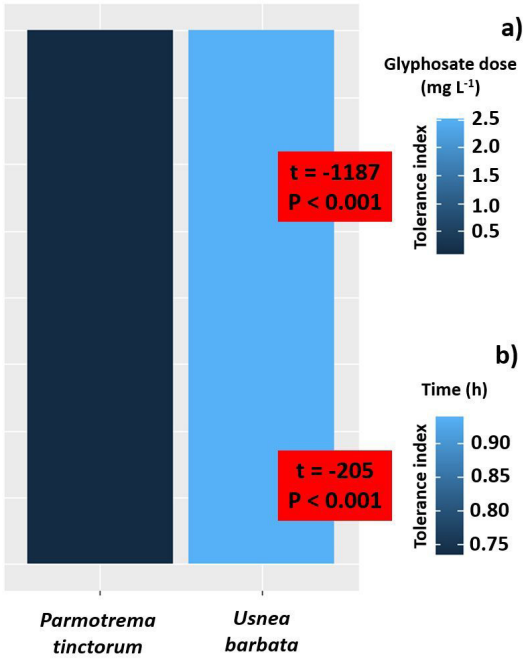

Figure 17. Tolerance index to high doses of glyphosate (19.2 mg L^{-1}) (a) and long exposure time (72 hours) (b) observed for thalli of the lichen species, *Parmotrema tinctorum* and *Usnea barbata*, submitted to the action of the herbicide. Student's *t*-test was used to indicate a difference at P <0.05.

This inhibition occurs by competition with the enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19), thus preventing the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan, and consequently, protein synthesis (Helander et al., 2012). In addition, glyphosate can produce harmful side effects on cell metabolism (Gomes et al., 2014).

4.1.2. Increasing glyphosate dose affects the primary photochemistry of lichens; however, the photosystem recomposes itself during exposure

Photochemical stress parameters in lichens increased as the exposure dose of glyphosate was increased. On the other hand, the efficiency parameters were reduced. The reduced Fv/Fm values induced by the action of the herbicide indicate chronic photoinhibition of photosystem II (Carmo Araújo and Deminicis, 2009; Oquist et al., 1992). The portion of energy that is actually being harnessed in the photochemical step (Φ_{II}), which in turn depends on ETR, also indicates damage to the photobiont, promoted by increasing the applied dose of herbicide. With increasing stress, the portion of electrons destined for the photochemical step tends to decrease, due to the increase in other pathways for dissipating excess energy, for example, photorespiration (Sunil et al., 2019). This can be proven by the increase in the observed values of DI_0 /RC. In this case, the anatomical damage caused by the herbicide seemed to compromise the diffusion of CO_2 into the stem interior. When small amounts of CO_2 reach the algal layer, inhibition of the Calvin cycle occurs, and with this, the photochemical

dissipation is impaired by the slow regeneration of its substrates (ADP and $NADP^+$) (Sharma et al., 2020). It is known that the sum of the quantum yields tends to be equal to 1. Therefore, we observed diffusion of the energy intended for ΦII and ΦNO in the stalks.

The photochemical damage was more profound in *U. barbata* than in *P. tinctorum*, which may be explained by the type of thallus. In foliose lichens, such as *P. tinctorum*, the upper cortex is formed by hyphae that protect the algal layer, while the lower cortex and pith form an extensive layer, which any pollutant must overcome to reach the photobiont. In fruticose lichens, like *U. barbata*, the algal layer is protected by a single cortex, which makes it more exposed to the action of pollutants and other stressing agents, like excessive light and heat. Beecraft and Rooney (2021) demonstrated that the photosynthetic efficiency of algae occurring inside biofilms was not affected by 24 h of controlled exposure to glyphosate, which induces us to conclude that the upper and lower cortical layers of *P. tinctorum* were indeed essential to isolating the photobiont from the action of the herbicide.

When we evaluated the effect of exposure time, the stress indicator parameters decreased over time, while the photochemical efficiency parameters increased in *P. tinctorum*. These data indicate a pattern of photochemical recovery over time. The principal component analysis also showed that the efficiency parameters were associated with a longer exposure time in both lichens. This recovery can be explained by the high growth rate of the algae and, consequently, by the appearance, in the thallus, of cells that did not suffer the initial impact of the glyphosate action. Despite the evident occurrence of meiotic genes and sexual reproduction, the green algae (Trebouxiophyceae, Chlorophyta) are primarily asexual (Fučíková et al., 2015), of simple reproduction, and constitute the best-known class due to their affinity for establishing symbiotic relationships in lichens (Leliaert et al., 2012).

4.1.3. Glyphosate herbicide promotes oxidative stress in the thallus of P. tinctorum and U. barbata

We found that the synthesis of oxidative stress-related enzymes was affected by increasing glyphosate dose and also by increasing exposure time. Other work has shown that herbicide-induced oxidative stress in lichens is dose- and time-dependent (Sujetovienė et al., 2019). If glyphosate exposure leads to the overproduction of reactive oxygen species (ROS) (Gill and Tuteja, 2010), this explains any increase in SOD, CAT, and APX activity in stalks. As CAT is strictly linked to H_2O_2 metabolism, high CAT levels were consistently associated with high peroxide concentrations a dose-dependent function in *P. tinctorum* and a time-dependent function in *U. barbata*. Catalase is considered as the first line of enzymatic antioxidant defense and reacts efficiently in peroxisomes with H_2O_2 to form water and molecular oxygen (Panchal et al., 2015). Reductions in the content of H_2O_2, which occurred in *U. barbata* thallus with increasing dose and exposure time to glyphosate, may have occurred in response to the efficiency of the antioxidant system in this lichen. In this lichen, low concentrations of H_2O_2 are always related to high APX activities; this enzyme can catalyze the breakdown of peroxide into H_2O (Caverzan et al., 2012) and may constitute an important route of elimination of this antioxidant from *U. barbata* stalks.

The content of MDA increased in the thallus of *U. barbata* with increasing dose of glyphosate, indicating that although the antioxidant system suppressed peroxide, lipid peroxidation (indicated by MDA) (Devasagayam et el., 2003) was not efficiently inhibited. In *U. barbata*, the decrease in peroxide concentration as a function of herbicide dose was accompanied by an increase in TSS concentration. The accumulation of sugars is a well-known adaptive mechanism against stressful conditions (Bolouri-Moghaddam et al., 2010; Pasbani et al., 2020). They are involved in responses to various stresses and act as nutrient and metabolite signaling molecules that activate specific transduction pathways or hormonal crosstalk, resulting in essential modifications of gene expression and proteomic patterns. Transcriptome analyses suggest that sugar signaling and sugar-modulated gene expression are closely related to the control of oxidative stress (Couée et al., 2006).

4.1.4. Biomarkers for the effect of glyphosate-induced stress on P. tinctorum and U. barbata

Besides lichen morphoanatomy, which was effective as a biomarker of the stress of herbicide dose in both lichens, for species *P. tinctorum,* vitality index and photosynthetic pigments were efficient as biomarkers of dosage and exposure time stress. These parameters are directly associated with the most sensitive component of the lichen, the algal component. Our results are significant because the techniques associated with obtaining vitality data and photosynthetic pigments were simple and inexpensive, based on dyes and extractants (Le Blanc, 1971; Barnes et al., 1992; Wellburn, 1994; Zetsche and Meysman, 2012), and therefore easily implementable in laboratories aiming to use lichens in the biomonitoring of glyphosate dispersal in agricultural areas. Port et al. (2018) evaluated photobiont vitality and metal concentration in *P. tinctorum* samples in urban and forested areas and concluded that vitality and chlorophyll contents are essential parameters for the biomonitoring of urban pollution. Photobiont vitality also decreased in *P. tinctorum* stalks exposed to carbon nanotube pollution. In this case, the photosynthetic efficiency parameters, measured by chlorophyll fluorescence, were not considered good stress markers (Viana et al., 2015). However, the chlorosis seen in the thallus of this species in urban and industrial environments (Raimundo-Costa et al., 2021) indicates impaired pigment synthesis.

In *U. barbata*, only the activity of the enzymes, SOD and APX, was able to jointly indicate the stress of dosage and exposure time to glyphosate. Stalks of *Usnea* spp. can accommodate a high activity of antioxidant enzymes such as SOD, CAT, and GST and can even inhibit lipid peroxidation (Sepahvand et al., 2021). Increased SOD activities indicate increased O_2^- production in stalks, and a good correlation between SOD activity and atmospheric concentrations of O_3 and SO_2 in the lichen, *Hypogymnia physodes*, was found (Egger et al., 1994). Increases in

SOD activity after exposure to pollutants has also been documented in the lichens, *Xanthoria parietina* and *Ramalina farinacea* (Silberstein et al., 1996). Besides SOD, Monnet et al. (2006) found increases in APX activity in the thallus of the lichen, *Dermatocarpon luridum*, when exposed to copper toxicity.

In general, MDA, TSS, and the phaeophytization quotient are considered less effective parameters as a biomarker of the effect of a herbicide. During lipid peroxidation, conjugation of ethylene groups of polyunsaturated fatty acids can be observed, which increases conjugated hydroperoxy dienes (HPDC). Rodriguez et al. (2007) suggested that HPDC are better estimators of lipid peroxidation than MDA. MDA is usually measured by its reaction with TBA, yielding MDA-TBA_2, which is detectable by spectrophotometry. However, this method is poorly specific because TBA can react with various compounds, leading to the overestimation of MDA values (Abeyrathne et al., 2021). Thus, tissue MDA values may not correctly reflect oxidative stress states.

As glyphosate affects the lichenic morphoanatomy and the vitality of the photobiont, the metabolism of carbohydrates, as well as their transfer between photo and mycobiont, is affected (Honegger, 1991), such that it was not possible to establish a relationship between an increase in TSS and higher levels of stress following dose or exposure time to the herbicide. Similarly, it was not possible to detect an increase in chlorophyll phaeophytization in the thallus as a function of the degree of stress imposed. Phaeophytization reactions, which include the replacement of magnesium from the center of the chlorophyll molecule by hydrogen and the removal of the phytol chain, forming chlorophyllide or pheophorbide, constitute the most important pathways of chlorophyll degradation (Streit et al., 2005). However, as our lichens were treated with acetone precisely to prevent chlorophyll phaeophytization (Barnes et al., 1992; Karakaş et al., 2017), the observed values of this quotient were not efficient as biomarkers of algal stress from glyphosate.

4.1.5. Parmotrema tinctorum is more sensitive to glyphosate stress than U. barbata

Although the photobionts of *U. barbata* suffered more glyphosate-induced photochemical damage than *P. tinctorum*, the data showed recovery of the photosynthetic apparatus of the former over exposure time. Thus, we found a greater sensitivity of *P. tinctorum* to dose- and exposure-time-induced stress. These results were surprising, given the classical conception that fruticose lichens are more sensitive than foliose lichens (Raven et al., 2007; Santos et al., 2018). When compared to foliose lichens, fruticose lichens are more sensitive to air pollution, being the first to disappear in heavily polluted areas (Martins-Mazzitelli et al., 2006). However, given the drift caused by spraying or aerial application of glyphosate in agricultural areas, *P. tinctorum* seems to be more suitable than *U. barbata* for air monitoring studies in urban and forest areas adjacent to agricultural environments.

5. Conclusions

The lichens, *P. tinctorum* and *U. barbata*, respond to glyphosate stress, generating options for the use of these lichens in the verification of pollutant dispersion from plantation areas. For *P. tinctorum* as bioindicator, the lichen morphoanatomy, the vitality of the photobiont, and the concentration of photosynthetic pigments were found to be efficient biomarkers for the effect of concentration and time of exposure to the herbicide. For *U. barbata*, the lichenic morphoanatomy and the activity of SOD and APX enzymes were important indicators of herbicide action. The species, *P. tinctotum*, however, was characterized as more sensitive to the action of glyphosate, constituting a good bioindicator for the presence of this herbicide and for diagnosis of air quality in urban areas or vegetational fragments adjacent to agricultural environments where glyphosate and other herbicides are commonly applied. The function of the lichen as a bioindicator is to indicate the dispersion of this herbicide, ensuring that the necessary decision-making follows to minimize the impact of agricultural pollution on plant communities in vegetational sectors and people in urban areas.

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