ORIGINAL ARTICLE

Pb-N complex stress mitigates the physiological damage of a single stress (Pb or N) on bryophytes

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Abstract

Nitrogen (N) deposition levels and the frequency of lead (Pb) contamination events are increasing globally. In an effort to improve our understanding of plant responses to these stressors, we investigated moss responses to single and combined Pb and N stress. Three mosses from different habitats (*Syntrichia caninervis*, *Bryum argenteum* and *Plagiomnium acutum*) were studied and simulated Pb/N single and complex stresses were applied to them indoors. The chlorophyll (Chl) content, osmotic adjustment substances content, and antioxidant enzyme activities were measured at 7, 14, 21, and 28 days. The results revealed that the tolerance of the three bryophyte species to Pb or N stress was in the order of *P. acutum* > *B. argenteum* > *S. caninervis*, which was closely related to the conditions of their respective natural habitats. *S. caninervis* and *B. argenteum* were stress tolerant for 7 days and *P. acutum* for 14 days. The bryophytes were tolerant to Pb or N stress after the contents of osmoregulatory substances and antioxidant enzyme activities increased; however, as toxicity accumulated over time, all three species suffered irreversible damage, as indicated by an abrupt decrease in the Chl content and osmoregulatory substances, as well as a sudden drop in antioxidant enzyme activities. Under the combined effects of Pb-N stress, the Chl content, osmoregulatory substance contents, and antioxidant enzyme activities were significantly higher in the N-loving *P. acutum* (N produced significant benefits) than in *P. acutum* exposed to Pb stress alone. This phenomenon is likely because Pb and N have antagonistic effects on the growth of *P. acutum*; thus, their recombination generates a counter-balancing effect. In the N-sensitive species, *S. caninervis* and *B. argenteum* (N caused obvious toxicity), the indicators were slightly better than under N tress alone (indicated by the reduction of membrane lipid peroxidation and increased osmoregulatory substance contents and enzyme activities), suggesting that there is a certain antagonistic effect exerted by the simultaneous addition of Pb and N. Therefore, the detrimental effects of a single abiotic stress (Pb or N) on bryophytes may be diminished under the combined conditions of N deposition and presence of the heavy metal, Pb.

Keywords Anti-oxidative response · Combined stress · Heavy metal · Moss · Nitrogen deposition

Communicated by S. Esposito.

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Abbreviations

Chl	Chlorophyll
CAT	Catalase
MDA	Malondialdehyde
Pro	Free proline
POD	Guaiacol peroxidase
ROS	Reactive oxygen species
SS	Soluble sugar
SP	Soluble protein
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

Introduction

In recent years, toxic metal pollution caused by mining and metal industries has become the main type of inorganic pollution of greatest concern worldwide. Lead (Pb) is one of the most toxic heavy metals and is widely distributed in the environment, listed by the United Nations World Health Organization as one of the top ten pollutants affecting human public health (Chang et al. 2022). The toxic effects of Pb on plant growth, metabolism, and enzyme activity have been demonstrated and are reflected by inhibiting growth (Chaves et al. 2011), interference with photosynthesis and sugar metabolism (Prado et al. 2010; Velikova et al. 2011), and generation of reactive oxygen species (ROS) that inhibit or stimulate the activities of several antioxidant enzymes; for example, the activities of superoxide dismutase (SOD) and catalase (CAT) decrease, while the activities of guaiacol peroxidase (POD), glutathione reductase, and ascorbate POD increase (Choudhury et al. 2005, Sun et al. 2009, Usman et al. 2020). As a non-essential element of biological growth, Pb contamination in the soil is toxic to plants, animals, and microorganisms, and can be harmful to human health through food consumption (Moustakas et al. 1994; Sharma and Dubey 2005).

Furthermore, as human activities have increased over time (e.g., burning of fossil fuels, production and application of chemical nitrogen (N) fertilizers, and the development of farming), atmospheric N deposition has increased substantially in recent decades and is expected to continue to increase in the near future (Liu et al. 2013, 2015; Li et al. 2016; Xu et al. 2018; Gao et al. 2019). Currently, East Asia (mainly China) has become one of three regions with the highest levels of N deposition on a global scale. N deposition is high in both rural and urban areas and the annual N deposition in cities is as high as 58–100 kg N ha^{-1} yr^{-1} (Galloway et al. 2008; Deng et al. 2009; Liu et al. 2013; Xu et al. 2018). N deposition improves photosynthesis and plant biomass under N deficient conditions (Gaju et al. 2016). However, excessive N deposition may disrupt the nutrient distribution in plants and the soil (Peng et al. 2019), thereby negatively affecting ecosystems (Roth et al. 2020). The harmful effects of N deposition include the disruption of cell membrane integrity (Pearce et al. 2003), lack of other nutrients (Elser et al. 2009), reduced photosynthesis (Tripodi and Sievering 2010), and oxidant retention (Medici et al. 2004; Koranda et al. 2007).

Both Pb stress and N deposition are threats to ecosystems; thus, it is important to understand and quantify plant responses to these two interacting environmental factors. Previous studies have shown that excessive N or Pb induces oxidative or osmotic stress in plants and affects their physiological and ecological performance. However, little is known about the effects of elevated N on plant responses to Pb stress. A limited number of studies have addressed these phenomena and only a few have demonstrated that Pb uptake and toxicity can be moderated in the presence of certain essential cationic nutrients, such as Ni, Zn, and Cu (Sun et al. 2009; Malecka et al. 2014; Mihailovic et al. 2015), and other non-essential nutrients, such as silicon (Si), have been reported to attenuate Pb toxicity (Bhat et al. 2019). In contrast, there are no reports on the possible role of N deposition in Pb tolerance. In previous studies, only the relationship between N deposition and cadmium (Cd) uptake has been reported, suggesting that moderate N application enhances Cd tolerance in vascular plants, such as *Morus alba* (Li et al. 2013) and *Toxicodendron verniciflи* (Yu et al. 2022). Thus, our lack of knowledge on the combined effects of elevated N deposition and Pb stress on plants hinders a more robust understanding of plant responses to Pb and other heavy metal stressors under different N deposition regimes.

Mosses are unique organisms that can be used to study this response, as they are small, relatively primitive terrestrial plants without cuticles; thus, they are more sensitive to environmental changes than vascular plants (Sun et al. 2009; Schröder et al. 2014; Agnan et al. 2015). The effects of atmospheric N deposition and heavy metal pollution have attracted considerable attention (Varela et al. 2017). Notably, different moss species have different responses (Sun et al. 2009; Liu et al. 2013; Chen et al. 2015). Mosses have been used to monitor heavy metals and N deposition (González-Miqueo et al. 2010; Basile et al. 2012; Izquieta-Rojano et al. 2016). However, little is known about responses after these plants are subjected to Pb stress or elevated N supply. Therefore, further studies on these responses, effects and associated mechanisms are needed to assess the fate of bryophytes in Pb/N complex stresses.

To improve our knowledge of these phenomena, we investigated the chlorophyll (Chl) content, osmotic adjustment substance contents, and antioxidant enzyme activities in three moss species (*Syntrichia caninervis*, *Bryum argenteum*, and *Plagiomnium acutum*) from three different habitats distributed across China and recorded their responses at different times (0, 7, 14, 21, and 28 days) under Pb (Pb(NO$_3$)$_2$), N (NH$_4$NO$_3$), and Pb-N (Pb(NO$_3$)$_2$-NH$_4$NO$_3$) stress treatments. The goals of this study were to (1) compare the tolerance of the three mosses to Pb and N stress; (2) investigate the physiological responses of these moss species after exposure to stress treatments at different times; and (3) comprehensively analyze the differences in the effects between combined Pb-N application and single stress application in the three moss species.

Materials and methods

Bryophyte materials and experimental design

Three common and widely distributed mosses, namely *S. caninervis*, *B. argenteum* and *P. acutum*, were collected from Gurbantunggut Desert, Xitianshan Nature Reserve and Kanas Nature Reserve in Xinjiang, China, respectively (moss species were identified by native Xinjiang taxonomists specializing in moss taxonomy) and brought back to the laboratory in uncontaminated envelopes (Tables 1, 2, Fig. 1). The phytoplasma of *S. caninervis* is densely clustered, with strongly dorsally rolled leaf margins and horseshoe-shaped warts on both sides. The phytoplasma of *B.*

Table 1 Basic information on the three moss species used in this study

Family	Species	Sampling site	Habitat	Latitude and longitude	Altitude
Pottiaceae	*Syntrichia caninervis*	Gurbantunggut Desert	Dry barren sand	44°22′N, 87°55′E	395 m
Bryaceae	*Bryum argenteum*	Kanas Nature Reserve	Moist fertile soil	48°51′N, 87°13′E	1,340 m
Mniaceae	*Plagiomnium acutum*	Western Tian Shan Nature Reserve	Spruce forest	43°10′N, 82°52′E	2,240 m

Table 2 Meteorological information and soil nutrient information of the three habitats

Sampling site	Nutrient information			Meteorological information		
	SOC (g·kg^{-1})	TN (g·kg^{-1})	TP (g·kg^{-1})	MAT (℃)	MAP (mm)	Aridity
Gurbantunggut desert	7.725	0.667	0.630	10.60	123.02	0.895
Kanas nature reserve	83.243	3.091	0.892	8.20	150.30	0.725
Western Tian Shan Nature Reserve	40.970	1.948	0.827	9.50	136.60	0.822

Fig. 1 Habitat photos of the tested mosses (**A–C**) and enlarged images of *Syntrichia caninervis* (**D**), *Bryum argenteum* (**E**) and *Plagiomnium acutum* (**F**) after acclimation (×16)
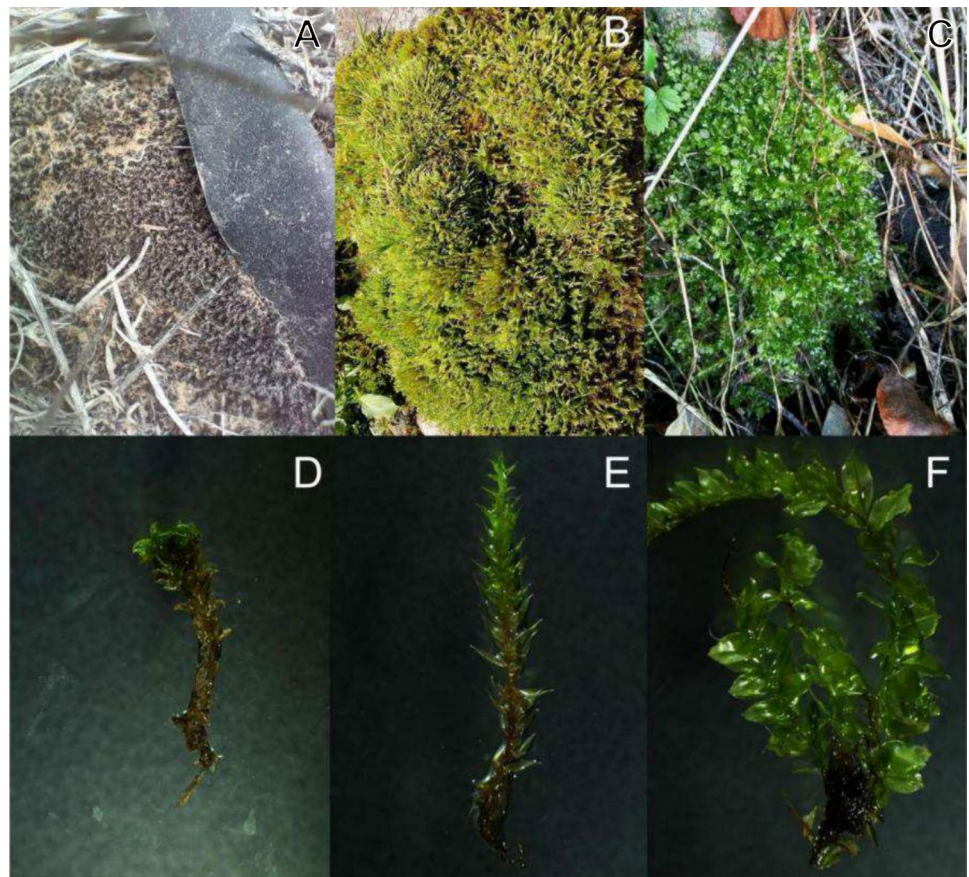
argenteum is mostly densely clustered. The leaves are often concave, broad-ovate, ovate, lanceolate, etc. The base of the leaf blade is often narrower and decurrent with more differentiated margins. The phytoplasma of *P. acutum* is lax and appears green or bright green. Its reproductive branches are erect, about 2–3 cm high, and are topped by dense clusters of leaves. Its nutritional branches are prostrate or bow-shaped.

Each moss sample consisted of a mix of 5–6 subsamples collected from a 10 m × 10 m area (Wei et al. 2003). To maintain the recovery of the "natural state" of the moss layer and avoid contamination during experimental sampling and handling, the mosses were soaked in 75% alcohol for 30 s, and washed with sterile water for 4 times, then soaked with 5% sodium hypochlorite for 60 s, and finally moss samples were washed 4 times with distilled water and acclimated in distilled water under natural conditions for 7 days prior to experimentation. The whole experiment followed the principle of aseptic operation. Our pre-experiments proved that this acclimation time was sufficient, as the physiological indicators of the mosses remained stable after 7 days of acclimation. After acclimation, ~ 10 g each of vigorous and morphologically similar mosses were used to reduce the interference of other factors. Samples were dried with filter paper, transferred to sterile petri dishes (9 cm diameter, 2 cm height), and immersed in a 50-mL culture solution. According to the content of airborne dust pollutants in Urumqi, the culture solution with a concentration of 100 μmol·L^{-1} Pb(NO$_3$)$_2$ was selected for the Pb stress treatment. Referring to the maximum N settlement rate of 4.6 g N m^{-2}a^{-1} in Urumqi, a culture solution with a concentration of 20 mmol·L^{-1} NH$_4$NO$_3$ was selected for the N stress treatment; the total amount of N applied was ~ 4.4 g N m^{-2}a^{-1}. In the combined stress treatment, NH$_4$NO$_3$ crystals were dissolved in a culture solution with a concentration of 100 μmol·L^{-1} Pb(NO$_3$)$_2$; the concentration of NH$_4$NO$_3$ in the mixed solution was adjusted to 20 mmol·L^{-1}. Our preliminary pre-experiments showed that all three mosses grew normally at the selected Pb/N concentrations. Culture solution was used as the blank control. Finally, the plates were transferred to a growth chamber under a 12/12 h photoperiod (50 μmol photons m^{-2} s^{-1}) at 20 ± 2 °C and 90% relative humidity. The materials used for the analyses were obtained from the same petri dishes. The liquid in each petri dish was changed weekly. The experiment consisted of five replicates per treatment and the moss samples were harvested on 0, 7, 14, 21, and 28 days after treatment for further analysis. The mosses were cultured in a concentration of 1/2 Hoagland nutrient solution, consisting of: 101.1 mg·L^{-1} KNO$_3$, 236.15 mg·L^{-1} Ca(NO$_3$)$_2$·4H$_2$O, 98.59 mg·L^{-1} MgSO$_4$·7H$_2$O, 16.01 mg·L^{-1} NH$_4$NO$_3$, 13.61 mg·L^{-1} KH$_2$PO$_4$, 1.345 mg·L^{-1} Na$_2$-EDTA, 1.112 mg·L^{-1} FeSO$_4$ 7H$_2$O, and trace elements (0.569 mg·L^{-1} H$_3$BO$_3$, 0.356 mg·L^{-1} MnCl$_2$·4H$_2$O, 0.043 mg·L^{-1} ZnSO$_4$·7H$_2$O, 0.01 mg·L^{-1} CuSO$_4$·5H$_2$O, 0.018 mg·L^{-1} H$_2$MoO$_4$·H$_2$O), pH value of 5.5.

The three mosses used in this study were selected for the following reasons: (1) these species are widely distributed throughout China; (2) the mosses were collected from protected areas where the air is clean and the atmosphere is not polluted; (3) the mosses are from different habitats and represent different ecosystems; and (4) the three bryophytes have different tolerance levels and were selected from 20 bryophytes used in the pre-experiment.

Determination of the Chl content

The Chl content was measured using the acetone extraction method (Arnon 1949). Approximately 0.2 g moss (fresh weight (FW)) was ground in a mortar, cooled on ice (away from light), homogenized with 3 mL 80% pre-cooled acetone, a small amount of quartz sand, and CaCO$_3$, washed twice with 7 mL 80% precooled acetone, homogenized with cleaning liquid, and rapidly transferred to a centrifuge tube. The content was centrifuged at 4 °C and 4000 rpm for 10 min. The supernatant was extracted and diluted to 10 mL; 80% acetone was used as the blank sample. The absorbance was read at 663 and 645 nm. Chl concentrations were calculated using the extinction coefficients and equation found in Porra et al. (1989).

Measurement of osmotic adjustment substances

We measured the free proline (Pro), soluble sugar (SS), and soluble protein (SP) contents in this study. The free Pro content was determined following previously described methods with minor modifications (Monreal et al. 2007). A total of 0.2 g stems and leaves (FW) from the mosses was weighed on an electronic scale (0.001 g precision), ground with 5 mL 3% sulfosalicylic acid, and extracted for 20 min in boiling water. The extract was transferred to a clean centrifuge tube and centrifuged at 8000 × g. The supernatant (2 mL) was mixed with 3 mL acetic acid and 3 mL acid ninhydrin. The mixture was oven-incubated for 40 min at 100 °C. The reaction mixture was extracted with 5 mL toluene and allowed to cool. The absorbance values were read at 517 nm; toluene was used as the blank. The standard curve was used to determine the free Pro content.

The SS content was determined using previously described methods (Lassouane et al. 2013). Frozen samples (0.2 g) were ground to a fine powder in liquid N and mixed with 7 mL 70% ethanol (V/V) for 5 min on ice. The mixture was centrifuged at 4 °C and 8000 × g for 10 min. After adding 1 mL anthrone solution to 200 mL extract, the mixture was shaken, heated in a boiling water bath for 10 min, and allowed to cool. The absorbance was read at 625 nm and the SS content was calculated from the standard curve.

The SP content was determined using previously described methods (Gonzalez and Pignata 1994). Fresh samples (0.2 g) were ground in 5 mL cold deionized water (4 °C) and centrifuged at 4 °C and 8000 × g for 30 min; centrifugation was repeated twice. The supernatant was added to a 10 mL volumetric flask. Precisely 200 μL protein extract was drawn from the flask and 5 mL Coomassie brilliant blue was added and then mixed well. The mix was allowed to stand for 3 min. The absorbance was read at 595 nm.

Measurement of lipid peroxidation

The malondialdehyde (MDA) content was used to represent the degree of lipid peroxidation. The MDA content was measured following the thiobarbituric acid (TBA) method (Dionisio-Sese and Tobita 1998). Approximately 0.2 g moss (FM) was homogenized in 2 mL 0.1% trichloroacetic acid (TCA) and centrifuged at 4 °C and 3000 rpm for 10 min; then, 2 mL supernatant was extracted and mixed with 2 mL 20% TCA containing 0.5% TBA. The mixture was placed in a water bath at 95 °C for 30 min, and then quickly cooled on ice to room temperature. The content was centrifuged at 4 °C and 3000 rpm for 10 min. The absorbance of the supernatant was read at 532 and 600 nm. The MDA content was calculated using an extinction coefficient of 155 L mM^{-1} cm^{-1}.

Antioxidant enzyme extraction and assay

The extraction procedure was performed following previously described methods with minor modifications (Sun et al. 2009). A total of 0.5 g fresh moss tissue was extracted by homogenizing 0.5 g fresh moss tissue under ice-cold conditions with a pre-chilled mortar and pestle in 10 mL extraction buffer solution containing 0.05 M phosphate buffer (pH, 7.8). The extract was centrifuged at 4 °C and 8000 × g for 20 min in a refrigerated centrifuge. The supernatant was collected for the SOD, POD, and CAT activity assays. The SOD activity was estimated following previously described methods (De Azevedo Neto 2006). Specifically, we measured the ability to inhibit the photochemical reduction of Nitro Blue Tetrazolium. The POD activity was assayed following previously described methods (Monnet et al. 2006). Briefly, we measured the rate of change in the absorbance at 470 nm using a spectrophotometer. The CAT activity was determined as the decline in absorbance at 240 nm due to H_2O_2 consumption following previously described methods (Patra et al. 1979).

Data analyses

The data were averaged from four replicates and the error bars corresponded with the standard error (SE) of the mean. Datasets were tested for normality and heterogeneity prior to the analyses. A one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine significant differences ($P < 0.05$) of individual moss species after treatment. Correlations between experimental parameters were performed using Pearson's correlation test. All analyses were performed using SPSS v20.0 (SPSS Inc., Chicago, IL, USA) and mapped with Origin v8.0.

Results

Changes in the Chl content

Both the treatment method and time significantly affected the Chl content of the three mosses (Table 3).

S. caninervis (Fig. 2A): both single and combined Pb and N treatments contributed to a sustained decrease in the Chl content. At 7 days, compared to the control group, the Chl content of the N-treated group significantly decreased to 38.67% ($P < 0.05$), while the Pb and Pb-N-treated groups decreased to 76.13% and 65.96%, respectively. The difference in the Chl content among the treatment groups decreased over time. At 28 days, the Chl content of the three treatment groups did not differ significantly and decreased to 9.97%, 12.96%, and 19.91%, respectively.

B. argenteum (Fig. 2B): at 7 days, the Chl content of the Pb-treated group significantly increased by 9.22% ($P < 0.05$)

Table 3 Multivariate ANOVA results (*P*-value) of the effects of species, time, treatment, and their interactions on the physiological indicators in the three moss species (*$P < 0.05$, **$P < 0.01$)

Effect	CHL	PRO	SS	SP	MDA	SOD	POD	CAT
Species	21,825.069**	534.180**	1369.974**	623.098**	1697.243**	421.680**	50.725**	178.721**
Time	454.081*	538.811**	293.274**	324.302**	219.563*	302.756**	159.173**	381.544**
Treatment	564.966*	44.037*	46.679**	148.890**	197.318**	49.940*	24.574**	108.723**
Species × Time	16.196*	66.436**	14.313*	9.496*	85.969**	15.427*	21.588*	11.152
Species × Treatment	521.799**	110.420**	108.202*	117.085*	30.996	59.491**	28.226*	46.469*
Times × Treatment	91.573*	78.130**	54.097*	37.027*	50.099*	33.505**	35.886**	81.055**
Species × Time × Treatment	49.384*	30.311**	10.054*	12.451*	38.701*	4.285**	7.303*	5.620*
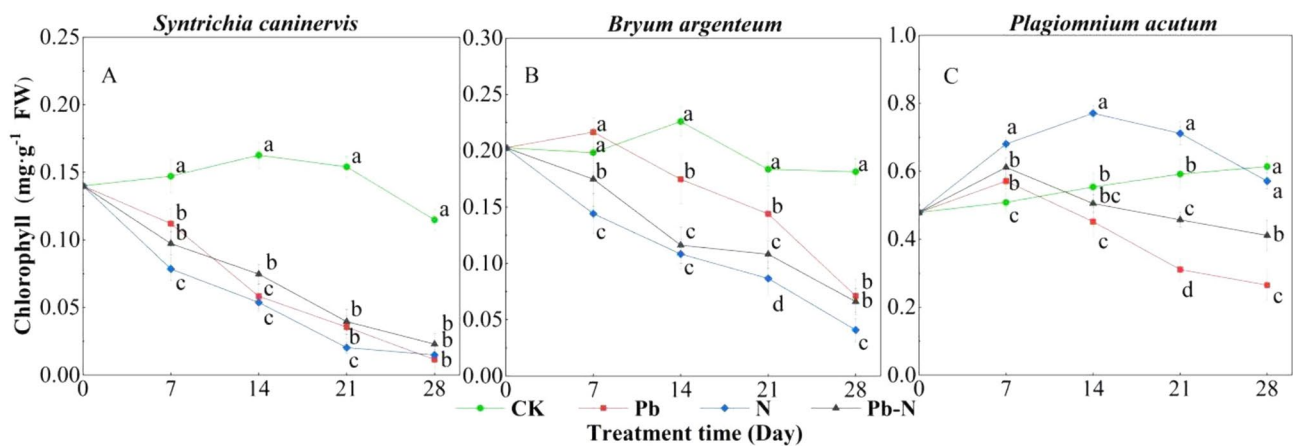
Fig. 2 Effects of single and combined Pb and N stress on the chlorophyll (Chl) content in *S. caninervis* (**A**), *B. argenteum* (**B**) *and P. acutum* (**C**). (FW): fresh weight. Each value represents the mean of 5 replicates ± standard error (SE). Different letters within each treatment indicate significant differences ($P < 0.05$, Tukey's test)

when compared to the control, while the Chl content of the N and Pb-N-treated groups significantly decreased to 72.68% and 88.12% ($P < 0.05$), respectively. After this point in time, the Chl content of all treatment groups gradually decreased. The rate of decrease was in the following order: N > Pb-N > Pb.

P. acutum (Fig. 2C): At 14 days, N treatment induced a significant increase in the Chl content, which reached a maximum of 39.19% ($P < 0.05$) when compared to the control. At 28 days, the Chl content decreased slightly and did not significantly differ from the control group. At 7 days, in the Pb and Pb-N-treated groups, the Chl content significantly increased by 13.17% and 20.26% ($P < 0.05$), respectively, when compared to the control. Then, the Chl content in both groups gradually decreased, where the Pb-treated group decreased more rapidly.

Osmoregulatory substances

The contents of three osmoregulatory substances (Pro, SS, and SP) were measured. The ANOVA results showed that treatment method and time significantly affected the contents of osmoregulatory substances (Table 3).

S. caninervis (Fig. 3A, D, G: the contents of three osmoregulatory substances in the Pb-treated group increased at first, and then decreased over time, reaching their maximum values at 7 days. The contents significantly increased to 56.14% (Pro), 36.10% (SS), and 37.51% (SP) when compared to the control ($P < 0.05$). The synthesis of the three osmoregulatory substances was continuously inhibited by N treatment and decreased to 30.62% (Pro), 25.29% (SS), and 17.12% (SP) when compared to the control at 28 days. The synthesis of the osmoregulatory substances in the Pb-N-treated group ranged between the Pb and N-treated groups.

B. argenteum (Fig. 3B, E, H: short-term Pb stress stimulated the synthesis of Pro and SS, which reached their maximum values at 14 and 7 days and significantly increased to 53.73% and 98.37%, respectively, when compared to the control ($P < 0.05$). Short-term N stress promoted the synthesis of the three osmoregulatory substances, which increased to 84.09% (Pro), 23.29% (SS), and 23.77% (SP) at 7 days when compared to the control. Subsequently, the content of the osmoregulatory substances decreased sharply in all treatment groups. Throughout the experiment, the Pro and SS contents were lower in the Pb-N-treated group than in the Pb-treated group, while the SP content was lower in the N-treated group.

P. acutum (Fig. 3C, , F, I: In the Pb and N-treated groups, the contents of the three osmoregulatory substances increased at first, and then decreased over time when compared to the control. The Pb-treated group reached its maximum value at 7 days, increasing by 106.72% (Pro), 49.52% (SS), and 23.77% (SP), while the N-treated group reached its maximum at 14 days and increased by 61.93% (Pro), 28.68% (SS), and 78.22% (SP). The Pro and SS contents of the Pb-N-treated group were lower than the Pb-treated group before 14 days, but higher after 14 days, while the SP content was always higher than the Pb-treated group.

Lipid peroxidation

Both treatment method and time significantly affected the MDA content (Table 3).

S. caninervis (Fig. 4A): the MDA content in the Pb and N-treated groups significantly increased, reaching its maximum values at 21, 7, and 14 days, which correspond to 2.08, 1.72, and 1.58× ($P < 0.05$) that of the control, respectively. After 21 days, the MDA content significantly decreased
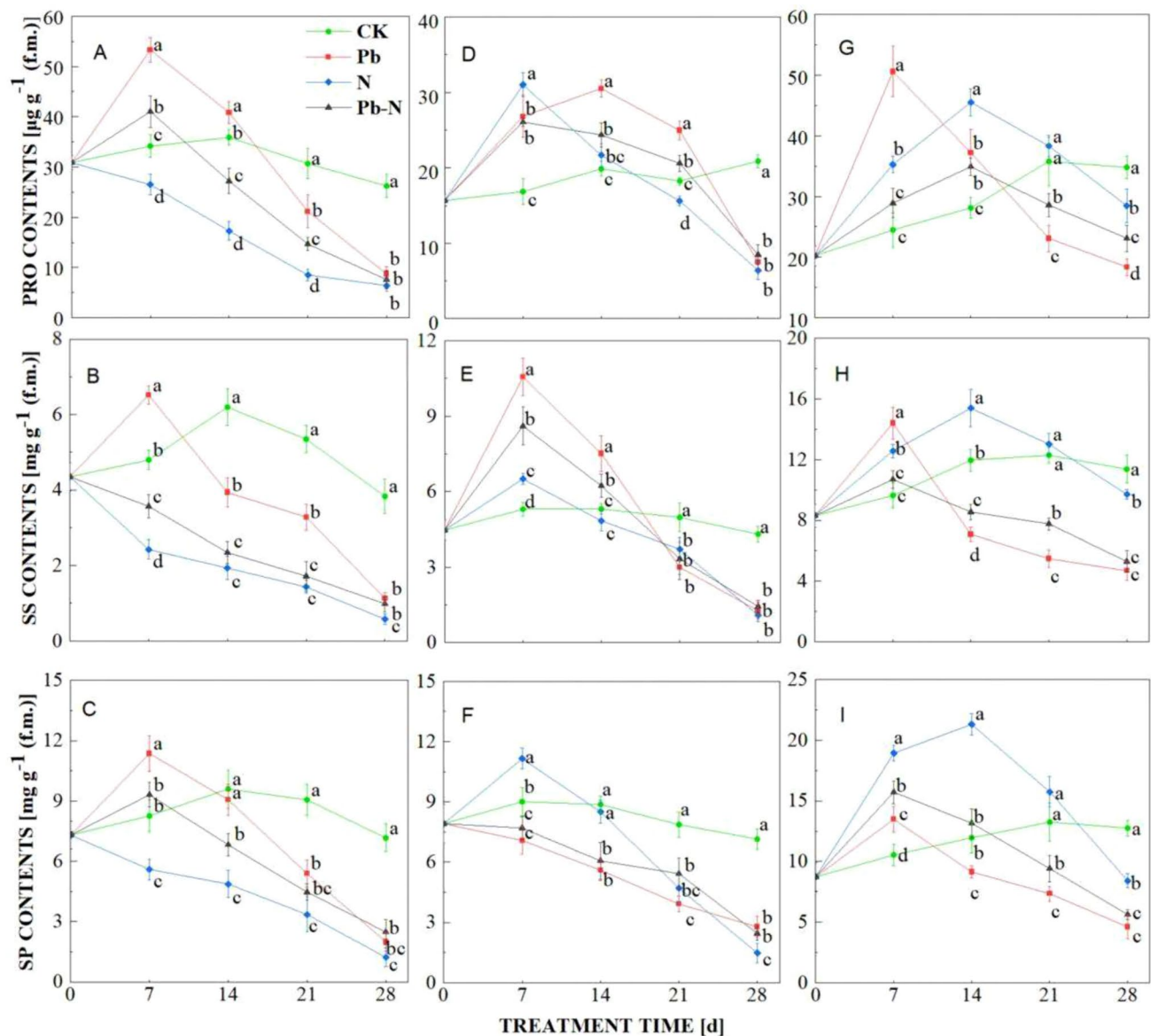
Fig. 3 Effects of single and combined Pb and N stress on osmoregulatory substance contents (Pro, SS, and SP) in *S. caninervis* (**A**, **D**, **G**), *B. argenteum* (**B**, *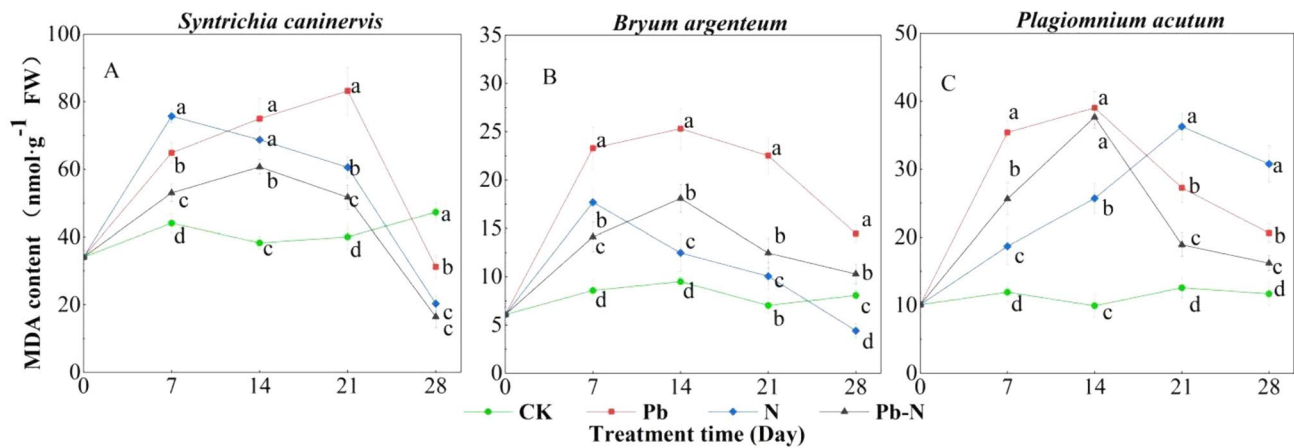*E**, **H**) *and P. acutum* (**C**, **F**, **I**). (FW): fresh weight. Each value represents the mean of 5 replicates ± standard error (SE). Different letters within each treatment indicate significant differences ($P < 0.05$, Tukey's test)

rapidly in all treatment groups, decreasing to 65.92%, 42.96%, and 34.78%, respectively, when compared to the control group at 28 days ($P < 0.05$).

B. argenteum (Fig. 4B): the MDA content in the Pb and Pb-N treated groups reached its maximum values at 14 days, which were 2.67 and 1.90 × that of the control group, respectively ($P < 0.05$). The MDA content of the N-treated group reached its maximum at 7 days and was 2.06 × that of the control group. Subsequently, the MDA content significantly decreased in all three treatment groups. At 28 days, the MDA content of the N-treated group was 45.32% significantly lower than the control

group ($P < 0.05$), while the Pb and Pb-N-treated groups were significantly higher than the control group ($P < 0.05$).

P. acutum (Fig. 4C): the MDA content of the Pb and Pb-N treated groups reached its maximum values at 14 d, which correspond to 3.93 and 3.79 × that of the control group, respectively ($P < 0.05$). The MDA content of the N-treated group reached its maximum at 21 days, which corresponds to 2.88 × that of the control group. Subsequently, the MDA content significantly decreased in all treatment groups and remained higher than the control group after 28 days ($P < 0.05$).

Fig. 4 Effects of single and combined Pb and N stress on the malondialdehyde (MDA) content in *S. caninervis* (**A**), *B. argenteum* (**B**) *and P. acutum* (**C**). (FW): fresh weight. Each value represents the mean of 5 replicates ± standard error (SE). Different letters within each treatment indicate significant differences ($P < 0.05$, Tukey's test)

Antioxidant enzyme activity

The activities of three antioxidant enzymes (SOD, POD, and CAT) were measured. The ANOVA results showed that treatment method and time significantly affected the activities of all three enzymes (Table 3).

S. caninervis (Fig. 5A, D, G): the SOD and CAT activities in the Pb-treated group reached their maximum values at 7 days, while the POD activity reached its maximum at 14 days, significantly increasing by 15.54% (SOD), 26.79% (POD), and 52.63% (CAT) when compared to the control. The POD and CAT activities increased in the N-treated group, which significantly increased by 14.68% and 32.25%, respectively, when compared to the control at 7 days ($P < 0.05$). In the Pb-N-treated group, the SOD and CAT activities were consistently higher than the N-treated group, while the POD activity was only elevated after 14 days when compared to the N-treated group.

B. argenteum (Fig. 5B, E, H): the enzyme activities of the three treatment groups increased at first and then decreased over time. Compared to the control, the SOD activity reached its maximum at 7 d and significantly increased by 5.43% (Pb), 17.04% (N), and 7.12% (Pb-N) ($P < 0.05$). The POD activity reached its maximum values at 14 (Pb), 21 (N), and 14 d (Pb-N), significantly increasing by 47.33%, 33.89%, and 41.07%, respectively ($P < 0.05$). The CAT activity reached its maximum value at 7 days and significantly increased by 63.72% (Pb), 47.92% (N), and 29.08% (Pb-N) ($P < 0.05$).

P. acutum (Fig. 5C, F, I): in the N-treated group, the activities of the three enzyme significantly increased, reaching their maximum values at 14 days, increasing by 21.54% (SOD), 42.60% (POD), and 66.17% (CAT) when compared to the control. The SOD and CAT activities in the Pb-treated group reached their maximum values at 7 days, significantly increasing by 12.79% and 16.81%, respectively, when compared to the control ($P < 0.05$). The POD activity reached its maximum at 14 days and significantly increased by 25.72% ($P < 0.05$). Subsequently, the enzyme activities in each treatment group gradually decreased. Throughout the experiment, the SOD and CAT activities were significantly higher in the Pb-N-treated group than in the Pb-treated group.

Relationship between physiological traits

The correlations between the physiological indicators of the three mosses differed across treatment groups and over time; the correlations also varied among species (Figs. 6, 7, 8). Specifically, there were more positive correlations detected between the three osmoregulatory substances (Pro, SP, and SS) and enzyme activities (SOD, POD, and CAT) of the three mosses in the pre-treatment period, suggesting that these moss species can resist stress through the synergistic effects of osmoregulatory substances and protective enzymes. Over time, the MDA content was more negatively correlated with other indicators, indicating that the accumulation of MDA resulted in the decreased Chl content and enzyme activities. At later stress stages, the indicators showed more positive correlations. Owing to severe damage to the moss cells, all the physiological indicators gradually decreased over time.

Discussion

Effects of Pb and N deposition on the Chl content

Chl is the main pigment of photosynthesis in plants. Its content reflects the strength of photosynthesis in leaves and
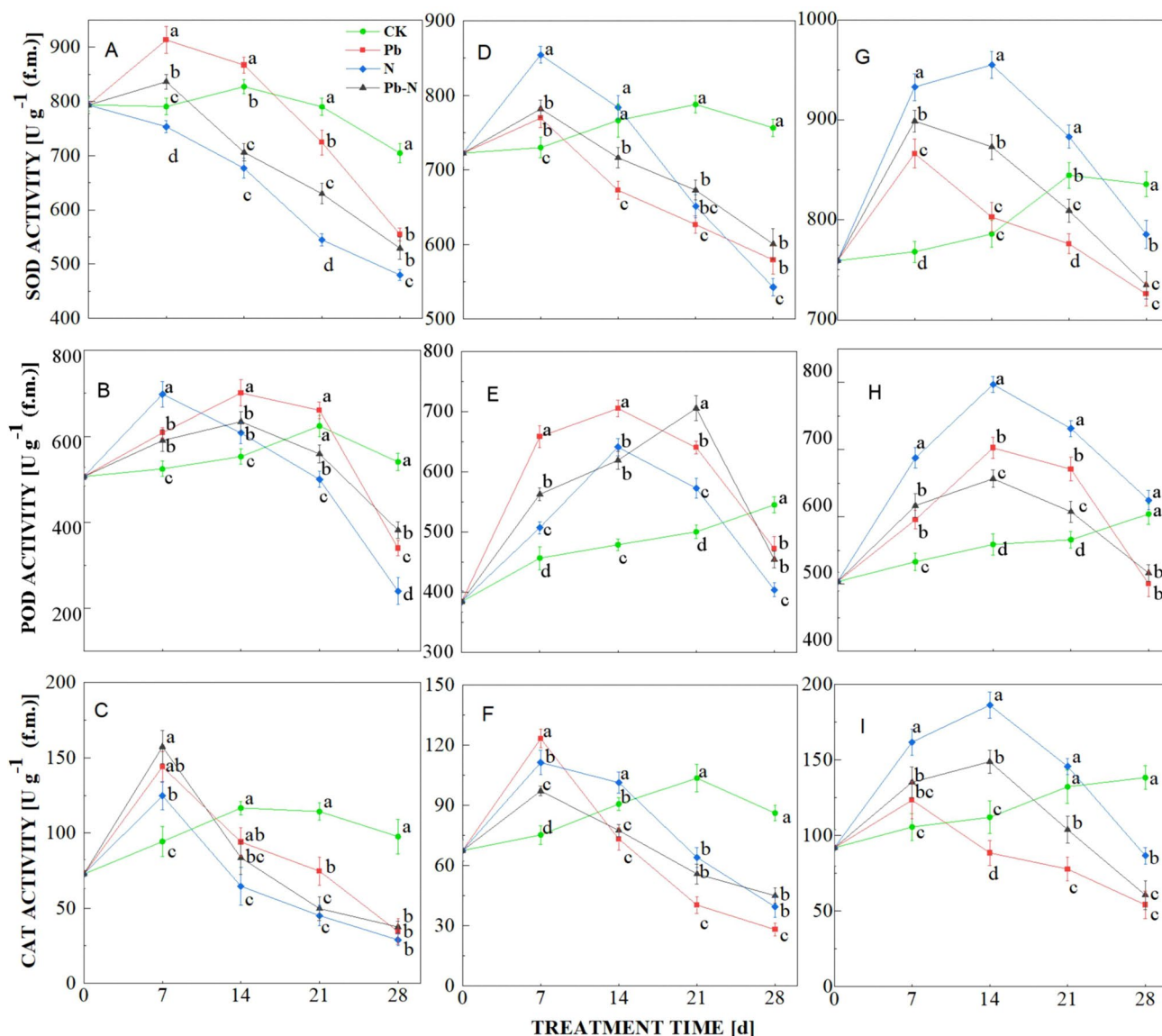
Fig. 5 Effects of single and combined Pb and N stress on antioxidant enzyme activities (SOD, POD, and CAT) in *S. caninervis* (**A**, **D**, **G**), *B. argenteum* (**B**, **E**, **H**) *and P. acutum* (**C**, **F**, **I**). (FW): fresh weight. Each value represents the mean of 5 replicates ± standard error (SE). Different letters within each treatment indicate significant differences ($P < 0.05$, Tukey's test)

characterizes the growth and development of plants under adverse conditions or stress (De Azevedo Neto et al. 2006). A previous study showed that adverse conditions or stress factors such as heavy metals (such as Pb, Cd, mercury (Hg), chromium (Cr), nickel (Ni), copper (Cu), and zinc (Zn)), inhibit or destroy the photosynthetic system by damaging the structure of plant cell membranes and chloroplasts, or affect Chl synthesis by disrupting the enzymes required for its synthesis (Shakya et al. 2008). In this study, changes in the Chl content varied with bryophyte species and exposure time. Under 100 μmol·L^{-1} Pb, the Chl content of *S. caninervis* continuously decreased over time, which was similar to *S. caninervis* under Hg Stress (Lin 2017), indicating that

S. caninervis from desert areas is sensitive to heavy metal stress. The Chl content in *B. argenteum* and *P. acutum* was higher than the control group up to 7 days, but gradually decreased afterwards. This result showed that the Chl content of *B. argenteum* and *P. acutum* have a stress response mechanism under short-term Pb stress, similar to *Hypnum plumaeforme* and *Timmiella barbuloides* from humid areas (Sun et al. 2009, Aydoğan et al. 2017), which showed similar increases in their Chl contents under low Pb concentrations.

We acknowledge that this short-term, rapid, and large-scale application of the N method is difficult and does not simulate natural N deposition. It is also more likely to cause severe damage than real N deposition. However,
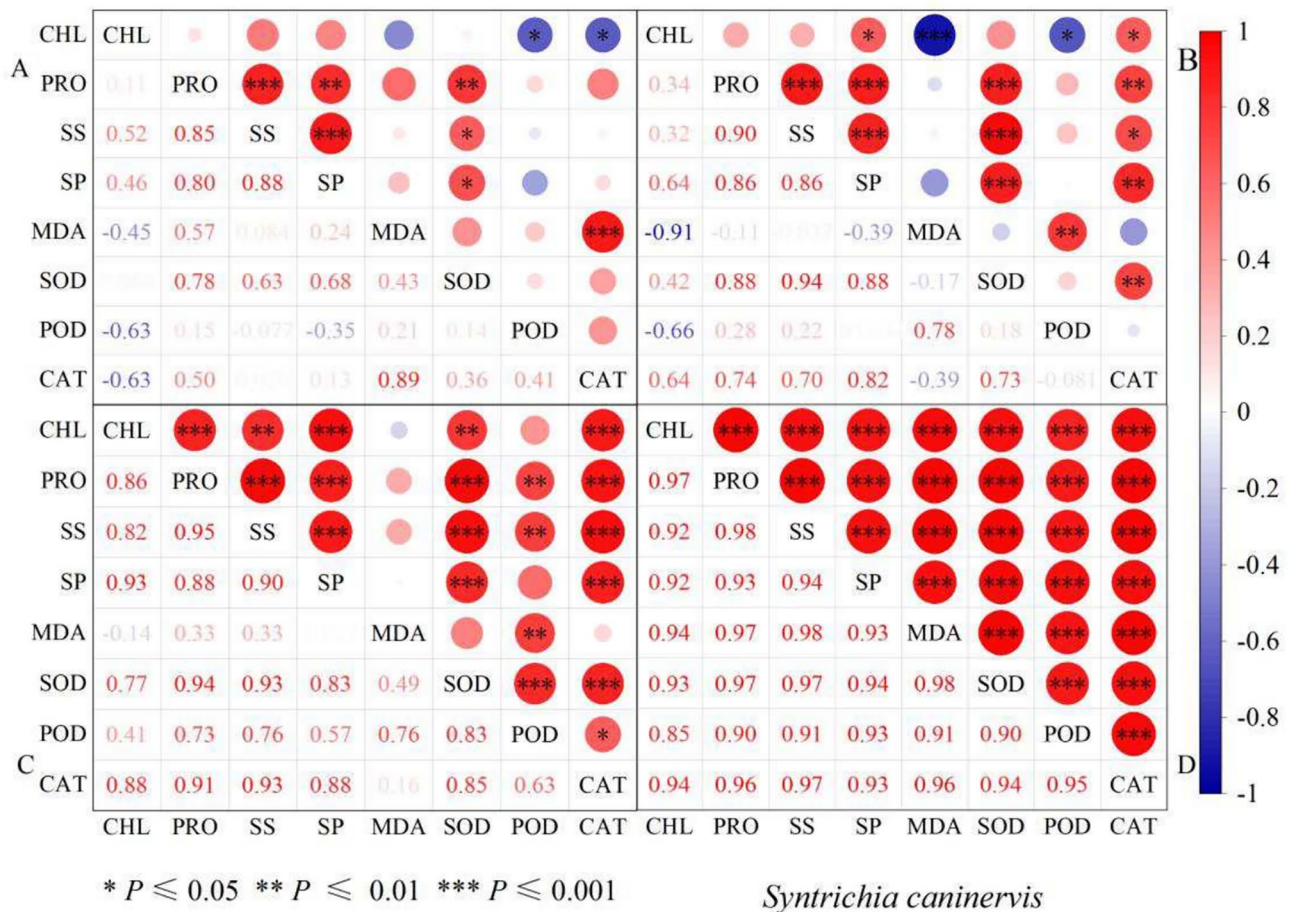
Fig. 6 Correlation analysis of physiological indexes of *Syntrichia caninervis* at different treatment stages

* $P \leqslant 0.05$ ** $P \leqslant 0.01$ *** $P \leqslant 0.001$

Syntrichia caninervis

the short-term addition of high N significantly increased the Chl content in *P. acutum* (plants were observed to be considerably greener during the experiment). Although the Chl content decreased slowly after 14 days, it was higher than the control group, indicating that *P. acutum* is an N-loving bryophyte. The short-term exposure to high N concentrations significantly promoted Chl synthesis, while long-term exposure exerted negative effects on growth and photosynthetic processes in *P. acutum*. In contrast, high concentrations of N had a strong inhibitory effect on the Chl content in *S. caninervis* and *B. argenteum* (the color of *S. caninervis* gradually turned red and *B. argenteum* gradually turned yellow over time). The negative effects of high N concentrations on these two bryophytes may be due to nutritional imbalances or cellular acid–base imbalances caused by these high concentrations (Pearson and Stewart 1993; Van der Heijden et al. 2000). Previous studies have shown that *S. caninervis* is an N-sensitive bryophyte (Zhang et al. 2016). Although the Chl content in *B. argenteum* decreased at a slower rate than *S. caninervis*, it

appeared be more tolerant to N; however, 20 mm N exposure had inhibitory effects on their Chl contents.

The Chl contents of the three bryophytes under each exposure treatment were ordered as follows: *S. caninervis* followed by *B. argenteum* (CK > Pb > Pb-N > N) and *P. acutum* (N > CK > Pb-N > Pb). Under Pb-N exposure, the Chl content slightly increased when compared to single stressor exposure, which was the most harmful to each bryophyte, indicating that the addition of Pb and N at the same time had a certain antagonistic effect. However, owing to stress exposure over time, different stressors gradually caused irreversible damage to the bryophytes and even death, resulting in smaller, insignificant differences between different exposure treatments.

Effects of Pb and N deposition on osmotic adjustment substances

Osmoregulation is an important physiological mechanism for plant adaptation to adverse stress. Under adversity stress,
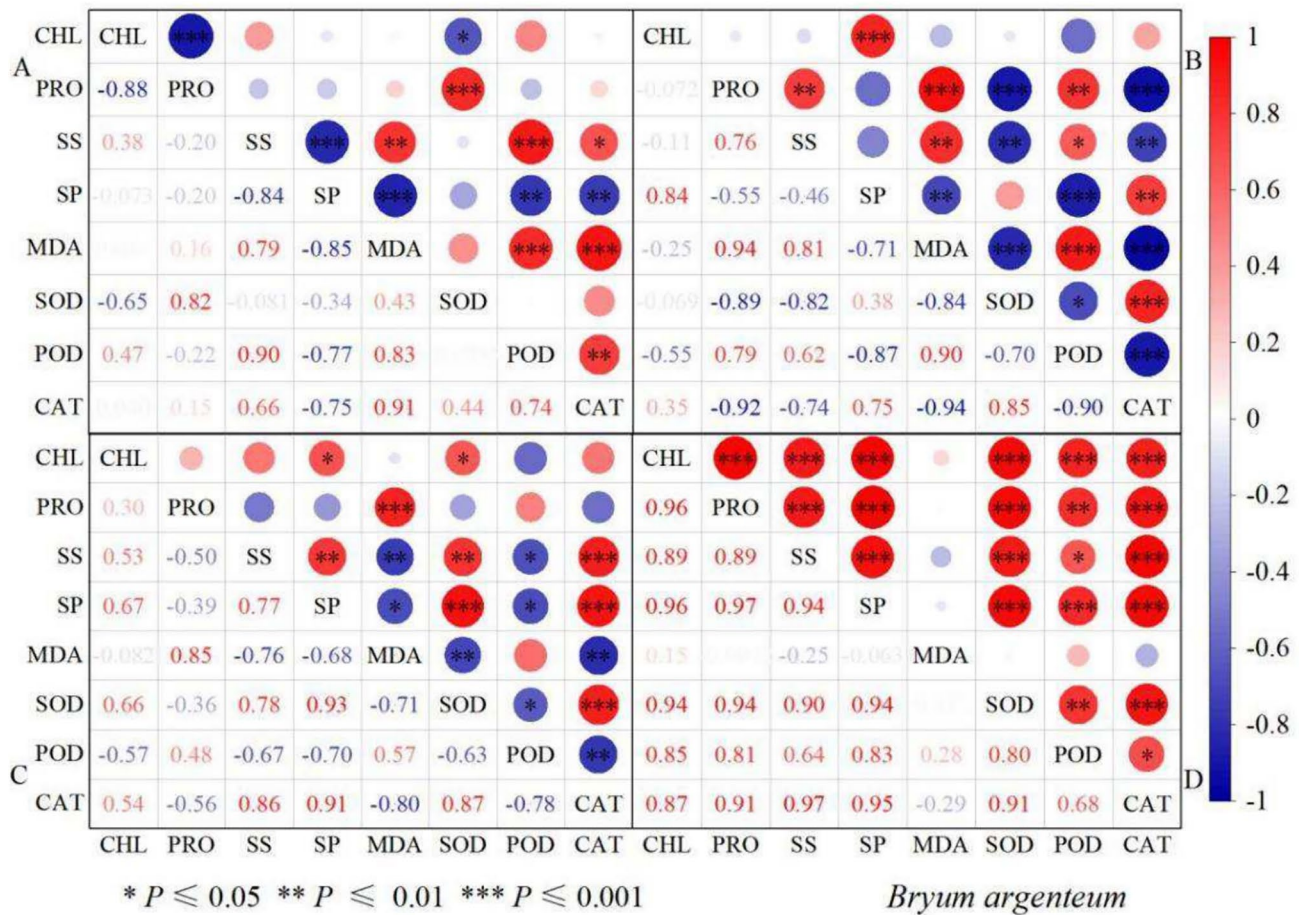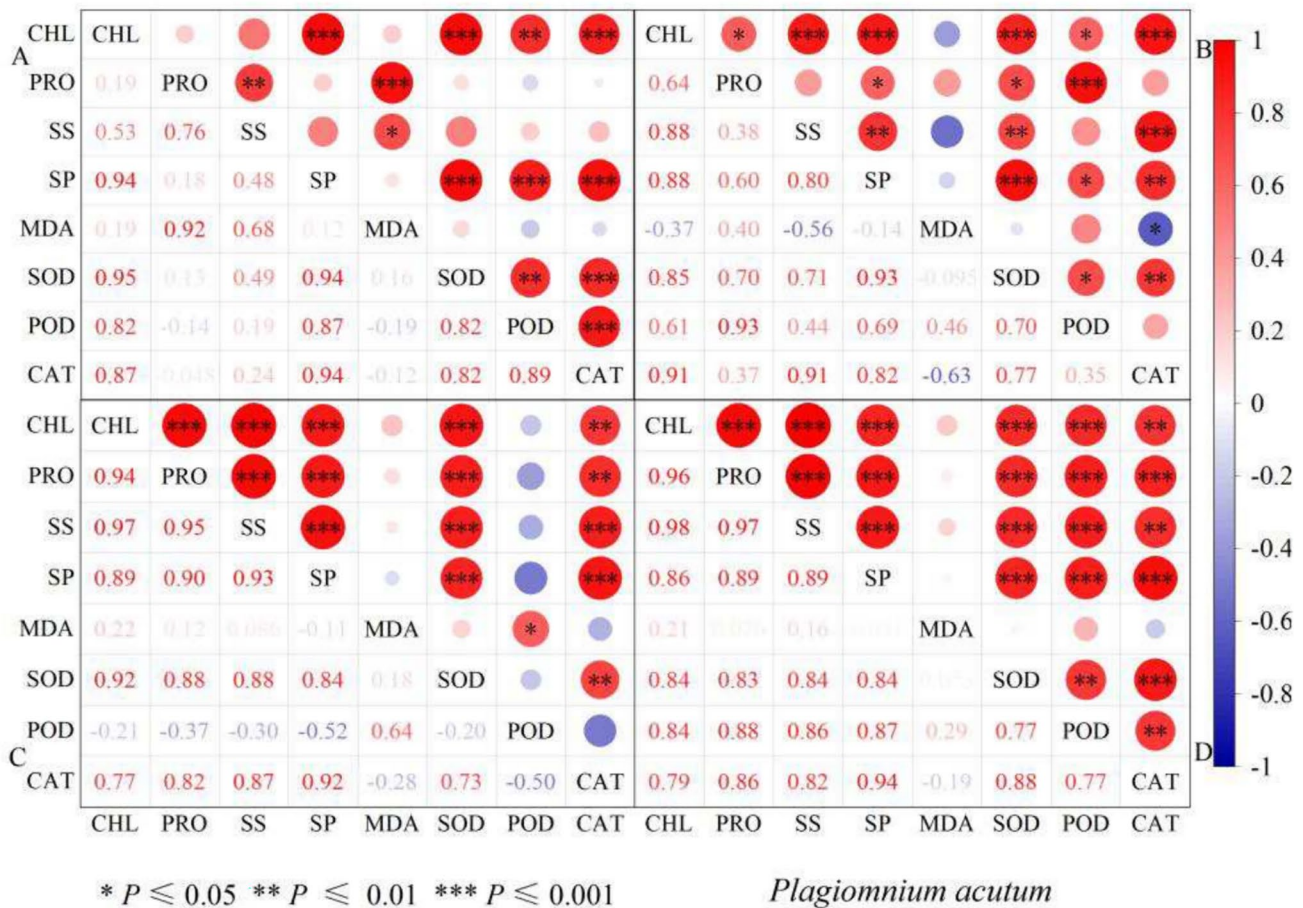
Fig. 7 Correlation analysis of physiological indexes of *Bryum argenteum* at different treatment stages

plants may increase the osmotic potential of the cytoplasm by accumulating osmoregulatory substances, such as proline, soluble sugars, and soluble proteins, to maintain cell expansion pressure and reduce water loss (Schobert 1977). The accumulation of osmoregulatory substances can, on the one hand, protect cell membranes and proteins from damage and maintain normal physiological activity; on the other hand, osmoregulatory substances can act as scavengers of reactive oxygen species and alleviate oxidative damage in plants (Malecka et al. 2014; Mihailovic et al. 2015; Usman et al. 2020; Zhang et al. 2020). Therefore, the accumulation of osmoregulatory substances is an important stress tolerance mechanism in plants. Moreover, several studies have found that plants accumulate a large amount of free Pro, SS, and other osmoregulatory substances to resist certain concentrations of heavy metals (Saxena et al. 2009, Sun et al. 2009, Zhang et al. 2016). Our study found similar results: the SS, free Pro, and SP contents in the three bryophytes under short-term heavy metal stress exposure (< 7 d) were significantly higher than in the control group. This finding may indicate that bryophytes are similar to vascular plants in

the cumulative concentration of osmoregulation substances in response to heavy metal exposure. Naturally, osmoregulatory resistance varies among species. We observed that the three bryophytes exhibited different increasing ranges of osmoregulatory substances in the early stages of stress, but decreasing ranges in the late stages, indicating that *P. acutum*, which had the smallest decline after 28 d, may have better stress tolerance.

Compared to heavy metal stress, there is a lack of research on the responses of bryophyte osmoregulatory substances to increased N deposition. Liu et al. (2017) investigated two bryophytes from South China (*H. plumaeforme* and *Pogonatum cirratum subsp. fuscatum*) under 0–60 kg N hm^{-2} N stress for 10 d and found that the SS and SP contents of both bryophytes increased as the N concentration increased. Zhang et al. (2016) found that under the exposure of simulated N deposition for three years (0–3.0 g N m^{-2} year^{-1}), the SP content of *S. caninervis* from a northwestern desert area increased at low N concentrations (up to N1), and then decreased at high N concentrations (N1.5 and N3), while the Pro and SS contents had an overall downward trend (a

A

	CHL	PRO	SS	SP	MDA	SOD	POD	CAT
PRO	0.19							
SS	0.53	0.76						
SP	0.94	0.18	0.48					
MDA	0.19	0.92	0.68	0.12				
SOD	0.95	0.13	0.49	0.94	0.16			
POD	0.82	-0.14	0.19	0.87	-0.19	0.82		
CAT	0.87	0.048	0.24	0.94	-0.12	0.82	0.89	

B

	CHL	PRO	SS	SP	MDA	SOD	POD	CAT
PRO	0.64							
SS	0.88	0.38						
SP	0.88	0.60	0.80					
MDA	-0.37	0.40	-0.56	-0.14				
SOD	0.85	0.70	0.71	0.93	-0.095			
POD	0.61	0.93	0.44	0.69	0.46	0.70		
CAT	0.91	0.37	0.91	0.82	-0.63	0.77	0.35	

C

	CHL	PRO	SS	SP	MDA	SOD	POD	CAT
PRO	0.94							
SS	0.97	0.95						
SP	0.89	0.90	0.93					
MDA	0.22	0.12	0.086	-0.11				
SOD	0.92	0.88	0.88	0.84	0.18			
POD	-0.21	-0.37	-0.30	-0.52	0.64	-0.20		
CAT	0.77	0.82	0.87	0.92	-0.28	0.73	-0.50	

D

	CHL	PRO	SS	SP	MDA	SOD	POD	CAT
PRO	0.96							
SS	0.98	0.97						
SP	0.86	0.89	0.89					
MDA	0.21		0.16					
SOD	0.84	0.83	0.84	0.84				
POD	0.84	0.88	0.86	0.87	0.29	0.77		
CAT	0.79	0.86	0.82	0.94	-0.19	0.88	0.77	

$* P \leqslant 0.05$ $** P \leqslant 0.01$ $*** P \leqslant 0.001$

Plagiomnium acutum

Fig. 8 Correlation analysis of physiological indexes of *Plagiomnium acutum* at different treatment stages

slight increase was observed at N0.3). Similarly, our results showed that the three osmoregulatory substances in *S. caninervis* decreased continuously under stress and in *B. argenteum* increased up to 7 d, while the Chl content in *P. acutum* remained higher than the control up to 21 days. These findings may reflect the fact that, although N is directly absorbed by bryophyte leaves and easily converted into protein and Pro (Soares and Pearson 1977), due to obvious differences in plant preferences for N, the N-loving bryophytes are more likely to absorb more N to synthesize these substances. In contrast, a large amount of N will impair the metabolic functions in the leaves of N-sensitive bryophytes, thus destroying the photosynthetic process (Zhang et al. 2016).

In this study, the three osmoregulatory substances did not synergistically coordinate in response to external stress, indicating that under limited resource conditions, plants sacrifice some osmoregulatory substances to preserve more important substances for plant survival (Yin et al. 2017). The Pb-N treatment caused severe damage to the cellular structure of the bryophytes and the metabolic processes that produce osmoregulatory substances were inhibited, resulting in a decrease in their contents (Saxena et al. 2009, Sun

et al. 2009, Zhang et al. 2016). Under the Pb-N treatment, the decline rate was slower and the range of osmoregulatory substances was lower in *S. caninervis* and *B. argenteum* than under the most harmful N concentrations, while the osmoregulatory substances in *P. acutum* were significantly lower than under in N exposure, indicating that the Pb-N treatment also had an antagonistic effect on the synthesis of osmoregulatory substances in the bryophytes.

Effects of Pb and N deposition on lipid peroxidation and enzyme activities

The cell membrane is a medium for plant cells to communicate with the external environment and consists of the most important receptors of external environmental signals. Its structural integrity is directly related to the ability of cells to perform their physiological functions properly (Dixon et al. 1990). Previous studies have shown that plants exposed to adverse environments (e.g., UV-B radiation, heavy metals, drought, high temperature, and high N) generate large amounts of ROS in their cells, causing an increase in cellular membrane lipid peroxidation and severe damage to

the composition and integrity of the cell plasma membrane, mainly in the form of a large accumulation of a membrane lipid peroxidation product, MDA (Sun et al. 2009; Koubouris et al. 2015; Liu et al. 2015). Choudhury et al. (2005) treated with 1000 μM Pb and Cd, MDA content of *Taxithelium nepalense* increased by 191% and 159%, respectively. Sun et al. (2009) found that the increase of MDA content in *Hypnum plumaeforme* under the treatment of 10 mM Pb and 1 mM Ni was 59% and 64% of the control, respectively, which was also confirmed in this study. We found that Pb, N, and Pb-N treatment resulted in a significant increase of the MDA content in all three bryophytes. Additionally, under Pb-N stress, the MDA content of the three bryophytes was lower than under Pb alone, indicating that the addition of N alleviated membrane damage caused by Pb stress, which may be because N is an essential element that is beneficial to plants. Moreover, the MDA content of the three bryophytes decreased after 21 days of stress, suggesting that the excessive accumulation of MDA in the plant body prior to this time had been disrupted and irreversibly damaged the internal defense system. Zeng et al. (2016) showed that a large amount of MDA crosslinked lipids, nucleic acids, sugars, and proteins, thereby adversely affecting the structure and function of the plasma cell membrane and leading to changes in the membrane structure, membrane fluidity, and its ability to bind to enzymes. This process destroys the structure and function of the cell membrane, resulting in the outflow of intracellular solutes, which leads to a depletion of MDA. This may explain the decrease of the MDA content in the three bryophytes investigated in this study.

In addition to osmoregulation, the plant body activates the antioxidant enzyme defense system to remove excess ROS, thereby enacting protection against stress. SOD, POD, and CAT are three important protective enzymes involved in the antioxidant enzyme system. SOD is the enzyme that specifically results in the dismutation of oxygen radicals (O^{2-}) to hydrogen peroxide (H_2O_2) in plants. POD and CAT further remove H_2O_2, which is toxic to plant cells; POD has more affinity for H_2O_2 and acts on low concentrations of H_2O_2 in plants (Bhaduri and Fulekar 2012). Sun et al. (2009) showed that the activities of SOD, POD, and CAT in *H. plumaeforme* increased to prevent oxidative stress caused by Pb or N. Saxena et al. (2011) found that the antioxidant enzyme activities in *Racomitrium crispulum* exhibited an increasing trend, and then decreased as heavy metal concentrations increased over time under Cu/Cd stress for 15 d.

The results of this study showed that Pb treatment increased the activities of SOD, POD, and CAT in the three bryophytes, but the increase of different enzyme activities differed and the peaks appeared on different days. There may be two explanatory reasons for these findings. First, we observed differences in the tolerance of the three bryophytes. Second, under stress, the enzymes

that play major roles in different species may be different, indicating that the changes in plant antioxidant enzymes vary in response to stress. Zhang et al. (2016) found that the moderate addition of N alleviated N deficiency and reduced the activities of POD, SOD, and CAT in *S. caninervis* by simulating N deposition. Large amounts of N had a negative effect on the growth and physiology of *S. caninervis*, resulting in an increase in the activities of these antioxidant enzymes. In contrast to the results of this study, N stress alone directly increased the activities of SOD, POD, and CAT in the three bryophytes, including the phenomenon of alleviating the lack of N and reducing enzyme activities in *P. acutum* with a more appropriate amount of N application. It is speculated that due to differences in N application methods, a single large amount of N application may more likely cause oxidative stress in bryophyte antioxidant enzymes than long-term simulated N deposition. Similar to changes in the Chl content and osmoregulatory substances, we observed an abrupt decrease in the enzyme activities during the late stages of stress, indicating that the three bryophytes are unable to prevent damage caused by excessive Pb-N through osmoregulatory substances and antioxidant enzymes. Additionally, we found that the effects of Pb-N on the antioxidant system in *P. acutum* reduced the inhibition of antioxidant enzyme activities under Pb stress, similar to the results of Yu et al. (2022), who found that N addition improved the tolerance of *M. alba* to Cd stress.

Conclusions

In this study, we found that: (1) Sensitivity: the sensitivity of three mosses to Pb/N single stress was: *S. caninervis* > *B. argenteum* > *P. acutum*. (2) Temporal effect: *S. caninervis* and *B. argenteum* for 7 days and *P. acutum* for 14 days could tolerate Pb-N stress by increasing their osmoregulatory substances content and antioxidant enzyme activity. (3) Combined effect: under the combined Pb-N stress, the indices of the three mosses were better than those under the single stress treatment (manifested by the reduction of membrane lipid peroxidation, the increase of chlorophyll content, osmoregulatory substance content and enzyme activity), which proved that there was a certain antagonistic effect of the simultaneous addition of Pb and N. In future studies, we will verify the responses of these physiological indicators in bryophytes to combined pollution conditions with higher gradients of heavy metal concentrations and N deposition. The feasibility of using bryophytes as biomarkers of urban air pollution and pollution alert thresholds will also be investigated.

Declarations

Conflict of interest The authors state no conflict of interest.

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