Characterization of three species of aquatic mosses in axenic culture for biomonitoring and biotechnological applications

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A B S T R A C T

Bryophytes are known bioindicators and are also emerging as effective tools for bioremediation. *In vitro* culture of bryophytes is an important tool for the implementation of several research and industrial applications but it is a poorly explored technology. In this study, we characterize in sterile conditions three aquatic moss species largely used all over the world for decoration but poorly studied: *Leptodictyum riparium*, *Vesicularia montagnei* and *Taxiphyllum barbieri*. They share interesting morphological traits that suggest their use as natural biofilters. Results include protocols for the establishment of axenic *in vitro* cultures, different for the different species because of their sensitivity to treatments, on which the morphological characters of the three species were described. The sporophytic generation was observed in *L. riparium* and *V. montagnei* but not in *T. barbieri* that may be unable to develop the diploid generation. The effect of plant growth regulators on gametophyte fragments was described applying 6-benzylaminopurine as cytokinin and α-naphthalene acetic acid as auxin. The absorption of several trace elements was measured in a mixed solution simulating environmental pollution, evidencing specie specificity toward the different elements. The possible applications for these mosses are not only in the field of bioindication but also in bioremediation and environmental restoration. Our study produced widely applicable protocols and basic information for further applications.

1. Introduction

Nowadays, mosses are widely used as plant biomass for several biotechnological applications and the properties of their extracts. They have been studied for antimicrobial and antifungal activity (Mishra et al., 2014; Valeeva et al., 2022), for applications in pharmacology and cosmetology (Decker and Reski, 2020), for their allelopathic effect and possible uses in agriculture (Mishra et al., 2014). Bryophytes are also known as bioindicators in environmental monitoring (Debén et al., 2015) and are emerging as effective tools for bioremediation (Papadia et al., 2020). As an application in biomonitoring of air and water, they are employed for their capacity to bioconcentrate pollutants, such as trace elements, as inorganic and organic contaminants (Debén et al., 2015; Mahapatra et al., 2019). Trace elements are a heterogeneous group of elements that can be beneficial to plants, such as B, Cu, and Zn (Barker and Pilbeam, 2015), but can also be potentially toxic such as heavy metals (Cd, Cr, Pb) and metalloids (As); Li is also a harmful trace

element, but some research indicates that it may increase biomass production, chlorophyll pigments and flavonoid synthesis (Shakoor et al., 2023).

Biomonitoring of air pollution was investigated in several species, for example in *Hypnum cupressiforme* (Mentese et al., 2021) and *Scorpiurum circinatum* (Brid.) Fleisch. & Loeske for its heavy metal bioconcentration capacity (Basile et at., 2008). For active biomonitoring of water pollution, the moss *Fontinalis antipyretica* has been amply studied also for emerging pollutants in river ecosystems such as mesoplastics and microplastics (Debén et al., 2020; Carrieri et al., 2022). The bioconcentration ability of aquatic moss *Leptodictyum riparium* (Hedw.) Warnst. has been investigated detecting toxic effects of metals and their tissue localization, antioxidant activity, ultrastructural damage, oxidative stress and HSP70 induction confirming its suitability for biomonitoring freshwater pollution (Basile et al., 2011; Esposito et al., 2012; 2018).

The absorbent capacity of mosses can then be extended in the

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purification of wastewater deriving from industries, this guarantees a lower cost, a high efficiency in the removal of heavy metals and dyes and the regeneration of the absorbent material, compared to the use of physical-chemical methods (Arafath et al., 2013). The substances are absorbed through the entire thallus and this increases the absorption capacity in proportion to the biomass (Degola et al., 2014, Bellini et al., 2020). Compared with other organisms, mosses possess significantly higher concentrations of surface reactive groups capable of binding divalent metals at the cell surface (González and Pokrovsky, 2014). Metals can also enter cells via transport proteins or channels located at the membrane level (Basile et al., 2012); the difference in the distribution of heavy metals in the cell is given by the duration of exposure. If the moss undergoes prolonged exposure, a larger proportion of metals will be observed inside the cell, otherwise, they will have an extracellular localization (Fernández et al., 2006). Using sequential elution technique (SET) main forms and locations of metals in bryophytes have been identified and not only intracellular and extracellular locations but also intercellular and particulate were distinguished (Pérez-Llamazares et al., 2011).

We already showed in the past how *Taxiphyllum barbieri* (Cardot & Copp.) Z.Iwats., a fast-growing moss, can be used as a biofilter for contaminated water as it can absorb heavy metals such as Pb, Cd, and Cr in a short time, in part absorbed to the walls, in part uptaken (Papadia et al., 2020). This makes *T. barbieri* a very good candidate for phytoremediation/phytofiltration of water polluted by specific human activities or for the complete remediation of water downstream of other purification processes.

We also studied how *T. barbieri* as well as *L. riparium* and *Vesicularia ferriei* (Cardot & Thér.) Broth. can actively capture inorganic nanoparticles becoming a potential tool capable of purifying water from nanostructured materials and reducing the toxicity associated with the ingestion of contaminated drinking water (De Matteis et al., 2021). Different potentials for the different species' biodiversity emerged from this study as well as from most of the literature cited here.

In light of these studies, we started to propagate several species of aquatic mosses under non-sterile conditions in growth tanks but also *in vitro*. The establishment of axenic conditions and the stable maintenance of cultures *in vitro*, are essential to allow investigation of the physiology of these and for their use as a source of plant biomass in contaminated water purification systems constituted by biofilters.

The aquatic mosses described in this study belong to two different families, Amblystegiaceae and Hypnaceae but share similarities that make them potentially useful for wastewater biofiltration.

They are well known in the aquarium hobby and not only for purely decorative reasons but also because provide oxygen, hiding places and substrates for spawning. *L. riparium* (Amblystegiaceae), also known as "Stringy moss", is widespread on all continents. The other two species belong to the family of Hypnaceae and are: *Vesicularia montagnei* (Schimp.) Broth., also known as "Christmas moss" native to Asia, is a semi-aquatic moss that grows on shady and humid banks; *T. barbieri*, also known as "Java moss" is a native species of South-East Asia and is known to adapt very easily to even extreme chemical-physical characteristics. Recently Wynns and co-workers (Wynns et al., 2018), suggested their inclusion on the genus Ectropothecium (*Ectropothecium barbieri* (Cardot & Copp.) J.T. Wynns).

In vitro culture of bryophytes is an important tool for studying the development of the species and for increasing our knowledge of some aspects of moss biology. The gametophyte of mosses, which is dominant during their life cycle, is a favourable model system for genetic, biochemical, metabolic and developmental studies (Cove et al., 2006). Furthermore, it is very important for biotechnological research and various experiments under controlled conditions, for example, effects of specific substrates, bioactivity of extracts, or response to endogenous phytohormones. Moreover, axenic cultures are required to obtain a large amount of biomass for analysis or production of specific chemical compounds (Sabovljević et al., 2012). The technique of culturing plant tissues and organs under axenic conditions was adapted and profitably used for the first time in bryophytes in 1913, on mosses (Sabovljević et al. 2012). One of the first bryophyte cultivated in vitro for cellular and molecular studies, *Physcomitrella/Physcomitrium patens* Hedw., turned into a scientific model organism (Schaefer and Zrÿd, 1997; Cove et al., 2006). Establishing axenic culture, the optimal culture medium and response to other supplements as additional carbon sources, phytohormones and biocides are the first conditions to be assessed for in vitro culture, which is relevant to boost biomass growth in the shortest time.

Furthermore, cultures of clonal strains of bryophytes are normally established starting from the sporophyte which, once collected from the environment, is carefully sterilized and opened to begin axenic *in vitro* culture from the spores which germinate form protonema and then the gametophyte (Heck et al., 2021). However, this is not always possible because there are species that are not found in the fertile phase of their life cycle, sometimes being naturally adapted to an exclusively vegetative propagation (Wang and Jia, 2019), therefore it is necessary to start from vegetative structures such as gametophyte fragments and gemmae (Duckett et al., 2004).

In the past, procedures have been developed to purify and sterilize mosses coming from the natural habitat to start cultures of clonal strains in axenic conditions from the gametophyte (Pereira et al., 2021) for example to provide biomass for environmental biomonitoring (Debén et al., 2020).

In this study, we set the technical bases to expand knowledge on three promising aquatic mosses that show to be perfectly adaptable to several applications in bioremediation and biomonitoring of trace elements.

We developed a sterilization protocol starting from gametophyte fragments of the three species of aquatic moss to obtain a considerable amount of axenic biomass to be used for future applications especially those based on the absorbent capacity of mosses. We propagated these species *in vitro* and evaluated possible biomass increases in different conditions. We made a morphological description to understand if the differences between the three species were constant in vitro and better understand the structure of the gametophyte, testing their response to different growth regulators and evaluating their general ability to accumulate trace elements.

2. Experimental

2.1. Plant material and gametophytes decontamination

T. barbieri and *V. montagnei* were obtained from a commercial source (Tropica Aquarium Plants; Mejlbyvej 200 8250 Egå, Denmark) while *L. riparium* derived from the Botanical Garden of the University of Naples "Federico II," Italy. Aquatic mosses belonging to three different species were grown in glass tanks used in aquariums (30 ×30×30 cm) submerged with fresh water (no supplements), constant air flow and incubated in the growing chamber under standard conditions ($22\pm2°$C, 16/8 h photoperiod, and light intensity 150 μmol m-2 s-1).

To induce axenic cultures, the mosses were washed with tap water to eliminate impurities and other organisms, then a specific sterilization *in vitro* protocol was applied to portions of, not too branched, gametophytes. The first wash was done in 15 mL of bidistilled water supplemented with 2 drops of Tween 20 (Polysorbate 20), stirring for 60 seconds; the second wash was done in a solution (1:5) of sodium hypochlorite (commercial bleach about 1.75% NaOCl) in bidistilled water, stirring for another 60 seconds. Subsequently, the gametophytes were treated with 50% ethanol solution for 60 seconds; two more washes in 50 mL of bidistilled water were used to remove any bleach and detergent residues; at the end of washing, mosses were exposed, out of the water, to UV rays for 10 minutes. The gametophytes of *V. montagnei* did not survive the described sterilization protocol therefore they underwent a different treatment: first a treatment with 0.08% PPM™ (Plant Preservative Mixture) under stirring (150 rpm) for 7 h then it was

transferred to agar medium containing 0.08% of PPM™ in the dark for a week before to be ready for use. After decontamination, mosses were cultured on solid medium $PpNO_3$ (Ashton et al., 1979) in a growing chamber under standard conditions ($22\pm2°C$, 16/8 h photoperiod, and light intensity 150 µmol m-2 s-1). During the first 2 weeks, it was possible to observe the regrowth of the filaments and eventually discard still contaminated plant material by checking under a stereo microscope.

Sterility check of the moss cultures was carried out estimating total numbers of microbes (the numbers of colony forming units, CFU) growing on plate count agar (PCA) at 25°C for 7 days without fungal antibiotics in order to estimate the growth of fungi as well as bacteria.

2.2. Axenic in vitro propagation

Axenic culture of the three species of mosses was established in solid into sterile Petri dishes using $PpNO_3$ and $PpNH_4$ media (Ashton et al., 1979) and in liquid into flask using $PpNO_3$, $PpNH_4$, dilution of $PpNO_3$ and $PpNH_4$ in bidistilled water or microfiltered and sterilized 60 µS/cm water (130 rpm); another propagation technique employed was Temporary Immersion bioreactors (TIBs) technology using Setis™ bioreactors with sterile bidistilled water supplemented with 5–10% of $PpNO_3$ or $PpNH_4$ media (immersion 2 min every 6 h). This method was developed for the start-up company Green Greener srl (Policoro, MT, Italy. https://www.greengreener.net/) now producing axenic biomasses of these organisms. In all cases, the propagation took place by cutting pieces of filaments with or without apex and transferring them to a new medium every month. All cultures were kept at $22\pm2°C$, 16/8 h photoperiod, and light intensity 150 µmol m-2 s-1. The growth rate was evaluated as fresh weight (FW) increase and expressed as Relative Growth Rate (RGR): $RGR = (\ln FW2 - \ln FW1)/(t2 - t1)$; where FW1 and FW2 are moss fresh weights at times t1 and t2.

2.3. Treatments with plant growth regulators

To study the influence of plant growth regulators on morphogenesis and growth of the three species of moss, 10/15 mm-long apical portions of shoots were used and placed in sterile Petri dishes with $PpNO_3$ solid medium added with two different plant growth regulators at different concentrations alone or in combination. We conducted the same treatment on $PpNO_3$ medium without plant growth regulators as the control. Specifically, it was used the cytokinin 6-benzylaminopurine (BAP) (Sigma-Aldrich) at 0.1 mg/L, 0.2 mg/L, 0.5 mg/L, 1 mg/L and the auxin α-naphthaleneacetic acid (NAA) (Sigma-Aldrich) at 0.1 mg/L, 0.2 mg/L, 0.5 mg/L, 1 mg/L (Table 1). The observation was made for 5 weeks on fragments treated continuously or fragments treated for two weeks and then moved to medium without plant growth regulators. The response of mosses to plant growth regulators was assessed visually through the use of images taken every week.

2.4. Microscopy

For the morphological characterization of the three species, the study of the growth in the different culture media and the response to plant growth regulators were used Stemi 508 stereo microscope (Carl Zeiss Microscopy GmbH, Germany) equipped with an Axiocam ERc 5 s.

Table 1
List of media used.

Treatment mg/L	Treatment mg/L	Treatment mg/L
$PpNO_3$ medium	$PpNO_3$ + NAA 0.2	$PpNO_3$ + BAP 1 + NAA 1
$PpNO_3$ + BAP 0.1	$PpNO_3$ + NAA 0.5	$PpNO_3$ + BAP 0.1 + NAA 0.2
$PpNO_3$ + BAP 0.2	$PpNO_3$ + NAA 1	$PpNO_3$ + BAP 0.2 + NAA 0.1
$PpNO_3$ + BAP 0.5	$PpNO_3$ + BAP 0.1 + NAA 0.1	$PpNO_3$ + BAP 0.5 + NAA 1
$PpNO_3$ + BAP 1	$PpNO_3$ + BAP 0.2 + NAA 0.2	$PpNO_3$ + BAP 1 + NAA 0.5
$PpNO_3$ + NAA 0.1	$PpNO_3$ + BAP 0.5 + NAA 0.5	

Microscopic optical analysis was performed with an optical microscope Orma Scientific OL201TL connected to a Camera Eurotek MDH5 (1080p).

2.5. Trace element accumulation treatment

Before the treatment with the selected trace elements, the three species were acclimated for two weeks in 60 µS water. A similar amount of mosses (1.00 – 1.30 g FW) were treated in a 50 mL falcon tube for 72 h with tap water enriched with As^{3+} ($NaAsO_2$ 4 µM), B^{3+} ($Na_2B_4O_7$ 300 µM), Cd^{2+} ($Cd(NO_3)_2$ 10 µM), Cr^{6+} ($K_2Cr_2O_7$ 12 µM), Cu^{2+} ($CuSO_4$ 4 µM), Li^{2+} ($LiCl_2$, 1 mM), Pb^{2+} ($Pb(NO_3)_2$ 100 µM) and Zn^{2+} ($ZnSO_4$ 40 µM), (see Supplementary Table S1 for more detailed concentrations). The pH variation in the solutions was measured at 0 h, 24 h, 48 h and 72 h for each species. After the treatment, the mosses were washed as described in Anglana et al., (2023). The element concentration in mosses after the two weeks of acclimation (t0, not treated samples), and from normal sterile culture, was also measured. Not treated samples were anyhow washed like the treated ones. 4 mL of tap water enriched with the element of interest described before were collected from each treatment at 0 h, 24 h, 48 h and 72 h. Mosses samples and water samples were then analysed with ICP-AES technique.

2.6. Samples mineralization and ICP analysis

The dry weight of the mosses was determined, and the samples were mineralized as previously described (Papadia et al., 2020); samples were mineralized in a microwave in the presence of concentrated ICP-grade HNO_3, 6 mL, and H_2O_2, 4 mL. At the end of mineralization, the volume was adjusted to 15 mL with ICP-grade water before filtration with 0.22 µm filter. Then 1 mL of each liquid sample collected, was diluted ten times using ICP-grade water containing ICP-grade HNO_3 (final concentration 2% v/v). All mineralized samples were analysed using iCAP 6000 ICP-AES (detection range: min. 0.001 mg/L, max. 10 mg/L) (Thermo Scientific, Waltham, MA, USA). To confirm the correct attribution of the metal values detected, the concentration of a commercial standard solution (Pachem, Bogomilovo, Bulgaria) containing known concentrations of Ca, Ba, Mg, Zn (2 mg/L), Al, Mn (10 mg/L), Ni, K (50 mg/L), and P (100 mg/L) was also evaluated during the analysis. This standard solution was tested in its pure and diluted (1:1, 1:5, and 1:10) forms.

2.7. Statistical analysis and figure manipulation

Statistical analysis and graphic rendering of the result were produced using R Core Team and R Studio Team software. The statistical analysis by a Kruskal Wallis test with a post hoc test using Fisher's least significant difference and a p value of 0.05 was applied to quantitative value of trace elements in moss biomass, expressed as efficiency in the uptake, as well as to parameters variation in the experimental water during treatments. The multiple comparison test "Bonferroni" was used for adjusting the p values. The n value of 3 correspond to 3 independent experiments with 3 replicas. Images were assembled using Adobe Photoshop CS6.

3. Results

3.1. Establishment of axenic in vitro cultures

Spores are usually used to establish axenic culture but this is not always possible therefore it is necessary to start from vegetative structures such as gametophytes. Diversified sterilization protocols applied to the three species of mosses proved to be valid; for 2 weeks the mosses were propagated on solid medium $PpNO_3$ to facilitate macroscopic observations that did not reveal the presence of algae and other microorganisms. Checks for contaminating aerobic microorganisms, carried out

by counting on PCA medium, did not highlight the presence of any colony-forming units. Subsequently, the propagation occurred mainly in a liquid medium.

RGR was calculated after 10 days of growth in flask (130 rpm) in three media: 60 μS/cm water, PpNO$_3$ and PpNH$_4$ (Fig. 1 A). *L. riparium* RGR for the different media was respectively 0.020, 0.034 and 0.047 g g^{-1}day^{-1}. Data also showed that it was the moss with the slower growth in all media. *V. montagnei*, had an intermediate RGR with significantly different growth in the different media (0.072 g g^{-1}day^{-1} in water, 0.028 g g^{-1}day^{-1} in PpNO$_3$ and 0.060 g g^{-1}day^{-1} in PpNH$_4$). *T. barbieri* had the best growth compared to the other species, especially in water (0.108 g g^{-1}day^{-1} in water, 0.085 g g^{-1}day^{-1} in PpNO$_3$ and 0.069 g g^{-1}day^{-1} in PpNH$_4$). Comparing the growth of moss *L. riparium* between the traditional propagation system in flask (130 rpm) and the TIB technology in water for 30 days data showed a significant increase in growth, with a RGR of 0.008 g g^{-1}day^{-1} in flask and 0.025 g g^{-1}day^{-1} in TIB (Fig. 1B).

3.2. Morphological characters

From the habit and overall size of the gametophyte we observed that both *L. riparium* and *V. montagnei* were pleurocarpous mosses with monopodial growth, very branched and prostrate. The gametophyte of *L. riparium* (Fig. 2A), of yellow-olive colour, formed mats with irregular ramifications (even secondary stems with ramifications) and anchored itself to the substrate with rhizoids; *V. montagnei* (Fig. 2B), of yellow-green-brownish colour, had dense and irregular branching and pinnate secondary branches forming glossy mats. *T. barbieri* (Fig. 2C) was a moss acrocarpous with sympodial growth and it was dark green to light yellow; it formed dense cushions with irregular branching.

The insertion of the leaves on the stem was different between the three mosses: in *L. riparium* the leaves were spreading (with the tips of the leaves pointing outwards from the plant and a 90° angle to the stem) and sub-complanate (inserted transversely to the stem, developed almost on one plane), therefore the general appearance of the leaf structure was flattened (Fig. 2D), in *V. montagnei* were spreading (Fig. 2E) while in *T. barbieri* they were erectopatent (angle less than 45° respect to the stem) (Fig. 2F). During the morphological characterization we also observed other structural elements along the stems such as bulbils, which are bulb-like vegetative propagules surrounded by leaf primordia, noted in *L. riparium* and *V. montagnei* (Fig. 2G, H) or foliose pseudoparaphylls (small filiform structures around the primordial

branch) noted in *T. barbieri* (Fig. 2I) and also present in *L. riparium*. In all three species, there were rhizoids observed along the stem in the form of micronemata (a set of thin, sparsely branched rhizoids produced on the stem in the area between the leaves). Furthermore, the tips of the filaments were distinguishable in shape and size among the three mosses (Fig. 2J, K, L).

The three moss species were distinguishable by the characteristics of the leaves. As for the shape, in *L. riparium* the leaves (2.5–3 mm) were oblong–lanceolate with acuminate apex and, when present, the midrib reached beyond the middle of the leaf; they were also decurrent (the alar cells, present at the lateral and basal ends of the leaf, formed two narrow elongated wings which extended on the stem, but briefly, below the point of insertion of the leaf, towards the base of the gametophyte) (Fig. 3A). In *V. montagnei* the leaves (1.5–2 mm) were ovate-concave, acuminate with apiculate - dentate apex and they were falcate (curved like the blade of a sickle) (Fig. 3B). The leaves of *T. barbieri* (2.5–3 mm) were ovate–lanceolate briefly acuminate with margins slightly denticulate and not decurrent at the base; sometimes we observed foliar heterophyllia with lanceolate apical leaves and oval median-basal leaves (Fig. 3C). Regarding the shape and size of the leaf cells, the apical cells in *L. riparium* were linear and slightly rhomboidal 18–42 μm (Fig. 3D), also in *V. montagnei* and *T. barbieri* apical cells were rhomboidal but with different sizes, respectively 26–38 μm (Fig. 3E) and 12–27 μm (Fig. 3F). Median cells were similar to the apical cells in *L. riparium* (Fig. 3G) while in *V. montagnei* they were rectangular and larger around 17–92 μm (Fig. 3H); also in *T. barbieri* they had a similar shape to the apical cells but larger up to 28–42 μm (Fig. 3I). Regarding the cells at the base of the leaf, in *L. riparium* they were hexagonal – square 20–24 μm, with alar cells similar but slightly hyaline (Fig. 3J), in *V. montagnei* they were similar to median cells with undifferentiated alar cells (Fig. 3K) while in *T. barbieri* were rectangular - squares 12–22 μm and alar cells shortly quadrate (Fig. 3L).

Sporophytic generation has been observed in *L. riparium* and *V. montagnei* but not in *T. barbieri* (observation span over 5 years of use). In *L. riparium* the sporophyte had a single seta 1.5–3.0 cm long, reddish dark brown with a capsule brown to reddish of 1.5–2.0 mm (Fig. 4A). *V. montagnei* had a seta 1.5–2.0 cm long brown yellow with an inclined capsule of 0.5–1 mm (Fig. 4B).

3.3. Influence of plant growth regulators on gametophyte fragments

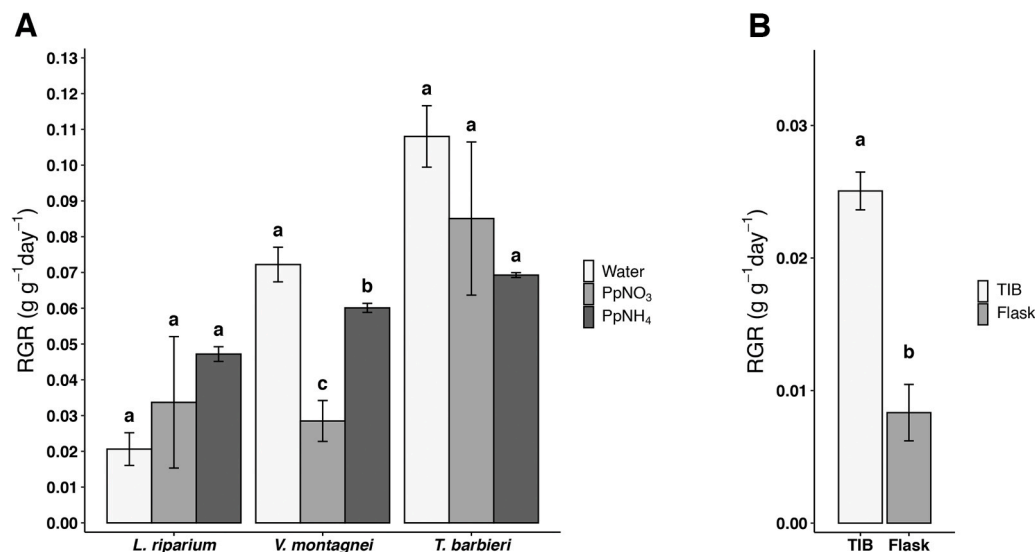To propagate the species in the shortest possible time, *in vitro*

Fig. 1. Mosses increase in biomass expressed as Relative Growth Rate (RGR g g^{-1}day^{-1}). A) Growth in 3 different liquid media water, PpNO$_3$ and PpNH$_4$ of *L. riparium, V. montagnei, T. barbieri* (in flask). B) Difference in growth of *L. riparium* between traditional flask method and TIB (temporary immersion bioreactor).
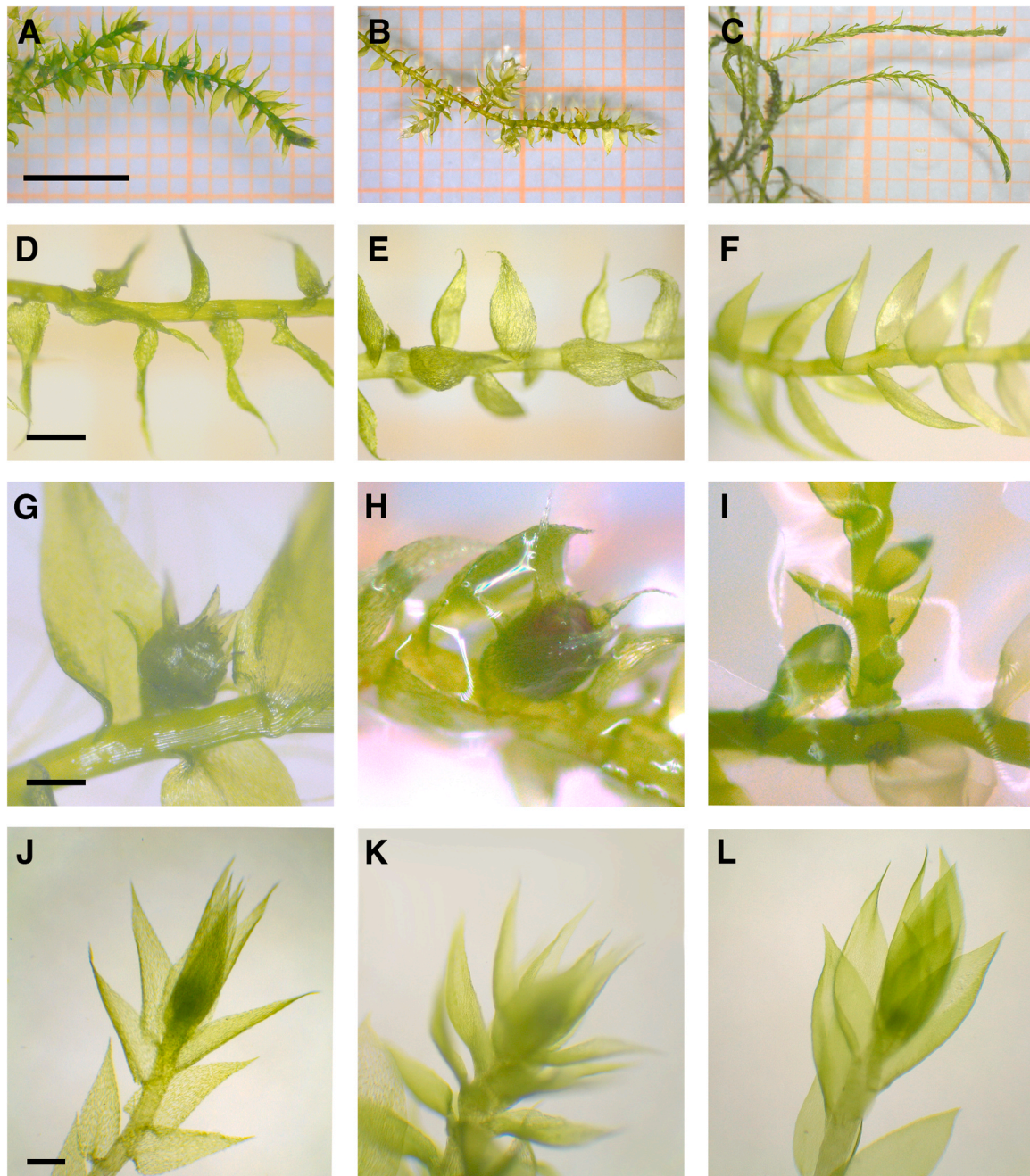
Fig. 2. Mosses morphology observed at different magnification. Gametophyte filament of (A) *L. riparium*, (B) *V. montagnei* and (C) *T. barbieri*. Leaves insertion on filaments of (D) *L. riparium*, (E) *V. montagnei* and (F) *T. barbieri*. Bulbils in (G) *L. riparium*, (H) *V. montagnei* and (I) *T. barbieri*. Tips of the (J) *L. riparium*, (K) *V. montagnei* and (L) *T. barbieri* filaments. Scale bar 5 mm for A, B, C; 1.5 mm for D, E, F; 1 mm for G, H, I; 0.5 mm for J, K, L.

micropropagation tests were carried out for the three species using two plant growth regulators: 6-benzylaminopurine (BAP, belonging to the cytokinins) and α-naphthalene acetic acid (NAA, belonging to the auxins), singularly or combined at different concentrations. The observation was carried out for 5 weeks. In one experiment, moss filaments underwent a two-week treatment and were then moved to a medium without plant growth regulators; in another experiment, filaments underwent a prolonged treatment on a medium supplemented with plant growth regulators. The evaluation of the response was made on apical gametophyte fragments, comparing them to the untreated control.

L. riparium, even if morphologically more similar to *V. montagnei* in control conditions, reacted to plant growth regulators in a different way that was more similar to *T. barbieri* (Fig. 5).

The fragments of the three species grown on PpNO$_3$ medium without plant growth regulators (control) maintained the green pigmentation over the 5 weeks and already after the second week new buds formed and gave rise to new ramifications (Fig. 5A, B, C), only in few cases, all referred to *T. barbieri* fragments, the elongation of the apex was observed.

When BAP was applied at low concentrations (0.1 mg/L and 0.2 mg/L) we observed in *L. riparium* a progressive loss of vitality, and already from the first week, the formation of gelatinous amorphous structures especially around the apex or the buds (already present before the treatment) which enlarged in time (Fig. 5D); on the contrary in *V. montagnei* BAP 0.1 mg/L led to the formation of new branches even more than in control conditions (Fig. 5E). Despite the closer
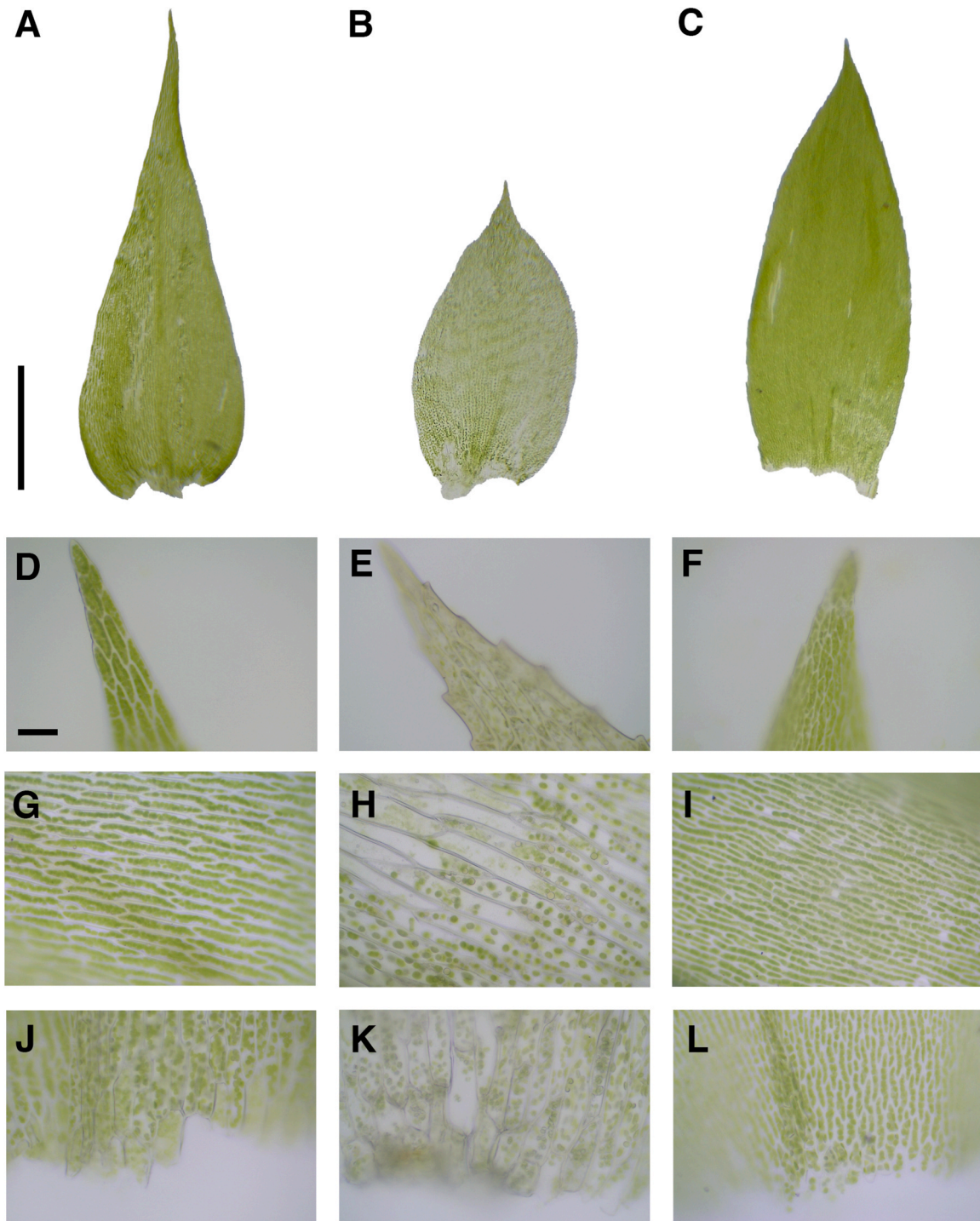
Fig. 3. Mosses leaves morphology. Leaf of (A) *L. riparium*, (B) *V. montagnei* and (C) *T. barbieri*. Leaf apical cells of (D) *L. riparium*, (E) *V. montagnei* and (F) *T. barbieri*. Leaf median cells of (G) *L. riparium*, (H) *V. montagnei* and (I) *T. barbieri*. Leaf basal cells of (J) *L. riparium*, (K) *V. montagnei* and (L) *T. barbieri* filaments. Scale bar 1 mm for A, B, C; 10 μm for D, E, F, G, H, I, J, K, L.

classification of *T. barbieri* with *V. montagnei* than to *L. riparium,* the fragments of *T. barbieri* also lost vitality with the formation of gelatinous amorphous structures, in a less evident way but similar way to *L. riparium* (Fig. 5F). When fragments were treated for only 15 days and then transferred to medium without plant growth regulators we observed a recovery of vitality in *L. riparium* and *T. barbieri,* with protonema formation (7 days after the transfer) and immediately new gametophytes (within 14 days after transfer) starting from the aberrant structures; in *L. riparium* 15 days treatments with BAP 0.2 mg/L, induced

more shoots after transfer (Fig. 5G) whereas in *T. barbieri* the best concentration was the BAP 0.1 mg/L (Fig. 5I). When moved to plant growth regulator-free medium, the *V. montagnei* fragments slowed development compared with prolonged treatment with no difference among the initial treatment (Fig. 5H).

Treatments with BAP 0.5 mg/L and 1 mg/L always induced the formation of amorphous gelatinous material, as swollen buds (especially in 0.5 mg/L), and over time the fragments lost their vitality; when transferred to plant growth regulator-free medium they did not recover
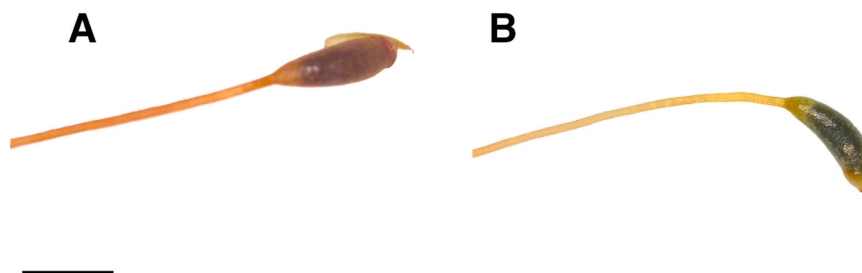
Fig. 4. Sporophyte of (A) *L. riparium*, (B) *V. montagnei* Scale bar 2 mm.

shortly; in *T. barbieri* in addition to these structures, protonema was formed especially after the transfer but it did not develop into new gametophytes. Also, in *V. montagnei* at these high concentrations, there was a loss of vitality of the fragments but they resumed growth with the formation of abundant protonema when transferred to medium without plant growth regulators.

In general, the effect of auxin NAA on *L. riparium* and *T. barbieri* induced rhizoids proportionally to increasing concentration (Fig. 5J, L). At all concentrations of NAA (0.1 mg/L, 0.2 mg/L, 0.5 mg/L and 1 mg/L) after the rhizoids appearance, fragments started losing their vitality, if transferred to medium without plant growth regulators after two weeks, the rhizoids persisted but, in one week, protonema and new ramifications (gametophyte) formed, to grow further in the following weeks (Fig. 5M, O). The induction of more gametophyte branches after transfer in NAA free medium was similar but more efficient than after cytokinin BAP treatment. Initial treatments with lower concentrations of NAA (0.1 mg/L and 0.2 mg/L) produced the best growth induction.

On *V. montagnei* 0.1 mg/L NAA induced new rhizoids and new ramifications both with the continuous treatment (Fig. 5K) and even more when the fragment was transferred to NAA free medium after 2 weeks (Fig. 5N); prolonged treatments at concentrations of NAA 0.5 mg/L and 1 mg/L led to abnormal formation of rhizoids and dark protonema (not shown).

When combined, the two plant growth regulators (Table 1) led to a progressive yellowing and loss of vitality in both the fragments of *L. riparium* and *T. barbieri* (Fig. 5P, R); when transferred to PpNO$_3$ without plant growth regulators, protonema started developing again. The best growth induction was observed after a limited 2 weeks' treatment with BAP 0.1 mg/L and NAA 0.2 mg/L (Fig. 5S, U). *L. riparium* recovered particularly well with the formation of new filaments. At higher concentrations, for example, BAP 1 mg/L – NAA 0.5 mg/L, the filament progressively lost vitality and hardly recovered. On the contrary, *V. montagnei*, except when treated with high concentrations, remained viable and produced ramifications even during prolonged treatments (Fig. 5Q, T).

3.4. Trace element uptake and biofiltration effect

The level of the trace element of interest in the three moss species grown in the same environment was determined prior to treatment (Fig. 6A). Cd and Li were found in low concentrations in the dry weight (DW) of the moss (less than 0.5 mg/kg DW), Cr was found in higher concentrations in *L. riparium* (3.4 mg/kg DW) than in *V. montagnei* and *T. barbieri* (0.6 and 0.9 mg/kg DW, respectively), and As and Pb was not found. B and Cu were more concentrated, with slight differences between species, but in the same concentration range. Zn concentrations were higher in *V. montagnei* and *T. barbieri* (755.2 and 617.2 mg/kg DW, respectively) than in *L. riparium* (174.5 mg/kg DW).

To prepare an environment to test the mosses' capacity to accumulate or remove trace elements and pollutants from water, we used an "experimental water" consisting of tap water supplemented with several elements As^{3+} (NaAsO$_2$ 4 μM), B^{3+} (Na$_2$B$_4$O$_7$ 300 μM), Cd^{2+} (Cd(NO$_3$)$_2$ 10 μM), Cr^{6+} (K$_2$Cr$_2$O$_7$ 12 μM), Cu^{2+} (CuSO$_4$ 4 μM), Li^{2+} (LiCl$_2$, 1 mM),

Pb^{2+} (Pb(NO$_3$)$_2$ 100 μM) and Zn^{2+} (ZnSO$_4$ 40 μM). To avoid saturating the mosses' absorption capacity, the concentration of the additional pollutants was one-fifth that utilized in previous studies (Papadia et al., 2020, Shakoor et al., 2023) or, in the case of B, present in contaminated environment (Caceres et al., 1992).

Since the effective amount of the trace elements absorbed by mosses is far from saturation, we estimated the removal capacity of 1 gr of moss from each species indicating the elements concentration after subtracting the initial values (Fig. 6A) as a percentage of the element present in the experimental water (Fig. 6B). The data then refer to a single gram of moss so that 2 g in the same volume of water may remove a double percentage of pollutants. Mosses did not efficiently acquire As, B, and Li. The three species had a similar attitude toward Cd, Cu, and Pb, with absorption rates of roughly 25%, 50%, and 30%, respectively. Cr was captured differently by the mosses; *L. riparium* can assimilate more of this trace element (53%) than *V. montagnei* and *T. barbieri* (34% and 47%, respectively). In the case of Zn, moss uptake efficiency was substantially different. *L. riparium* was able to absorb around 17% of the Zn present in the treatment solution, whereas *V. montagnei* and *T. barbieri* showed a negative balance (−6% and −22%, respectively), which could be attributed to a release of this element in the treatment solution.

To further understand the biofiltration activity of the three mosses we monitored the pH daily changes in the experimental water at 24 h intervals, observing that *V. montagnei* and *T. barbieri* are able to reduce pH of about 2 units in 72 h. The natural logarithmic plot of this kinetic shows that the plot angle for *L. riparium*, 0.26, is 62% (slower) than the others (Fig. 7A).

The variation of trace elements in the experimental water was evaluated 72 hours after immersion of the small amount of moss (Fig. 7B). Pb was almost eliminated from the experimental water and there was also a substantial reduction of Cd (−88% *L.r.*, −72% *V.m.*, −81% *T.b.*), Cr (−74% *L.r.*, 56% *V.m.*, 65% *T.b.*), and Cu (−70% *L.r.*, −66% *V.m.*, −70% *T.b.*). A poor uptake was observed for As (−3% *L.r.*, −3% *V.m.*, −4% *T.b.*), B (−9% *L.r.*, −10% *V.m.*, −8% *T.b.*), and Li (−5% *L.r.*, −1% *V.m.*, −4% *T.b.*). In the case of Zn, the decrease in concentration varied considerably between species; *L. riparium* reduced the treatment medium by 85%, *T. barbieri* reduced it by 68%, and *V. montagnei* reduced it by 39%. *L. riparium* appears to be the most performant moss in terms of element reduction in the medium in all circumstances where the reduction is statistically different between the mosses.

4. Discussion

Essential trace elements are typically present in plants at concentrations lower than 100 mg/kg DW (Barker and Pilbeam, 2015). It is important to have tools for the monitoring of their presence in the environment and bioindicators are among them. Bryophytes are efficient bioindicators (Debén et al., 2015) and, for the same characteristics, are also emerging as tools for bioremediation (Papadia et al., 2020). When dealing with trace elements, concentration variability among species may be relevant. It is essential to characterize the different capacities of bryophytes to accumulate the elements in traces. It is a
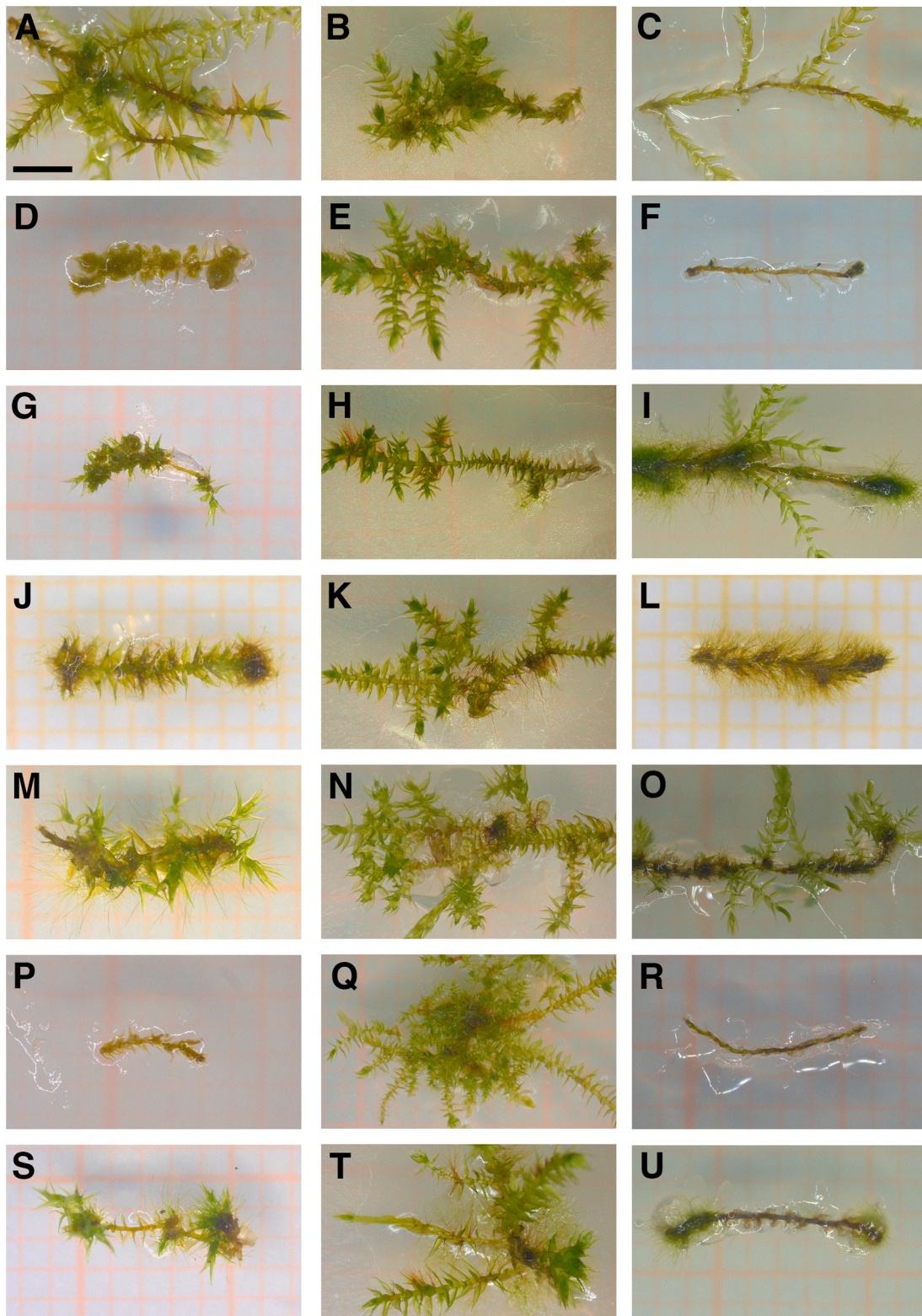
Fig. 5. Mosses fragments incubated on PpNO3. (A) *L. riparium*, (B) *V. montagnei* and (C) *T. barbieri* grown on medium without plant growth regulators (control). (D) *L. riparium*, (E) *V. montagnei* and (F) *T. barbieri* grown on medium with 0.1 mg/L BAP. (G) *L. riparium*, (H) *V. montagnei* and (I) *T. barbieri* treated for 15 days with 0.1, 0.2 and 0.1 mg/L BAP respectively and then transferred to medium without plant growth regulators for 3 more weeks. (J) *L. riparium*, (K) *V. montagnei* and (L) *T. barbieri* grown on medium with 0.2 mg/L NAA. (M) *L. riparium*, (N) *V. montagnei* and (O) *T. barbieri* treated for 15 days with 0.2 mg/L NAA and then transferred to medium without plant growth regulators for 3 more weeks. (P) *L. riparium*, (Q) *V. montagnei* and (R) *T. barbieri* grown on medium with with BAP 0.1 mg/L and NAA 0.2 mg/L. (S) *L. riparium*, (T) *V. montagnei* and (U) *T. barbieri* treated for 15 days with with BAP 0.1 mg/L plus NAA 0.2 mg/L and then transferred to medium without plant growth regulators for 3 more weeks. Scale bar 2 mm.
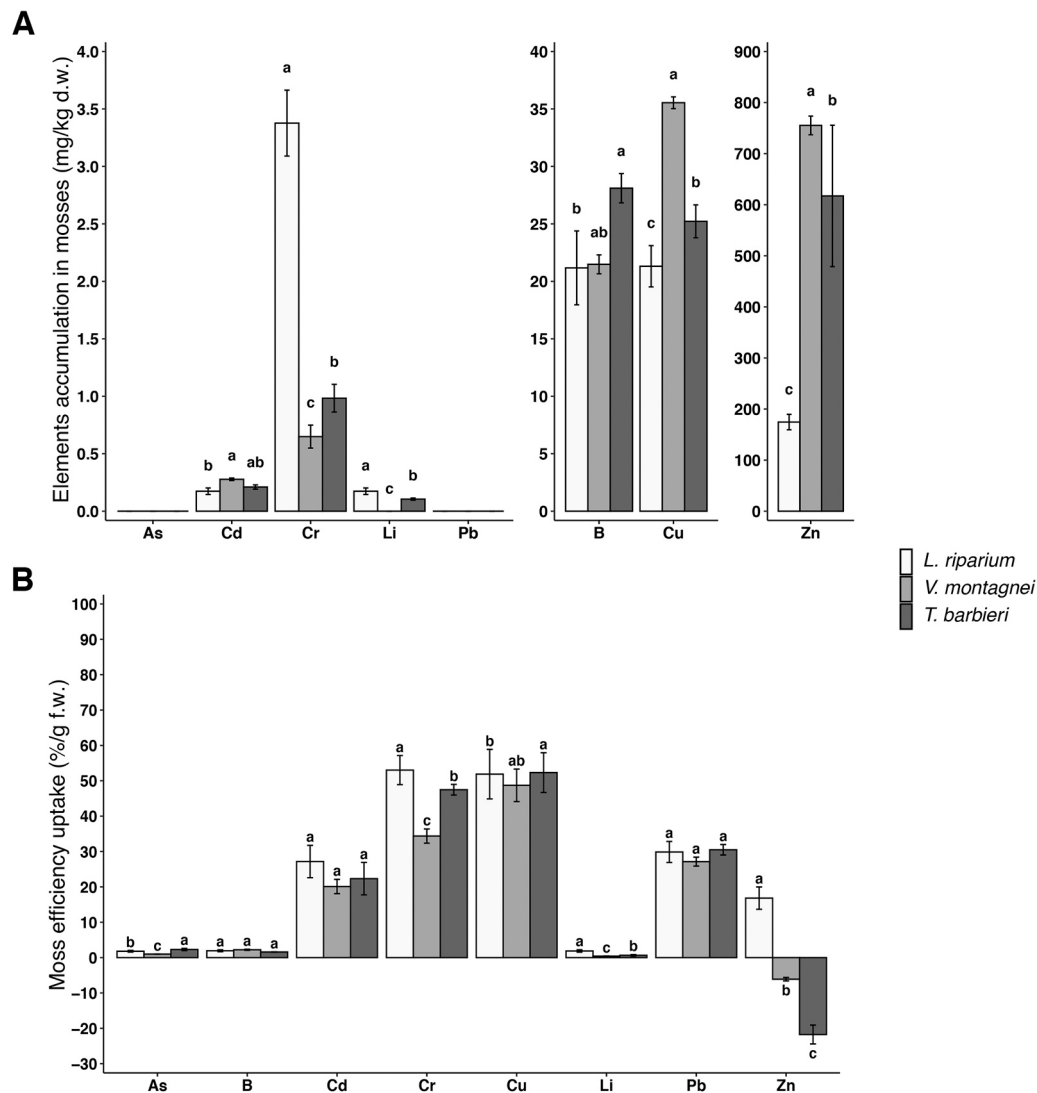
Fig. 6. Trace elements quantification in moss biomass. A) As, B, Cd, Cr, Cu, Li, Pb and Zn elements accumulation in the three moss species grown in control conditions before any treatment. B) Elements quantity variation in mosses after treatment, expressed as efficiency in the uptake. Statistical analysis performed by Kruskal Wallis test with a post hoc test using Fisher's least significant difference and a *p* value of 0.05. "Bonferroni" was used for adjusting the *p* values. Bars with same letters are not significantly different. Vertical bars show standard deviation. n = 3.

problem well distinct from the uptake or adsorption or bioaccumulation of pollutants.

To evaluate very low concentrations of trace elements, it is essential to work with axenic cultures of the moss to rule out the possibility of measuring elements contained in other organisms normally populating the moss mat, such as algae and bacteria. Then we established axenic cultures of three aquatic moss species, very common but not fully characterized. It is difficult to sterilize gametophyte explants because surface sterilization often causes cell death (Duckett et al., 2004). Starting from the capsule of the sporophyte is usually preferred since, after superficial sterilization, it can be opened and used as inoculum for sterile culture. The sporophytic generation may not be available (as in the case of *T. barbieri*), so we established protocols for gametophyte filament sterilization. One protocol proved to be very effective for *L. riparum* and *T. barbieri* but not for *V. montagnei* which required a different treatment, with lower efficiency. Several media have been used for bryophyte propagation *in vitro* (Duckett et al., 2004; Sabovljević et al. 2012). Based on our experience PpNO₃ and PpNH₄ medium, were preferred in solid and in liquid but also 60 µS/cm water was used with good results. The difference between the two PpN media lies in the addition of ammonium tartrate as a nitrogen source in PpNH₄ that favors

the growth of the chloronema and delays the differentiation into caulonema while, in PpNO₃ the lack of nitrates improves the appearance of the caulonema and the rhizoid (Jenkins and Cove, 1983). As predictable we noticed that this process depends largely on phytohormones and species (data not shown). *T. barbieri* had a higher relative growth rate compared to the other species in all conditions (PpNO₃, PpNH₄ and 60 µS/cm water); this was also found to have good growth in bidistilled water and this suggests that it could be propagated easily and at relatively low cost. *L. riparium* had the slowest growth comp slightly stimulated by the addition of ammonium (PpNH₄). *V. montagnei* grew better, with a preference for PpNH₄. To search a valid method to rapidly grow also *L. riparium* we tested SetisTM bioreactors. This technology was introduced a few years ago for mass propagation in several plant species and it is an approach that we also appreciated (Anglana et al., 2023) for distinctive benefits such as yield improvement during plant micropropagation, high-quality production and reduced impact on the environment. Furthermore, TIB allows to obtain plants free from most pathogens in controlled in vitro systems and can be also used in advanced techniques for genetic improvement. We found a clear improvement using the TIB system compared to the traditional liquid culture in flasks. However, grown mosses' macroscopic appearance is
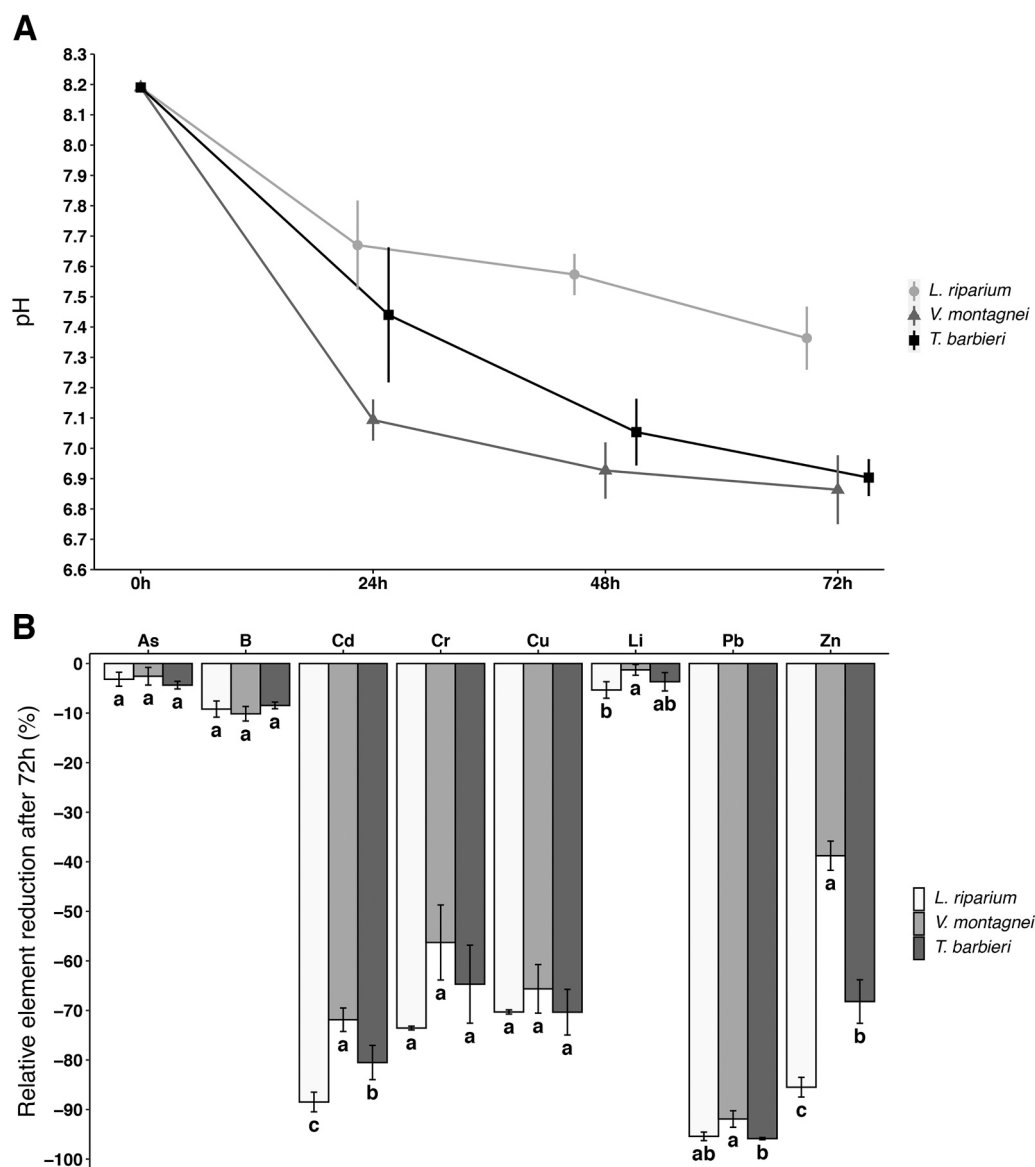
Fig. 7. Variation in the experimental water during moss treatment. A) pH variation in the time. Vertical bar indicates the standard deviation. n = 3. B) Relative reduction of the trace element from the experimental water after 72 h of treatment with the mosses. Statistical analysis performed by Kruskal Wallis test with a post hoc test using Fisher's least significant difference and a *p* value of 0.05. "Bonferroni" was used for adjusting the *p* values. Bars with the same letters are not significantly different. Vertical bars show standard deviation. n = 3.

similar.

We describe their essential traits because, despite their general characteristics may appear similar, each of them is different, with peculiarities for technological applications such as water and air purification or in the production of substrates for agriculture and plant nursing. In these moss species, after growing in optimal laboratory conditions, only Zn was above 100 mg/kg concentration showing the first differences. Zn concentration was 2 times higher in *L. riparium*, 7.5 times higher in *V. montagnei*, and 6 times higher in *T. barbieri*.

Usually, the identification and characterization of mosses take place through the recognition of characteristics of gametophytes such as habit and overall size, the position of the leaves in relation to the stem and their details, specific structures (bulbils, paraphyllia, buds and rhizoids) and characters of sporophyte such as seta, capsule, calyptra, operculum (Buck and Goffinet, 2000). During in vitro propagation of the three species, we obtained sporophytic generation in both *L. riparium* and *V. montagnei* but in this study, we focused on the characterization of the gametophytic phase only since in laboratory conditions these plants may

be maintained indefinitely in the gametophytic generation.

Systematic classification does not necessarily guarantee the possibility of using gametophyte morphological differences for classification. Despite belonging to two different families, *L. riparium* and *V. montagnei* showed similar characteristics regarding habit and overall size, they are both pleurocarpous mosses with monopodial growth while *T. barbieri*, more related to *V. montagnei*, is a acrocarp moss with sympodial growth. These differences were evident in all of our propagation systems, despite them may influence size and branching. During the observations, we detected the presence of rhizoids to a limited extent in all species. Remarkable differences were observed in the morphology of the leaf: relative position and distribution, shape and size. In microscopic observations, we also detected cellular differences in shape and size.

Considering the characteristics relevant to technological applications, it is to be noted that *T. barbieri* was growing in water much faster than the other studied species. This difference was then reduced in enriched media. This good growth rate may be related to the lack of sporophyte formation in *T. barbieri* in a very long time of laboratory use

in different conditions. In fact, it was hypothesized that this moss is, all over the world, a sterile clonal population (Wang and Jia, 2019).

The clear characterization of mosses in vitro is necessary for their use in laboratory for molecular biology or bioproduction applications. It is not necessary to respect natural conditions so that growth, as well as development, may be modified by the application of phytohormones (von Schwartzenberg, 2009). A few species have been used to study the effect of plant growth regulators on development in vitro, for example, *Funaria hygrometrica* Hedw. and *Physcomitrella/Physcomitrium patens* (Hedw.) Bruch & Schimp (Bijelović et al., 2004) but not all bryophyte species tested react in the same way to exogenously applied stimuli.

Treatments with plant growth regulators, specifically the cytokinin BAP and the auxin NAA, led to a different growth of the treated filaments compared to the controls. *L. riparium* and *T. barbieri* showed a similar response which was not advantageous but, on the contrary, detrimental when compared with growth on medium without plant growth regulators. On *V. montagnei* the treatments gave a different response favouring the growth even when applied for prolonged periods.

In some species of moss, including *P. patens*, the use of the cytokinin BAP has been shown to inhibit the growth of new rhizoids (Ashton, et al., 1979). It was also highlighted in some studies the abnormal growth of buds and the formation of callous structures when the treatment was prolonged with inhibition of gametophyte growth (Bijelović, et al., 2004). Similarly, we observed similar responses, with the formation of gelatinous amorphous structures, especially in *L. riparium* and less in *T. barbieri* with a general loss of vitality and with the formation of protonema and new gametophytes only after transfer of the moss fragment to medium without plant growth regulators.

The stimulant effect of NAA on rhizoid formation was already known and discussed (Bijelović, et al., 2004) and in fact, in the three species, the rhizoid formation was induced proportionally to NAA concentration and time of exposure; only in *V. montagnei* prolonged treatments at low concentrations did not alter growth and did not induce the abnormal growth of rhizoids. These effects appeared at concentrations higher than NAA 0.5 mg/L. After transfer on medium without NAA, in *L. riparium* and *T. barbieri* the formation of new gametophytes restarted from the protonema with a greater effect than treatments with BAP. Treatments carried out with both plant growth regulators did not give any positive effect except in *V. montagnei* at low concentrations.

In conclusion, for two of the species, the control medium, plant growth regulators-free, proved to be optimal for obtaining sufficient quantities of biomass in a short time and in these cases, the use of plant growth regulators could be justified only by different purposes, for example, treatments with BAP to induce callus or to induce and prolong the protonema stage on moss fragments or low viable material; treatments with NAA may be finalized to promote the growth of rhizoids for anchoring on specific substrates or to obtain a highly branched gametophyte.

Once axenic culture was established it was possible to perform experiments with an experimental water where trace elements were added as known concentrations of their salts: As^{3+} 4 µM, B^{3+} 300 µM, Cd^{2+} 10 µM, Cr^{6+} 12 µM, Cu^{2+} 4 µM, Li^{2+} 1 mM, Pb^{2+} 100 µM and Zn^{2+} 40 µM. These concentrations were arbitrarily determined in the range of 20% of concentrations normally used in studies on HMs uptake or concentration in soil considered polluted. We intended to investigate the performance of the mosses on "low" concentrations of trace elements. These conditions are normally not investigated but the possible differences in the accumulation of trace elements in different species are essential to use such organisms as bioindicators.

To evidence trace elements accumulation, it was essential to quantify the starting amount of the elements in the organisms. Even if grown in the same conditions the element's concentration was diversified. This may be due to the undefined age of the mats that, being very resistant and vital, may be not distinguishable if one or six months old. Occasional contaminations during culture can also be responsible of the differences. Anyhow the starting situation showed that only Zn was found at high concentration, higher than 100 mg/kg DW and differentiated among mosses. *L. riparium* had less Zn than the other mosses at the start of the experiment. The other investigated elements were not abundant, even if Cr appeared more present in *L. riparium*. Given this initial concentration, we were able to measure the variation of the elements concentration, as moss efficiency uptake (Fig. 6B).

Cu and Cr are the two contaminants removed more efficiently from the experimental water; Cu is an essential element that reached, at the end of the experiment, the threshold level of about 100 mg/kg DW in all the mosses; Cr is a toxic not essential element that can interfere in the accumulation of other elements, like Cu and Zn (Sharma et al., 2020), but this does not appear evident since these two elements concentrations remained stable or increased (Supplemental Figure S1). Heavy metals Cd and Pb were also removed efficiently from experimental water. It is well known that Cd interferes with Fe and Mn accumulation (Takahashi et al., 2011; Chang et al., 2020); in our system where the low concentrations may be below the threshold with which interference effects can be observed, Fe grew while Mn remained constant or slightly decreased (data not shown). Pb is a heavy metal that is commonly found in soils. Plants suffer severely from its toxicity (Zeng et al., 2007), yet they can tolerate large amounts (Collin et al., 2022). Because of this, Pb was one of the most abundant pollutants in the experimental water (100 µM), and the mosses appeared to tolerate and accumulate the element at high concentrations (Supplemental Figure S1), confirming results that emerged from prior work (Papadia et al., 2020). Interestingly, mosses may have shown a reaction to actively reduce Pb absorption since in our treatment, P concentration increased in the experimental water while decreasing in the mosses (data not shown). This may be related to the known phenomenon that adding phosphate to the soil reduces Pb bioavailability (Strawn, 2018). The mechanisms bringing P outside the moss cells remain to be investigated.

Strawn (2018) found that adding phosphate to the soil, on the contrary, increased As bioavailability; however, we did not observe this situation in our treatment because As was accumulated in the mosses in very low amounts, as previously reported (Papadia et al., 2020). B was found to reach about 100 mg/kg in all mosses when considering the total amount including pre-existing elements and the new accumulation (Supplementary Figure S1 repropose the data in Fig. 6 before subtraction of elements concentration before treatment) but its accumulation during the experiment was not efficient nor it was for Li, even if both elements were in high concentrations (300 µm and 1 mM, respectively) in the experimental water; B accumulation could have been disturbed by the amount of Zn in the experimental water (Long and Peng, 2023), and uptake of Li could have been influenced by the pH of the media (Aral and Vecchio-Sadus, 2008) or by unexplored differences between the mosses and vascular plants.

Zn showed the most diverse profile; *L. riparium* was able to absorb more Zn from the experimental water than the other two mosses in the experimental time, but the absolute concentration remained anyhow lower. As for Li, also for Zn, behavior could be explained by the high pH and bioavailability of Zn in the experimental water; in fact, for better absorption of Zn, a pH below 7.7 is required (Balafrej et al., 2020) and, since *L. riparium* was unable to quickly acidify the experimental water like the other mosses (Fig. 7A), this may be part of the diversification. In a previous work (Papadia et al., 2020) it was used a concentration of 200 µm of Zn and in 24 h *T. barbieri* was able to accumulate different amounts depending on its physiological state. Moss growth in low light was able to uptake more than 3000 mg/kg DW of Zn but the moss growth in conditions similar to the present study showed uptake of about 750 mg/kg DW, similar to the present outcome. This reminds us that all considerations have to be related to the specific growth conditions.

When the efficiency of the mosses' uptake (Fig. 6B) is compared to the relative element reduction in the experimental water after 72 hours (Fig. 7B), it is obvious that the mosses were not only able to actively accumulate some of the trace elements used but also to bind them at the

cell wall. Except for As, B, and Li, the concentrations of Cd, Cr, Cu, and, in particular, Pb were significantly reduced at the end of the treatment in the experimental water but not all can be found inside the moss biomass after washing. Other studies have demonstrated the ability of mosses to bind contaminants to their cell walls and washing in distilled water removes most of it (Papadia et al., 2020). Because mosses were washed before ICP analysis after the treatment, the difference in trace element absorption efficiency and relative element reduction in the experimental water after 72 hours is explained. Moreover, additional critical matters emerged.

It was previously shown in different studies that *L. riparium* efficiently accumulated Cu, Cd, Zn, Pb (Basile et al., 2012) and *T. barbieri* accumulated Pb, Cd and Cr (Papadia et al., 2020) but a comparative study was missing. In the conditions described here we observed significant accumulation of Cu, Cd, Zn and Pb for all species evaluated. The magnitude of the uptake was different from earlier studies, although this discrepancy may be explained by differences in the experimental conditions, such as treatment duration, metal concentration and combination and sample processing after treatment. However, the relative reduction of the metals from the media in our study was comparable the others (Basile et al., 2012, Papadia et al., 2020) and can help to make some consideration; Pb and Zn are very efficiently removed from the media independently of experimental conditions, on the contrary Cd and Cu uptake vary with parameters.

If we consider Zn, as an example, we note that at the initial conditions shown in Fig. 6A, *L. riparium* has less Zn than *V. montagnei* and *T. barbieri;* this may suggest that these species accumulate better the element but the variation during the experiment shows the opposite: *L. riparium* accumulates more Zn while *V. montagnei* and *T. barbieri* release it in the medium (Fig. 6B). Looking at the total amount of Zn in the mosses after the experiment we anyhow see that *V. montagnei* and *T. barbieri* contain a higher absolute amount of metal (Supplemental Fig. 1S). This means that the elements' accumulation depends on other parameters than medium composition in the short period. For example, it may depend on the relative age of the moss mat which is very difficult to estimate because of the extreme longevity of these organisms. Most likely the *V. montagnei* and *T. barbieri* biomass were more aged and we hypothesized that they reached already saturation for this specific element. On one side it reduces the value of the experimental data, on the other side it evidences important aspects of mosses biology that have to be taken into account.

In conclusion, we investigated three interesting aquatic mosses with great potential in the field of biomonitoring and bioremediation. We indicated hints for their cultivation to produce biomasses for further applications and characterized their behaviour in the absorption of common trace elements with further hints on their measurement. The protocols can be widely applied since some ecological concerns can be allayed. *L. riparium* is widespread on all continents and its biomass could be collected in any geographical region; *T. barbieri* appears sterile and can be easily contained.

It appears extremely interesting the complexity of the situation that requires the measure of elements before treatments and to take into account interspecific differences and the influence of pH and other elements concentration. The availability of standardized biological material appears crucial. These are simple but essential steps for the technological use of these organisms.

CRediT authorship contribution statement

Danilo Migoni: Methodology. **Piergiorgio Capaci:** Methodology, Investigation, Formal analysis. **Fabrizio Barozzi:** Writing – original draft, Supervision, Investigation, Formal analysis. **Chiara Anglana:** Writing – original draft, Methodology, Investigation, Formal analysis. **Gian Pietro DI SANSEBASTIANO:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Francesco Paolo Fanizzi:** Writing – review & editing, Supervision. **Makarena Rojas:** Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2024.103762.

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