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The ecology of peatland bryophytes – adaptations and competition in alkaline fens

Ph.D. Thesis

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Annotation

This dissertation thesis studies adaptations and competition of peatland bryophytes in alkaline fens. Specifically, it examines the existence of plant–plant interactions through volatile organic compounds in peatland bryophytes and surveys impacts of water chemistry on composition of bryophyte communities and species survival. It also aims to resolve principles behind calcicole–calcifuge behaviour of peatland bryophytes and mechanisms behind calcium toxicity.

Cover photo

Brown moss carpet with invading *Sphagnum* species. Photo by Eliška Vicherová.

Declaration [in Czech]

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

Vicherová E., Hájek M., Hájek T. 2015. Calcium intolerance of fen mosses: Physiological evidence, effects of nutrient availability and successional drivers. Perspectives in Plant Ecology, Evolution and Systematics 17, 347–359. (IF = 2.5)

EV and TH designed the experiments, EV conducted larger part of laboratory experiments, evaluated the data and wrote the manuscript with the assistance from TH and MH.

Vicherová E., Hájek M., Šmilauer P., Hájek T. 2017. *Sphagnum* establishment in alkaline fens: Importance of weather and water chemistry. Science of the Total Environment 580: 1429–1438. (IF = 5.6)

EV, TH and MH designed the experiments, EV conducted most of laboratory and field experiments, analysed the data with assistance from PS, and wrote the manuscript with the assistance from TH and MH.

Vicherová E., Kahoun D., Trebacz K., Fojtíková P., Hájek T. New insights to the mechanism of calcicole–calcifuge behavior in bryophytes (manuscript).

EV designed and conducted the experiments, measured membrane potential and Ca^{2+} accumulation, analysed data, assisted with GSH/GSSG measurements, and wrote the manuscript with the assistance from TH.

Vicherová E., Glinwood R., Hájek T., Šmilauer P. Ninkovic V. (in prep). Bryophytes can recognize their neighbours through volatile organic compounds (accepted to Scientific Reports, IF = 4.1)

EV, TH, RG and VN designed the experiment, EV conducted the experiment, made VOCs collection with assistance from RG, analysed data with assistance from PS, and wrote the manuscript with the assistance from TH, RG and VN.

Contents

Chapter 1. General introduction

Boreal and temperate peatlands are one of the few ecosystems dominated by bryophytes. Acting as an ecosystem engineers, peatland bryophytes shape the composition of plant communities, influence peatland hydrology and water chemistry and are responsible for peat accumulation (Granath et al. 2010, Hájek et al. 2011, Singh et al. 2019). Mineral-rich mires (i.e. alkaline fens) are one of the species richest but also the most threatened of these ecosystems (Hájek et al. 2006). Land use changes of past century connected with intensive agriculture caused a severe destruction of alkaline fens, the destruction being recently enhanced further by a climate change. As a result, they are protected across Europe through EU Habitat Directive (Council Directive 92/43/EEC), including the Czech Republic.

According to the Species pool hypothesis (Pärtel et al. 1996; Zobel 1997; Hájek et al. 2007), composition of bryophyte communities is determined by three filters: local history and species migration, characteristics of the environment and species interactions (Udd et al. 2015). Asuming that the local history and species migrations do not pose limitations, the ability of species to survive in the peatland (species fundamental niches) is determined solely by environmental factors (hydrology and water chemistry being the most prominent), while the final composition of the bryophyte community (species realised niches) is affected by species interactions (particularly by competition and *plant communication*).

The aim of this dissertation is to examine the most important features that govern composition of bryophyte communities in peatlands. The study aims particularly on topics overlooked by peatland ecologists or those that are, in the long term, unresolved. The **Papers I, II and III** examines the impact of water chemistry on composition of the bryophyte community and survival of individual species, and aims to resolve principles behind calcicole–calcifuge behaviour of peatland bryophytes and behind calcium toxicity. **Paper IV** examines plant interactions in bryophytes, i.e. interactions that were found crucial for functioning of vascular plant communities and have never been studied in bryophytes.

The Papers are preceded by a review that introduces readers to the problematics of species survival in bryophyte communities. It introduces peatland habitats and concentrates particularly on species survival and adaptations evolved along the gradient of calcium availability (calcicole–calcifuge behaviour), mechanisms behind calcium toxicity and on species interactions that are important for survival of individual species in the bryophyte community.

1.1 Peatland habitats

Based on hydrological conditions, peatlands are divided into ombrotrophic bogs fed by precipitation and minerotrophic fens fed by groundwater. The fens can be separated further by groundwater chemistry, especially by the concentration of calcium ions and pH (i.e. concentration of calcium bicarbonate, Rydin et al. 1999, Hájek and Hájková 2007). While bog and poor fen waters have low concentrations of calcium ions ($[Ca^{2+}] < 5$ mg/L) and low pH (< 5.5), pH of rich and alkaline (extremely rich and calcareous) fens usually exceeds pH 7. $[Ca^{2+}]$ in rich and extremely rich fens ranges between 30–90 mg/L. $[Ca^{2+}]$ in calcareous fens are even higher and calcium ions precipitate into calcium carbonate (Hájek et al. 2002, Rydin and Jeglum 2006). In extremely rich and calcareous fens, calcium is the most abundant metallic ion and species that are not adapted to such high concentration, e.g. species of the genus *Sphagnum*, suffer from calcium toxicity, especially when the high $[Ca^{2+}]$ is accompanied by high pH (Clymo 1973). In contrast, the low $[Ca^{2+}]$ in bogs or poor fens might lead to calcium deficiency in species not adapted to these conditions. The minimum concentration of Ca^{2+} that limits growth of peatland bryophytes is not known, but the importance of calcium on cell wall formation and cell metabolism was shown on the example of several bryophytes (Fulford et al. 1947, Geldreich 1948, Nakajima et al. 1981).

Apart from toxicity or deficiency of Ca^{2+}, peatland bryophytes must deal with toxicity or deficiency of other metals or phosphorus. In acidic peatlands, species must cope with metal toxicity, especially Fe^{2+} (Rozbrojová and Hájek 2008). The solubility of iron depends mainly on its reduction state. The toxic concentration of the reduced form Fe^{2+} on

peatland bryophytes is not known, neither are the mechanisms that allow calcifuge bryophytes to survive high [Fe^{2+}]. In lichens and vascular plants, calcifuge species have lower intracellular uptake of iron ions (Fe^{2+}or Fe^{3+}) than calcicole species, especially in low pH (Snowden and Wheeler 1993, Paul et al. 2009). The possibility that calcifuge bryophytes might have similar mechanisms of dealing with high [Fe^{2+}] as calcifuge vascular plants and lichens was examined in **Paper I.**

Calcareous peatlands have low concentration of accessible phosphorus and micronutrients, especially iron, since these elements are usually in precipitated form (Hájek and Hájková 2007). The phosphate precipitates with calcium, while iron mainly as bicarbonate or (hydr)oxides but also as phosphate. By that means, bicarbonate inhibits iron uptake by plants (Woolhouse 1966). We do not know the minimal concentration of both elements that are required for growth of peatland bryophytes; neither do we know the mechanisms by which calcicole bryophytes overcome possible iron and phosphorus deficiency. The significance of iron and phosphorus deficiency on species survival was studied in **Paper I.**

1.2 Species survival along the gradient of calcium availability

Calcifuge–calcicole behaviour (i.e. reaction on Ca^{2+} availability and pH) is a very prominent phenomenon in peatland ecosystems, connecting "behaviour" of bryophytes with vascular plants and phylogenetically basic animals. The universal response of organisms to calcium ions is probably caused by integration of calcium into cell signalling; the cells react on developmental or environmental stimuli through specific changes in concentration of calcium ions ([Ca^{2+}]) in cytoplasm (White and Broadley 2003). Any violation of intracellular calcium homeostasis thus results in cell death. Additionally, calcium has an important structural role in membranes and the plant cell wall (Hepler and Winship 2010).

An extensive research of calcifuge and calcicole strategies in the past 100 years brought about several hypotheses dealing with adaptations of calcicole plants to calcareous habitats, which could be thought relevant also for bryophytes. They relate either to direct calcium toxicity (hypotheses 1–3

listed below) or emphasize the importance of other nutrients on species survival (4–5).

1) High extracellular [Ca^{2+}] disrupts intracellular calcium homeostasis, which lead to plant death.
2) Calcium ions associated with cell wall pectins block cell wall loosening in high pH. The plant growth is thus inhibited and consequently the plant dies or is outcompeted (Proseus and Boyer 2006, Hepler and Winship 2010).
3) Cation exchange sites may buffer excessive concentrations of toxic elements (Ca or other ions) by binding them and thus reducing the amount that reaches the plasma membrane (Brown 1982).
4) In a solution of high pH and [Ca^{2+}], Ca^{2+} "condense" (i.e. is bound in higher concentration than would correspond to the cation exchange capacity) on the charged binding sites of the cell wall creating a barrier at the cell surface and blocking absorption of cations with lower valency to cation exchange sites. Those ions then might become deficient (Dainty and Richter 1993).
5) The calcicole and calcifuge species differ in the uptake of iron and phosphate; the low nutrient uptake in calcifuges leads to Fe and P deficiency in calcareous habitats and cause chlorosis (Snowden and Wheeler 1993, Zohlen and Tyler 2000, Paul at al. 2009).

All the mentioned hypotheses comprising processes either on the cell wall or in cell protoplast, will be discussed in the following chapters of the review and were approached in **Paper I**. The first hypothesis was more thoroughly studied in **Paper III**. The last hypothesis (5), was discussed in a previous chapter of this review and was approached in **Paper I**.

1.2.1. Mechanisms behind calcium toxicity: cell wall properties – possible link with calcium tolerance

Negatively charged pectic polysaccharides are essential components of the plant cell wall. In bryophytes, pectins are rich in galacturonic acid and when completely ionized (at pH>6) they are present as long chains of COO^-

groups (Dainty and Richter 1993). The chains of polygalacturonic acids are able of crosslinking to each other through hydrogen bonding and divalent cation cross-bridging (mainly by Ca^{2+}), creating the 3D structure of the cell wall (Hepler and Winship 2010). Without calcium cross-bridging, the cell wall does not have necessary firmness (Fulford et al. 1947, Geldreich 1948). However, in solutions of high [Ca^{2+}] and pH, the saturation of COO^- by calcium ions leads to cell wall rigidity (Fraeye et al. 2009), precluding cell wall loosening and thus supressing growth (Proseus and Boyer 2006, Hepler and Winship 2010).

The rigidity of the cell wall can be weakened if some carboxyl residues of polygalacturonic acids remain esterified and the de-esterification is finished after the cell ceases to grow. This strategy is used during pollen tube growth (Bosch et al. 2005) and might be frequently used by apical meristematic cell of bryophytes growing in hollows in calcareous peatlands.

The loosening of the cell wall is also affected by expansines. These proteins, active in low pH (McQueen-Mason and Cosgrove 1994), loosen noncovalent adhesion between polysaccharides, specifically cellulose and hemicellulose (Yennawar at al. 2006). The apoplastic pH can be to some extend lowered by H^+-ATPase (Frías et al. 1996), however, the extensive buffering capacity of calcium bicarbonate-rich waters of alkaline fens would seem to make the pH lowering impossible. Therefore, growth limitations were suggested as one of possible reasons behind the absence of non-specialized bryophytes from hollows and pools in calcareous fens. Moreover, it may explain the small growth rate of *Sphagnum* species in solutions of high pH, even when that was accompanied by minimal concentration of calcium ions (Clymo 1973). The possible limitations of cell wall loosening were examined and discussed in **Paper I.**

High cation exchange capacity (CEC) may also represent some advantage in calcareous habitats since calcicole species growing on limestone had 3–4 times higher CEC than calcifuge species (Bates 1982). The higher CEC of calcicole species was observed also in other studies (Koedam and Büscher 1983, Büscher et al. 1990, Soudzilovskaia 2010 – after exclusion of *Sphagnum* species and allowing for some exceptions). It is possible that species of rich and calcareous fens could use high CEC as a buffer binding excessive Ca^{2+} (Bates 1982, Brown 1982). The binding might also lower

cell wall pH if Ca^{2+} are exchanged for H^{+}. This hypothesis, however, may work only if the incoming concentration of calcium bicarbonate does not exceed charge-equivalent concentration of the new COO^{-} groups created by plant growth. In fens, this can occur in hummocks, i.e. above the influence of the fen-water table. The high CEC of *Sphagnum* (Clymo 1963) might help this species to lower extracellular Ca^{2+} concentration to an acceptable level, notably in dry summer conditions when the upward capillary flow is the only water source. In **Paper II,** we examine the acidifying ability of *Sphagnum* species and other characteristic fen species (brown mosses) and discuss the relevance of this ability in the concept of species survival and expansion in alkaline fens.

Cation exchange sites in the cell wall might also affect the cell nutrition in calcareous mires. It was suggested by Dainty and Richter (1993) that Ca^{2+}, as a bivalent cation with high affinity to negatively charged cell wall pectins, may condense (bind) on this polyanion and thus blocks the adsorption of cations with lower valency to the cation exchange sites. Those might then become deficient (Dainty and Richter 1993). This hypothesis corresponds with the results of Robson and Loneragan (1970) that showed that Mn toxicity was alleviated by high [Ca^{2+}]; the presence of Ca^{2+} also lowered the uptake of Mn^{2+} in protoplasts. However, the same results were observed for Al^{3+} and other metals (Kinraide et al. 2004, Guo 2006) with higher valency to cation exchange sites than calcium has. We have shown in **Paper I** that cation exchange sites in the cell wall of living cells are not saturated by Ca^{2+} under the field condition of alkaline fens.

Metabolic pathways concerning calcium homeostasis are probably much more important for species immediate survival in calcareous habitats than processes in the cell wall. The regulation of calcium homeostasis and the intracellular adaptations to calcium toxicity are described in the following chapter of the review and were studied in **Papers I** and **III**.

1.2.2. Mechanisms behind calcium toxicity: intracellular processes

The signalling function gives calcium ions a special position among other plant nutrients. Consequently, its intracellular concentration must be strictly regulated, free calcium in cytoplasm is kept in very low concentration (~

200 nM; Trebacz et al. 1994) despite its high (mM) concentration in apoplast or mM/μM concentration in vacuole. Any disruption of Ca^{2+} homeostasis is directly linked with pathways leading to apoptosis. Since Ca^{2+} can coordinate six to eight uncharged oxygen atoms that change conformation upon binding with calcium (Sanders et al. 1999) it makes the Ca^{2+} ideal for cell signalling (White and Broadley 2003, Spalding and Harper 2011).

The electrochemical gradient of Ca^{2+} between cytosol and apoplast is very steep in calcareous environment. Considering cytosolic–apoplastic [Ca^{2+}] difference, it is 500 times higher in poor fens, but 10 000 times higher in calcareous fens. If the cells of calcifuge species managed to regulate calcium homeostasis and cellular signalling in such a high [Ca^{2+}], even the calcifuges should survive in calcareous habitats for at least a limited period of time (before being eliminated by competition or factors indirectly connected with calcium toxicity). Since a number of calcifuge *Sphagnum* species do not survive in calcareous habitats for more than two weeks (results described in **Paper I**), the inability of strict calcifuges to survive in rich and calcareous fens is likely given by a disruption of calcium homeostasis, involving Ca^{2+} influx and/or efflux.

Ca^{2+} influx through ion channels

Calcium enters cytosol through channels in plant membranes, predominantly tonoplast, endoplasmatic reticulum and plasma membrane (PM). Given the Ca^{2+} concentration gradient and negative membrane potential, the Ca^{2+} flow to cytoplasm is passive, not requiring energy supply. The PM Ca^{2+} channels regulate overall influx of calcium to the cell, Ca^{2+} channels in other membranes are used only for signalling. Since the membrane identity, local elevation in [Ca^{2+}] and the type of perturbation are fully or partly responsible for the activation of a defined signal pathway (Plieth 2001, Webb at al. 2001, White and Broadley 2003), the correct function of all Ca^{2+} channels is important.

Calcium channels can be classified according to their voltage dependence and may be regulated by other factors: membrane-stretching, interaction with the cytoskeleton, the ligand binding or by covalent modification (White

2000). Moreover, some of them can be activated or inhibited by increased cytosolic [Ca^{2+}]. If the calcareous environment has some effect on calcium channels, it should be in plasma membrane since those channels are in direct contact with apoplast.

Calcium enters cytosol through three types of PM Ca^{2+} channels: depolarization-activated (DACC), hyperpolarisation-activated (HACC) and voltage-independent (VICC) cation channels. The VICC channels are opened at all physiological membrane potentials. They are considered to procure basal Ca^{2+} influx necessary for calcium homeostasis in unstimulated cells (White 2004). Contrary to DACC and HACC, they are permeable not only for Ca^{2+} but also for other bivalent or monovalent cations and as mostly open, they mediate Na^+ or toxic metal infux, causing metal toxicity in a saline or heavy-metal-polluted environment (White 1999, White and Broadley 2000). The regulation of VICC channels by increased cytosolic [Ca^{2+}] has never been tested, as well as its possible role in enhancement of calcium toxicity in a calcareous environment.

Contrary to VICC, the DACC and HACC are strictly controlled by membrane voltage (V_m) although their type of regulation is quite different. The DACC channels are activated upon depolarization (V_m> resting potential of about –180 mV), and Ca^{2+} influx is compensated by the K^+ efflux through the outward-rectifying K^+ channels (Miedema et al. 2001). The K^+ efflux have hardly any effect on cytoplasmic [K^+] since, as a major cation and main osmoticum, K^+ is present at cytoplasm in mM level (Trebacz et al. 1994, Miedema et al. 2001).

The HACC channels are activated upon membrane hyperpolarisation (V_m< resting potential), e.g. during apical cell growth or in stress signalling. Their function is closely tied with H^+-ATPases that counterbalance change in membrane potential caused by HACC opening. It seems that calcicole and calcifuge species may use this diversity of calcium channels in regulating the amount of Ca^{2+} entering to cytosol. We have examined this hypothesis in **Paper III.**

Ca^{2+} efflux through Ca /H exchangers and Ca-ATPases

The elevated concentration of Ca^{2+} in cytosol can be reduced by means of two different transport systems: Ca^{2+}-ATPases and Ca^{2+}/H^{+} antiporters. The transporters actively remove Ca^{2+} from cytosol against its electrochemical gradient, requiring energy of ATP. Ca^{2+}-ATPases are high affinity (K_m = 1–10 μM), low capacity pumps, thought to function mainly in fine regulation of low cytosolic [Ca^{2+}] (Geisler at al. 2000), whereas Ca^{2+}/H^{+} antiporters with low affinity (K_m = 10–15 μM) and high capacity for Ca^{2+} transport can quickly remove elevated calcium concentration. Thus, they are likely to modulate cytosolic [Ca^{2+}] perturbations (White and Broadley 2003).

The pumps and exchangers transport Ca^{2+} to apoplast or to intracellular organelles. There, the calcium ions are either used in various biochemical functions or are stored and used in signalling (Sanders et al. 1999). The main storage compartment in plants is vacuole where the Ca^{2+} can be accumulated even to a mM concentration (Dunn at al. 1994). Many vascular plants can immobilize Ca^{2+} in vacuoles by making oxalacetate crystals (Webb 1999). Yet this ability was not detected in bryophytes.

Plant cells have many different types of Ca^{2+}-ATPases and Ca^{2+}/H^{+} antiporters. While their main function remains the same, they may differ in regulation, allowing cells to adapt to environmental changes (Garciadeblas 2001, Manohar 2011).

The species survival in calcareous habitats is linked with expression of Ca^{2+}-ATPases and Ca^{2+}/H^{+} exchangers in cell membranes. High external [Ca^{2+}] stimulate expression of both transporters in vacuolar membrane (Garciadeblas 2001, Kamiya et al. 2006), increasing Ca^{2+} accumulation in vacuoles (Conn 2011). The importance of Ca^{2+}/H^{+} antiporters for species survival in a calcareous environment was demonstrated on single-celled apicomplexan parasites, *Plasmodium* and *Toxoplasma*. When the function of antiporters was fully eliminated and calcium homeostasis regulated only by Ca-ATPases, the survival of parasites was fully dependent on external [Ca^{2+}] (Guttery at al. 2013). Similar results were found in *Arabidopsis*, where partial elimination of Ca^{2+}/H^{+} antiporters (knock-out of CAX1) lead to sensitivity to high levels of Ca^{2+} (Hirschi 2001). Moreover, the

elimination of CAX1 alleviate Ca^{2+} deficiency in Mg^{2+}-rich environment (Bradshaw 2005).

The reason why plants accumulate Ca^{2+} in vacuoles instead of transporting them back to apoplast is not known but it seems that the accumulation rate of calcicole and calcifuge species differ (results provided by and discussed in detail in **Paper III**).

The importance of Ca^{2+} efflux in calcium homeostasis and the difference in the vacuolar Ca^{2+} accumulation rate suggests that calcicole fen bryophytes might differ in regulation or composition of Ca^{2+}efflux complexes, and this hypothesis is studied and discussed in detail in **Paper III.**

1.3 Plant interactions affecting species survival in peatland bryophyte community

Interactions are crucial for the survival of individuals in plant communities, thus effecting composition of the plant community itself. Surprisingly, the study of plant interactions in peatland bryophyte ecology was focused almost solely on competition; other interactions (e.g., allelopathy in bryophytes, Whitehead et al. 2018; bryophyte–fungi or microbe interactions, Davey and Currah 2006, Carella and Schornack 2018) were studied rarely. Even after the discovery of *plant communication* three decades ago that led to an increasing comprehension of the significance the interactions have on functioning of ecological communities, *plant communication* has never been studied in peatland ecosystems or bryophyte communities in general. Hence, we have decided to examine this neglected topic in **Paper IV**.

The following subchapters discuss the main particularities of bryophyte competition in peatlands and aspects of plant communication that could have crucial impact on peatland ecosystems and functioning of peatland bryophyte communities.

1.3.1. Competition among peatland bryophytes

Bryophytes are a phylogenetically basic group of land plants lacking developed vascular tissues that enables efficient water conductance and

water economy. Therefore, bryophytes tolerate desiccation (Hájek and Vicherová 2014) and in response to this limitation they form uniform, relatively compact bryophyte layer with minimal vertical differentiation.

Competition among peatland bryophytes reflects the growth strategy and water economy of the individual species. It is generally referred to as competition for space (Rydin 1997), where individual bryophyte shoots compete for available resources (e.g. light, nutrients) while the compactness and uniformity of the layer protect the whole community against desiccation.

The opinions about competitive exclusion in peatland bryophyte communities differs according to peatland type, the amount of available resources and a type of study. Considering species with similar ecological requirements (species with wide overlapping niches) and similar competitive abilities in a given peatland type, the small occasional disturbances and changes in environmental factors are generally sufficient to enable species coexistence and prevent competitive exclusion (Rydin 1993, Mälson and Rydin 2009). In contrast, competitive exclusion can occur when environmental changes facilitate survival and expansion of a strong competitor into the bryophyte community. If the stronger competitor can also act as an ecosystem engineer, changing the environment to its own ecological requirements, the competitive exclusion and substantial changes in the bryophyte (or even vascular plant) community can be very swift. This ecological switch can occur when *Sphagnum* species (e.g. *S. teres, S. flexuosum*) expand to alkaline or rich fens of *Caricion davallianae* or *Sphagno warnstorfii-Tomentypnion nitentis* association when the mineral rich fen groundwater is absent from bryophyte layer due to drought. The competitive dominance of a stronger competitor can be bolstered further by nutrient enrichment or other factors (Kooijman and Bakker 1995). The conditions required for successful establishment of *Sphagnum* in alkaline fens and the subsequent initiation of succession to poor fens was studied in **Paper II,** while **Paper I** examines fundamental niches of peatland bryophytes along the gradient of Ca^{2+} availability which stands behind the classification of peatland ecosystems.

1.3.2. Species interactions that affect competition, survival and tolerance to the environment

Plants interact with other plants or organisms in variety of ways, some known for decades or centuries (e.g. pollination, Lovell 1912; perception of light cues, useful e.g. for neighbor detection, Smith 1982). Our understanding of plant interactions changed with a discovery of plant communication (Baldwin and Schultz 1983, Rhoades 1983). With increasing knowledge, it became gradually evident that plants use light (Keuskamp et al. 2010), touch (De Wit et al. 2012, Elhakeem et al. 2018, Markovic et al. 2016, 2019), vibrations (Appel and Cocroft 2014) and various means of chemicals (volatile organic compounds, Tumlinson 2014; chemicals transported via mycorrhizal network, Babikova et al. 2013; exudates, Biedrzycki et al. 2010) to communicate in an intricate web of multitrophic interactions that condition ecosystems functioning.

Apart from light cues, volatile organic compounds (VOC, air-borne chemicals) are nowadays the best-known cues in plant interactions, generally called *plant communication* (Ninkovic et al. 2016). VOCs are lipophilic, low molecular-weight chemicals with high vapour pressure at ambient temperature, produced by organisms in all terrestrial and marine ecosystems as part of species secondary metabolism (Dudareva et al. 2013, Fink 2007). Considering the chemistry, plant VOCs are very close to pheromones produced by insects (Reddy and Guerrero 2004). The blend of volatiles produced by plant individuals depends upon species identity, life history and health, thus carrying important information for neighboring plant individuals or other groups of organisms. Those organisms that can detect and decipher this information can use VOC for *communication*.

The fitness of vascular plant individuals is largely affected by herbivores and parasites. Consequently, the ability to attract predators of these herbivores or parasites, by changing VOCs profile, has an important ecological advantage. Bryophytes are generally considered poorly digestible organisms with low herbivory or predation pressure (Haines and Renwick 2009). Therefore, even if the ability to attract predators is shared with vascular plants, it has probably no crucial effect on species survival in peatland bryophyte communities.

In contrast, the fitness of peatland bryophytes, similar to vascular plants in general, is greatly affected by competition and by the stress caused by environmental factors. Since plant-to-plant communication in vascular plants has the form of eavesdropping (Ninkovic et al. 2016), it provides warning against superior competitors in near proximity or warns against environmental stress. Eavesdropping thus provides vascular plant individuals time to adjust their growth or to adapt to the oncoming stress (by metabolism changes in a process of hardening, Caparrotta et al. 2018) and to survive. The ability to recognize warning cues against strong competitors or oncoming environmental stress would present a strong ecological and evolutionary advantage for survival in the bryophyte community. We demonstrate in **Paper IV** that plant–plant communication exists in bryophytes and discuss the importance of this discovery in the concept of peatland bryophyte ecology.

1.4. Aims of the thesis

The thesis studies adaptations and competition of peatland bryophytes in alkaline fens.

Paper I was aimed at studying competitive hierarchies of peatland bryophytes along the gradient of calcium availability, on calcicole–calcifuge behaviour of peatland bryophytes and on selected aspects of the mechanisms behind calcium toxicity.

Paper II was aimed at the expansion of *Sphagnum* species to alkaline fens.

Paper III was aimed at finding cellular mechanisms behind calcium toxicity and tolerance.

Paper IV was aimed at finding evidence of plant–plant communication in bryophytes and at describing its impact on peatland bryophyte communities.

1.5. References

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Chapter 2.

Calcium intolerance of fen mosses: Physiological evidence, effects of nutrient availability and successional drivers

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Abstract

Species composition of peatlands is determined by the dominance of either *Sphagnum* or non-sphagnaceous (brown) mosses. *Sphagnum* species are more or less intolerant to alkaline waters rich in calcium bicarbonate, but the physiological background of this intolerance is poorly understood. Recently, sphagna have been widening their realized niches, expanding to alkaline brown-moss fens and altering their functioning. One possible reason is increased nutrient availability, but existing evidence is equivocal. We approached this problem by a series of laboratory experiments with 15 fen moss species cultivated submerged in solutions corresponding to natural poor- to rich-fen waters. We tested basic ecophysiological mechanisms of calcium tolerance (ion compartmentalization, cell-wall cation-binding properties, phosphorus and iron uptake), the breadth of fundamental pH/calcium niches for protonemata and adult plants, interspecific competition, and relationships between nutrient availability and pH/calcium tolerance. Our results suggest that calcium toxicity in calcifugous bryophytes is caused by insufficient control over the balance of intracellular Ca^{2+} uptake/efflux. Cell-wall cation-exchange sites of living mosses remain unsaturated with Ca^{2+} even in calcareous solutions, contradicting the proposed inhibitory effect of Ca^{2+}-oversaturation on cell-wall expansion and monovalent cation uptake. Growth and biomass accumulation of brown mosses was highest in alkaline fen waters, but they could also survive and germinate in poor-fen waters. Calcium-tolerant sphagna survived along the entire poor–rich gradient, but their growth was inhibited by calcium bicarbonate. The three most obviously expanding sphagna produced protonemata even under calcareous conditions. Flowing but not stagnant alkaline fen waters were toxic for calcifugous sphagna, the strongest competitors in poor-fen waters. Increased potassium availability facilitated the survival of calcifugous sphagna in alkaline fens, corroborating field observations that potassium facilitates sphagnum expansion. Surprisingly, the rare and declining moss *Hamatocaulis vernicosus* was supported by nitrogen and phosphorus more than its competitors. Our comparison of fundamental and realized niches suggests that the dominance of particular moss functional groups in fens is governed by a competitive hierarchy altered by different calcium levels. The expansion of calcium-tolerant sphagna into brown-moss fens therefore requires perturbation that weakens

competition. Additionally, expansion of calcifugous sphagna to alkaline environments may be stimulated by potassium availability.

Keywords

Calcicole–calcifuge, Cation exchange capacity, Cultivation, Fundamental niche, Orthovanadate, Sphagnum

Introduction

Two contrasting ecosystems occur within peatlands. One is dominated by *Sphagnum* species and one by so-called brown mosses, i.e. non-sphagnaceous, usually weft-forming calcium-tolerant bryophytes (Vitt et al. 2000). Brown-moss fens (calcareous and extremely rich fens, referred to as alkaline fens in the present study) are usually an earlier successional stage and may turn into *Sphagnum* fens rather rapidly. Because this successional transition is connected with great changes in species composition, species richness, conservation value, nutrient cycling and carbon dynamics, it turned out to be an attractive topic of current peatland ecology (Granath et al. 2010; Soudzilovskaia et al. 2010, Tahvanainen 2011; Laine et al. 2015). However, little is still know about the mechanisms triggering this important ecosystem change, and its physiological background has not yet been studied.

The ecosystem shift between brown-moss fens and *Sphagnum* fens is generally linked with altered hydrology and expansion of *Sphagnum* mosses. The genus *Sphagnum* represents mostly calcifugous species that are well adapted to acidic, ion-poor conditions (bogs and poor fens), which they help to create through acidification, water retention, and production and accumulation of poorly decomposable peat (Rydin and Jeglum 2006). If environmental conditions in fens allow the establishment and spreading of *Sphagnum* at the expense of dominant brown mosses, their ecosystem engineering features could isolate fen vegetation from the influence of calcareous ground water and consequently speed up the succession from alkaline fens to acidic poor fens, i.e. from species-rich to species-poor habitats (Kooijman 2012). In addition, *Sphagnum* expansion is associated with the disappearance of rare, EU-protected species (Štechová et al. 2012) and short-lived fen specialists (Peterka et al. 2014).

The range of conditions under which fen mosses can establish and expand their fundamental niches with respect to water alkalinity (i.e. calcium bicarbonate concentration) is still unknown. Consequently, we lack a fundamental comparison of realized niches that would allow us to separate the effects of competition and environmental toxicity on species distribution – information valuable for the conservation of the rich fen biota. As bryophytes generally show great dispersal ability, limitation by water chemistry has a crucial effect on their distribution (Hájek et al. 2011); spore

germination and protonema development thus may be the critical phase of initial species recruitment (Forman 1964).

The alkaline to acidic fen transition had occurred frequently during the entire Holocene (Kuhry et al. 1993; Wehrli et al. 2010); it is, however, more frequent and more rapid in modern agricultural landscapes. The reasons are not entirely clear. Experimental and correlative studies provide somewhat equivocal results, stressing the role of different factors alleviating the influence of alkaline water on late-successional species. These are either water table decline, or increasing availability of phosphorus, ammonium, potassium or iron (van Diggelen et al. 1996, 2015; Hájek et al. 2002, 2014; Kooijman and Paulissen 2006; Navrátilová et al. 2006; Kooijman 2012; Peterka et al. 2014). The lack of a thorough understanding of successional drivers in fens is, among others, caused by our poor understanding of basic physiological mechanisms that may be involved in calcium (in)tolerance of fen mosses (i.e. their calcifuge–calcicole behaviour). In this study, we therefore aimed to elucidate the ecophysiological mechanisms behind calcifuge–calcicole behaviour of fen bryophytes, to test their possible modifications by altered nutrient availability and finally to understand the observed successional patterns.

Although the mechanisms behind calcium toxicity in bryophytes are unknown, it is well understood that calcifugous species (i.e. *Sphagnum* mosses) are intolerant to high concentrations of the calcium cation ($[Ca^{2+}]$) when combined with high pH, while the separate effects of the two factors seems to be negligible (Clymo 1973). In alkaline fen waters, high pH is maintained by buffering properties of bicarbonate anions, associated with Ca^{2+}. Flooding by alkaline water should thus have a strong negative effect on *Sphagnum* species. Yet, it had a strongly negative effect on the competition ability and survival of the transplanted bog species *Sphagnum fuscum*, but not on rich-fen sphagna (Granath et al. 2010). Studies of calcifuge–calcicole behaviour of vascular plants and other eukaryotes have revealed that nutrient deficiency, processes at the cell-wall level and/or intracellular metabolism may be involved in calcium toxicity. As concerns the intracellular compartment, the intolerance seems to be linked with regulation of calcium homeostasis. The calcium cation is a key component of signalling pathways in the plant cell (White and Broadley 2003). Hence, its cytosolic concentration (the influx–efflux balance) must be strictly regulated. Because Ca^{2+} cannot be accumulated in the cytosol, high external

[Ca^{2+}] enhances Ca^{2+} accumulation in vacuoles (Conn 2011) and stimulates the expression of transporters responsible for extracellular Ca^{2+} efflux (Ca^{2+}-ATPases and Ca^{2+}/H^+ exchangers; Garciadeblas 2001; Kamiya et al. 2006). Consequently, calcicoles may differ from calcifuges in their tolerance of intracellular Ca^{2+} accumulation (Zohlen and Tyler 2004) and linked expression of calcium transporters in the vacuolar or plasma membrane. Moreover, since the function of Ca^{2+}/H^+ antiporters is regulated by pH (Pittman et al. 2005), the increased toxicity of Ca^{2+} under high pH, enhanced by flooding, might be explained by increased intracellular Ca^{2+} accumulation.

The involvement of the apoplast (cell wall) compartment in calcium toxicity has been studied mainly with respect to inhibition of plant growth. The plant cell wall is interwoven by unesterified pectin chains bearing negatively charged carboxyl groups. Cross-linking of pectic carboxyls by Ca^{2+} is fundamental for maintaining the dimensional structure and firmness of the cell wall (Hepler and Winship 2010). However, full saturation of pectic residues by Ca^{2+} under high pH makes the cell wall more rigid (Fraeye et al. 2009), inhibiting its loosening necessary for cell growth (Proseus and Boyer 2006; Hepler and Winship 2010). Aside from growth, cation exchange sites might interfere with nutrients uptake. In Ca^{2+}-rich solution of high pH, Ca^{2+} may condense (in terms of Manning's counterion condensation theory) on carboxyl cation-exchange sites of the cell wall and create an apoplastic barrier that prevents intracellular uptake of univalent cations, such as K^+ and NH_4^+ (Dainty and Richter 1993).

Nutrient deficiency due to low iron and phosphorus availability has been suggested as the crucial reason why calcareous environments are unfavourable for vascular plants (Zohlen and Tyler 2004) or lichens (Paul et al. 2009), as these elements become insoluble (Boyer and Wheeler 1989; Zak et al. 2010). Phosphates precipitate with calcium whereas iron precipitates mainly as carbonate or (hydr)oxides, but also as phosphate. Calcicoles are able to make these nutrients available by exuding low-molecular-weight acids and phenolics (Tyler and Ström 1995; Paul et al. 2009; Ishimaru et al. 2011), but this mechanism has not been described in bryophytes even though they exude the same organic acids as tracheophytes (Lenton et al. 2012). Recent nutrient analyses of fen mosses revealed that potassium availability may facilitate the expansion of poor-fen sphagna into alkaline fens (Hájek et al. 2014; Hájek et al. (unpublished results)); a

physiological explanation based on experimental evidence is missing, however.

In our study, we conducted a series of greenhouse cultivation experiments in order to assess fundamental niches of dominant fen mosses (in the protonema and the gametophore developmental stage) with respect to water chemistry. We tested whether nutrient deficiency and/or processes on levels of the cell wall and intracellular metabolism may be involved in calcium (in)tolerance of fen bryophytes, and discuss the results in the context of *Sphagnum* establishment in alkaline fens.

Specifically, we tested the following hypotheses concerning calcium intolerance of fen mosses exposed to alkaline fen water:

- Tolerance of fen mosses to calcium bicarbonate in artificial rich fen waters reflects the calcifuge–calcicole pattern observed in the field. Cells are unable to maintain a calcium balance, which results in Ca^{2+} accumulation.
- Saturation of cell-wall pectic residues by Ca^{2+} inhibits plant growth and/or uptake of other nutrients.
- As in vascular plants, low availability of iron and/or phosphates limits moss growth (production).
- Increased nitrogen, phosphorus and/or potassium availability may alleviate the effect of alkaline fen water.

Materials and Methods

Moss material

Species of the genus *Sphagnum* (*S. contortum*, *S. flexuosum, S. fimbriatum, S. russowii, S. subsecundum*, *S. squarrosum*, *S. teres, S. warnstorfii*) and brown mosses (*Aulacomnium palustre*, *Bryum pseudotriquetrum, Calliergonella cuspidata, Hamatocaulis vernicosus*, *Scorpidium cossonii*, *Tomentypnum nitens;* species nomenclature follows Kučera et al., 2012) used in cultivation and spore germination experiments were sampled mainly from Czech and Slovak fens (Central Europe), each species from several localities with contrasting ground water [Ca^{2+}] (Tables A.1, A.2). Three or more patches per locality were sampled. The mosses were kept in a greenhouse at 12–24 °C under a 12–15 h daylight regime (winter days were prolonged to 12 h using high pressure discharge lamps providing light of photosynthetic photon flux density (PPFD) of about 200 µmol m^{-2} s^{-1}). The mosses were watered by rain water or artificial rain water alternated with distilled water. The mosses grew well and did not exhibit any stress symptoms. They were kept for a maximum of 8 months; the material was thus renewed several times during the experimental period (2012–2014).

Cultivation experiments

All cultivations were conducted in a greenhouse using either flowing or stagnant solutions of artificial fen waters (Fig. A.1) with alteration of pH, Ca^{2+}, Mg^{2+}, Fe^{3+} and nutrient (N, P, K) concentrations. Nitrogen was supplied as NH_4NO_3. The concentrations of microelements were uniform: Mn – 5.4 µg L^{-1}, B – 5.3 µg L^{-1}, S – 1.4 mg L^{-1}, Na – 1 mg L^{-1}, I – 1 µg L^{-1}, Zn – 1 µg L^{-1}, Br – 0.9 µg L^{-1}, Co – 0.8 µg L^{-1}, Cu – 0.7 µg L^{-1}. Apical (15 mm) shoot segments were grown submersed, attached to 96-well microplates, 1.5 cm apart from each other (Fig. A.1). Seven cultivation experiments lasting from three to nine weeks were conducted successively from July 2012 to June 2014. In cultivations 1–5 (flowing solutions), approximately 2 L of solution was exchanged every 2 h between a stock solution (52 L in an air-tight barrel) and a 2.5 L container with the mosses, using automated pumps (BrightLogic, Brightwell Dispensers Ltd, UK). In Cultivation 6, the mosses were submerged in 20 L of stagnant solution in air-tight HDPE transparent containers; the solution was changed every 5

days and no calcium carbonate precipitation occurred. In cultivation 7, about ten shoots were incubated in 4.8 L of stagnant solution inside air-tight 5 L glass bottles. Such experimental setups allowed precise regulation of pH thanks to large volume of the solution and the small weight of mosses (about 1 g of dry mass per 10 L of solution). The pH of the bicarbonate solutions was adjusted with CO_2 whereas 1 M HCl or NaOH was used in the chloride solutions. We decided to use submerged cultivation because of the direct effect of water chemistry on moss physiology. The calcium bicarbonate solutions contained excess free CO_2, whose availability does not limit photosynthesis rates in submerged mosses under neutral pH.

Cultivation 1 – Effects of [Ca^{2+}] and pH

The mosses were grown for three weeks in July 2012, submerged in five solutions of calcium bicarbonate simulating the poor–rich fen gradient of [Ca^{2+}]: (i) 4 mg L^{-1} (0.1 mM $Ca(HCO_3)_2$; poor fen conditions; fen terminology follows Hájek et al., 2006 to which the [Ca^{2+}] were assigned following Sjörs and Gunnarsson, 2002), pH 6.3 and (ii) 7.1; (iii) 32 mg L^{-1} (0.8 mM $Ca(HCO_3)_2$; moderately rich fen conditions), pH 7.1; (iv) 60 mg L^{-1} (1.5 mM $Ca(HCO_3)_2$; extremely rich fen conditions), pH 7.1; (v) and 96 mg L^{-1} (2.3 mM $Ca(HCO_3)_2$; calcareous fen conditions), pH 7.1. The concentrations of N and P (1.41 and 0.41 mg L^{-1}, respectively) corresponded to the higher concentrations found in our rich fen sites; we assumed that such N and P availability does not limit species growth (Rudolph et al. 1988). The concentrations of Mg, Fe and K were 0.5, 0.1 and 1.25 mg L^{-1}, respectively. The concentrations of N and P were lowered to < 0.01 and 0.2 mg $L^{-}1$ after 2 weeks of cultivation in order to suppress algal growth.

Cultivation 2 – Effects of [Ca^{2+}] and pH

The mosses were grown for two months in September and October 2012 submersed in solutions identical with Cultivation 1, extended with an additional concentration of 60 mg L^{-1} (1.5 mM $Ca(HCO_3)_2$; extremely rich fen conditions, pH 6.3). In addition to submerged cultures, some moss-holding microplates were floating, so the moss shoot apices were grown 1

cm above the water surface at the beginning of the experiment and up to 5 cm above the water surface at the end of it. The concentrations of Mg, Fe, K, P and N were 0.5, 0.1, 1.25, 0.2 and < 0.01 mg L^{-1}, respectively. To suppress algal expansion, additional N and P was provided once a week by submerging the mosses into 1.41 mg L^{-1} (N) and 0.41 mg L^{-1} (P) solutions for 1.5 h. The limited nutrient supply reduced the growth and production to about half of that in Cultivation 1.

Cultivation 3 – Effects of [Ca^{2+}], [Mg^{2+}] and [K^{+}]

The mosses were grown for three weeks in July 2013 in six solutions of calcium and/or magnesium bicarbonate in order to compare the effect of allied cations with contrasting physiological roles and to examine the effect of K^{+} addition. The solutions (all pH of 7.1) were: (i) 1.2 mM $Ca(HCO_3)_2$; (ii) 1.2 mM $Mg(HCO_3)_2$; (iii) 1.2 mM $Ca(HCO_3)_2$ + 1.2 mM $Mg(HCO_3)_2$; (iv) 2.4 mM $Mg(HCO_3)_2$; (v) 2.4 mM $Ca(HCO_3)_2$; and (vi) 2.4 mM $Ca(HCO_3)_2$ + 1.2 mM $KHCO_3$.

Moreover, the effect of low [Ca^{2+}] of 0.8 mg L^{-1} (0.02mM $Ca(HCO_3)_2$, extremely poor fen conditions) was tested in combination with low (5.0) and high (7.1) pH.

Concentrations of N, P and Fe of all eight solutions were 1.4, 0.5 and 0.1 mg L^{-1}, respectively. In solutions where Ca, Mg and K concentrations were not specified, they were 0.8, 0.5 and 1.25 mg L^{-1}, respectively.

Cultivation 4 – Effects of [Ca^{2+}] and pH at low N and P

The mosses were grown for three weeks in November 2013 in eight solutions of calcium and magnesium bicarbonate with low N and P concentrations (0.05 and 0.1 mg L^{-1}), corresponding with lower values found in Czech and Slovak fens (Hájek et al. 2014), so as to prevent the growth of algae that would otherwise contaminate the moss biomass used for analysis of intracellular ion concentrations. The nitrogen concentration in the solution was replenished once a week. Otherwise, the solutions composition corresponded with that described in Cultivation 3, only the 0.02 mM $Ca(HCO_3)_2$ solution of pH 5.0 was replaced by 1.2 mM $CaCl_2$, pH

5.0 to test the effect of low pH and high [Ca^{2+}] (the chloride was used because the buffering ability of bicarbonate is out of of the required range at low pH).

Cultivation 5 – Effects of low and high [Fe^{3+}]

The mosses were grown for three weeks in March 2014 in nine solutions of calcium bicarbonate or calcium chloride with low, high and extra-high [Fe^{3+}]: 0.002, 0.02 and 0.2 mM. The low concentration corresponded with [Fe^{3+}] used in the other cultivations. The solutions were: (i–iii) 2.4 mM $Ca(HCO_3)_2$, pH 7.1 with either low, high or extra-high [Fe^{3+}]; (iv–v) 2.4 mM $CaCl_2$, pH 5.0 with low or high [Fe^{3+}]; (vi–vii) 0.02 mM $CaCl_2$, pH 5.0 with low or high [Fe^{3+}]; (viii) 0.02 mM $Ca(HCO_3)_2$, pH 7.1 with high[Fe^{3+}]; and (ix) 2.4 mM $CaCl_2$, pH 6.7 with low [Fe^{3+}]. The extra-high [Fe^{3+}] solution was stagnant as described for Cultivation 6. Although the Fe^{3+} was provided in chelated forms (Fe^{3+}-EDTA and Fe^{3+} citrate), the iron precipitated in high and extra-high [Fe^{3+}] in 2.4 mM Ca^{2+} solutions within 10 and 3 days, respectively (the final concentration was around 0.5 µM). Therefore, the solutions were changed once (low/high [Fe^{3+}]) or every 3 days (extra-high [Fe^{3+}]). The concentrations of nutrients and other ions were as described for Cultivation 4.

Cultivation 6 – Effect of N and P

The mosses were grown for three weeks in May 2014 in four stagnant solutions of 2.4 mM $Ca(HCO_3)_2$ with a factorial combination of low and high concentrations of N (0.05 and 1.4 mg L^{-1}) and P (0.1 and 0.5 mg L^{-1}). The concentrations of other ions corresponded with Cultivation 3. Apart from standard shoot organization in wells, *Hamatocaulis vernicosus* was grown mixed with its frequently occurring competitor *Calliergonella cuspidata* (both species in one well, six replicates). The wells were surrounded by additional shoots of *C. cuspidata* to evoke the competition.

Cultivation 7 – Effect of small pH changes on Sphagnum *survival*

The three calcifugous species (*S. russowii, S. subnitens, S. fimbriatum*) were grown in June 2014 in three stagnant solutions of 2.4 mM $Ca(HCO_3)_2$ with

pH: (i) 6.7–6.8, (ii) 7.0–7.1, and (iii) 7.3–7.4. After two weeks, the solutions were changed to 5 mM $Ca(HCO_3)_2$, in which the species grew for additional 16 days at the same pH levels. The whole experiment was conducted in three replicates.

Evaluation of shoot survival and growth

Species survival was estimated using an optical microscope as the percentage of living cells in capitula or upper shoot parts. Shoot growth was evaluated from the length and weight increment. To reduce and homogenize the variable content of external capillary water prior weighting, fresh shoots were gently but thoroughly blotted between sheets of cellulose filter paper using constant pressure. The variability in water content of blotted shoots was very low within the groups of *Sphagnum* and brown mosses. The fresh-mass weight (FW) was converted to dry-mass weight (DW) using the formula: FW = 5.16 × DW (R^2 = 0.99) for *Sphagnum* species and FW = 3.38 x DW (R^2 = 0.99) for brown mosses.

Spore germination and cultivation of protonemata

The ability of *Sphagnum* and brown mosses to germinate under contrasting pH and [Ca^{2+}] was tested in solutions of: (i) 2.4 mM $Ca(HCO_3)_2$, pH 7.1; (ii) 1.2 mM $Ca(HCO_3)_2$, pH 7.1; (iii) 0.02 mM $Ca(HCO_3)_2$, pH 6.3; (iv) 2.4 mM $CaCl_2$, pH 5.0; (v) 1.2 mM $CaCl_2$, pH 5.0; (vi) 0.02 mM $CaCl_2$, pH 5.0; and (vii) 4.8 mM KCl, pH 6.0. The concentrations of other ions and nutrients were the same as in Cultivation 1, but without N and P depletion. The last solution was used for verifying that Cl^- ions are not toxic for spore germination and development of protonemata.

Mature capsules sampled in the field before rupturing were stored in sealed petri-dishes at 15 °C in the dark for 2–12 months, where they opened. The storage had no notable effect on spore germination. Spores were sealed in small polyester mesh bags (10 µm openings) and germinated submerged in sealed transparent containers for 6 weeks. The solutions were changed once a week and were kept free of algal contamination. Additionally, we germinated spores of three *Sphagnum* species (*S. squarrosum, S. flexuosum* and *S. fimbriatum*) on 1.5% agar medium (nutrient concentration as

described for Cultivation 1) with (i) 2.4 mM $CaCl_2$, pH 6.5 or (ii) 0.1 mM $CaCl_2$, pH 6.5 in sealed Petri dishes. The protonemata were then cultivated in 2.4 mM and 1.2 mM $Ca(HCO_3)_2$, pH 7.1 to test their acclimation to high $[Ca^{2+}]$ and pH.

The germination rate, protonema size and shoot formation were evaluated under a stereomicroscope (Olympus SZX 10, maximal magnitude 65×) after two, four and six weeks.

Inhibition of ATPases by sodium orthovanadate

Sodium orthovanadate (Na_3VO_4, 0.1 mM, pH 7.0; Sigma-Aldrich) acts as an ATPase inhibitor (Gallagher 1982; DuPont et al. 1990). It was prepared from an activated (depolymerized) 100 mM stock solution stored at –20 °C (Gordon 1991). To activate the solution, it was briefly subjected to five fast boiling–cooling cycles. The pH of the solution was adjusted to 10.0 using 1 M HCl or NaOH before and after each cycle. The inhibitory effect of orthovanadate on ATP-ases of moss plasmalemma was tested by the leakage technique: intracellular Ca^{2+} that had accumulated inside the cells during incubation in calcareous solutions did not leak out of the cells in 0.02 M HCl when the shoots were submersed in 0.1 mM orthovanadate for 24 h before the acid treatment. Without orthovanadate, the intracellular $[Ca^{2+}]$ equalled the pre-treated value within 50 minutes of the acid treatment. The slow K^+ leakage in HCl did not change after orthovanadate application.

We tested the effect of 0.1 mM orthovanadate on intracellular Ca^{2+} accumulation and species survival in 9-day cultivation experiment. Moss shoots were submerged in calcareous solutions (pH 6.8 and 7.2, with or without orthovanadate in factorial design with two replicates, i.e. eight containers in total) and distilled water (with/without orthovanadate in two replicates, i.e. four containers in total) in 20 L air-tight HDPE transparent containers in shade (PPFD < 50 µmol m^{-2} s^{-1}). Prior to the orthovanadate treatments, the mosses were kept in distilled water with 0.1 mM orthovanadate for 24 h.

Photosynthetic measurements

The effects of Ca^{2+} and orthovanadate on intracellular metabolic processes were assessed using measures of photosynthesis and respiration. Photosynthetic CO_2 assimilation and chlorophyll fluorescence was measured using a gas exchange fluorescence measurement system (GFS-3000, Walz GmbH, Germany) equipped with fluorescence imaging (LED-Array/PAM module 3055-FL). The standard chamber was adapted for bryophytes, whose shoots were arranged in removable sample cuvettes (inner size of 40 × 20 × 10 mm) with mesh bottoms. The shoots were partly blotted with filter paper before the measurement to minimize diffusive resistance caused by a large amount of capillary water. Air flow was set to 400 μmol s^{-1}, incoming CO_2 concentration to 400 ppm, cuvette temperature to 25 °C and RH of incoming air to 70–80%, resulting in 90–95% RH of outcoming air. The internal chamber fan was switched off to reduce sample desiccation. We verified that the water loss during measurement was low enough not to affect CO_2 exchange. After the measurement of F_v/F_m (maximum quantum efficiency of PS II photochemistry; measured after 10–15 h of dark acclimation), the shoots were exposed to PPFD of around 200 μmol m^{-2} s^{-1} in a greenhouse. We then measured the photosynthetic CO_2 assimilation during the light period (PPFD of 200 μmol m^{-2} s^{-1} for 10 min and 20 μmol m^{-2} s^{-1} for 5 min) and dark respiration (5 minutes).

Determination of cell-wall-bound cations and cation exchange capacity (CEC) by elution

Extracellular exchangeable Ca^{2+} and Mg^{2+} bound to negatively charged cell wall sites were eluted by 30 mM $NiCl_2$ (pH adjusted to 7.0) for 100 min to separate the intracellular and extracellular fraction. Before the elution, shoots were washed with distilled water (5 times for 6 min). We verified that the washing was sufficient to remove unbound extracellular Ca^{2+}. The minimum volume of the elution solution was proportional to 1 L per 1 g of shoot dry mass in order to ensure an excess amount of Ni^{2+} over the cation exchange capacity of the cell walls (about 1 meq g^{-1}). Intracellular cations accumulated during the cultivation were not leaking out of the cell during the elution (verified by changing elution times and applying orthovanadate). The concentrations of residual ions bound in the cell wall after elution by $NiCl_2$ or 20 mM HCl were 0.10–0.15 mg L^{-1} for Ca^{2+} and 0.04–0.06 mg/L

for Mg^{2+} (tested on dead material saturated by 30 mM $CaCl_2$ or $MgCl_2$ at pH of 7.0; acid-digested shoots were analysed by FAAS – see below).

The CEC (always expressed for given pH of the saturation solution) was determined in dead moss material. The protonemata (placed in polyester mesh bags with 150 μm openings) and shoots were eluted by 20 mM HCl (45 min) to remove all extracellular cations and then saturated in 30 mM $CaCl_2$ at pH 7.0 for 5 days (protonemata) or 30 hours (shoots). The solution was changed three times. The samples were then washed with distilled water (5 times for 1 day or 6 min for protonemata and shoots, respectively) and eluted by 20 mM HCl for 30 min. For the construction of CEC–pH curves, dead moss shoots of four species (*Sphagnum flexuosum*, *S. squarrosum*, *Scorpidium cossonii* and *Tomentypnum nitens*) were placed in three replicates into mesh bags. Exchange sites were saturated by 100 mM $CaCl_2$ for 8 hours at given pH (8.0, 7.0, 6.0, 5.0, 4.0, 3.0), washed with and acid-eluted as described above.

Chemical analyses of moss biomass

Eluted shoots were acid-digested, either with HNO_3 (0.5 ml of concentrated acid per 3–20 mg of dry mass at 130 °C for 2–3h; samples only for cation analysis) or $HClO_4$ (0. 25ml of concentrated acid per 6 mg of dry mass at 180 °C for 12 h; samples for cations and phosphorus analysis). Metallic cations were analysed using flame atomic absorption spectrometry (FAAS) and phosphorus colourimetrically using flow-injection analysis.

Since calcium carbonate precipitated on moss shoots in flowing calcareous solutions, we used only samples from stagnant solutions for the calcium analysis. The amount of precipitated $CaCO_3$ was estimated from the amount of CO_2 released after carbonate dissolution by 1M H_2SO_4, measured by an Agilent 6850 gas chromatograph (Agilent technology, USA). The precipitation of $MgCO_3$ was negligible.

Statistical analysis

Calcium tolerance of fen mosses was assessed based on their survival in calcareous solutions (2.4 mM $Ca(HCO_3)_2$) by one-way analysis of variance

(ANOVA) followed by Tukey's (HSD) post hoc test to evaluate differences among species. The data on species survival in all analyses were transformed using an arcsine transformation ($y = \arcsin(x^{0.5})$). The effect of *nutrients* on the growth of individual *Sphagnum* and brown-moss species in poor to rich solutions (factor *solution*) was evaluated by two-way ANOVA (factors: *nutrients*, *solution*). The responses of *Sphagnum* species and brown mosses to increased nutrient concentration (interaction *Sphagnum/brown-moss* × *high/low nutrients*) were compared by nested ANOVA (factors *species, high/low nutrients, Sphagnum/brown-moss*; random factor *species* nested in *Sphagnum/Brown-moss*), individually for each poor to calcareous solution. The difference between biomass production of *Hamatocaulis vernicosus* and other brown mosses (*H.v/brown-moss*) in moderately rich to calcareous solutions (*solution*) after three weeks cultivation was tested by two-way ANOVA. The effect of P addition on biomass accumulation of *H. vernicosus* or *C. cuspidata* during nine weeks of cultivation was tested by one-way ANOVA. Because the addition of P had no effect, the contrasting effect of added N on biomass accumulation of *H. vernicosus* and *C. cuspidata* (interaction *species* × *low/high N*) in nine weeks cultivation was evaluated by nested ANOVA (factors *solution, species, low/high N*; random factor *solution* nested in *low/high N*). The effect of iron on species survival, growth and biomass accumulation was tested by one-way ANOVA for calcareous solutions (separately for each species) and by two-way ANOVA (factors *species*, *Fe*) for low pH solutions (separately for 0.02 and 2.4 mM [Ca^{2+}]). The effect of additional K^+ on calcifugous *Sphagnum* survival was evaluated by nested ANOVA (factors *cultivation* (Cultivation 3 and 4)*, species, low/high K*; random factor *cultivation* nested in *low/high K*). The effects of pH and nutrients on changes in CEC of moss shoots in calcareous solutions were evaluated by nested ANOVA (factors *species, pH* or *low/high nutrients*; random factor *species* nested in *pH* or *low/high nutrients*; high P included in high nutrients). The effect of pH on CEC of moss protonemata in 1.2 mM [Ca^{2+}] solutions was tested by one-way ANOVA. The difference in intracellular phosphate accumulation between *Sphagnum* and brown mosses was tested by nested ANOVA (the random factor *species* nested in factor *Sphagnum/brown-moss*; factor *solution* (1.2/2.4 mM $Ca(HCO_3)_2$) was added to the model when testing the accumulation of P in calcium bicarbonate solutions). Differences in growth among protonemata, whose germination in Ca^{2+} rich/poor conditions (*Ca*) was followed by growth in solutions of calcium bicarbonate (*solution*), was

evaluated by three-way ANOVA (factors *solution* (random), *species* (random), *Ca*). The effect of orthovanadate on species grown in distilled water was tested by a t-test. The relationship between biomass and growth increments was evaluated by regression. The software package Statistica ver. 8 (Statsoft, USA) was used for all data analyses. The data generally met the assumptions of residuals normality and of homoscedasticity for running ANOVA.

Results

Effects of [Ca^{2+}] and pH – flowing solutions of calcium bicarbonate and chloride

The survival and growth of fen sphagna, but not brown mosses, submerged in flowing artificial poor- to rich-fen waters was limited by increasing [Ca^{2+}] and pH (Fig. 1, Tables B.1, B.2). All species survived and grew in solutions of low [Ca^{2+}] and low pH (0.02 mM $CaCl_2$, pH 5.0; poor fen water). Increased [Ca^{2+}] and pH (solutions of calcium bicarbonate) were lethal for calcifugous *Sphagnum* species (the less tolerant *S. russowii* and *S. subnitens* died sooner than the more tolerant *S. flexuosum, S. fimbriatum* and *S. subsecundum*). The growth of sphagna that are traditionally referred to as calcium-tolerant (*S. teres, S. squarrosum, S. warnstorfii* and *S. contortum*) was suppressed by high pH and [Ca^{2+}]; however, all these mosses survived well even in calcareous fen water (2.4 mM $Ca(HCO_3)_2$). Calcium ions were lethal for calcifugous sphagna in calcareous solutions buffered by bicarbonate, while all species survived well in solutions of 2.4 mM $CaCl_2$, pH 5.0 and 6.7. Consequently, it seems that bicarbonate plays a specific role in the toxicity of high [Ca^{2+}] under high pH. Brown mosses *Scorpidium cossonii* and *Tomentypnum nitens* and partly the calcium-tolerant *S. contortum* had lower survival in acid solutions of high [Ca^{2+}]. This might be caused by their possible intolerance to Cl^-, yet under higher pH (6.7) the Cl^- did not have a negative effect on the survival and growth of any of the species (Tables B.1, B.2).

Brown mosses and sphagna exhibited contrasting growth patterns in response to [Ca^{2+}] and pH. Brown mosses grew best under high Ca^{2+} and pH (chloride and bicarbonate solutions), both in terms of length (Fig. 1, Table B.1) and biomass (Figs. A.2, A.3). On the contrary, sphagna grew well only in solutions without calcium bicarbonate. Most had their optimum at low pH (5.0) with either high or low [Ca^{2+}]. Accordingly, the growth of brown mosses was superior down to the calcium bicarbonate concentration of 1.2–1.5 mM at pH 7.1 (the border between extremely rich and rich fens), where it levelled with quickly growing calcitolerant sphagna (*S. teres* and *S. squarrosum*). By contrast, sphagna overgrew brown mosses under the calcium bicarbonate concentration of 0.1 mM, pH 7.1 (*S. cossonii, T. nitens*) or pH 6.3 (the lowest pH of rich fens, Sjörs and Gunnarsson (2002);

Hamatocaulis vernicosus, Calliergonella cuspidata, Aulacomnium palustre, Bryum pseudotriquetrum).

Flooded conditions enhanced the growth of brown mosses. When shoot apices were kept 1–5 cm above the water surface, *H. vernicosus* grew more slowly than quickly growing *Sphagnum flexuosum* and *S. squarrosum*, which can cause *H. vernicosus* to be outcompeted in the field.

Nutrient (N, P) availability played a significant role in interspecific competition. Higher nutrient concentrations increased the growth and biomass increment of most species in poor- to alkaline- fen water (Figs. 1, A.2). The growth increment was higher in *Sphagnum* species in poor-fen solutions (0.02 mM $Ca(HCO_3)_2$, pH 5.0; $p = 0.045$). It did not differ between sphagna and brown mosses in moderately rich and rich fen solutions (0.1 and 0.8 mM $Ca(HCO_3)_2$, pH 7.1, $p = 0.062$ and 0.78, respectively) and was higher in brown mosses in extremely rich and calcareous fen solutions (1.5 and 2.4 mM $Ca(HCO_3)_2$, respectively, pH 7.1; $p < 0.001$). The biomass increment was significantly higher in brown mosses compared to sphagna in calcareous solutions ($F_{1,118} = 79.8$, $p < 0.001$); in other solutions it could not be tested.

Nutrient availability changed competitive relations between rare *Hamatocaulis vernicosus* and other brown mosses in calcareous solutions. Under low nutrient concentration, biomass production of *H. vernicosus* was slightly lower or equalled that of *C. cuspidata* (its leading competitor in fens), which was the best-growing species in nutrient-poor calcareous solutions (Fig. A.3). Elongation growth was greater in *H. vernicosus*, as *C. cuspidata* allocated more resources to lateral growth (side branches). Biomass production of *H. vernicosus* exceeded that of *C. cuspidata* under increased nitrogen concentrations (nine weeks of cultivation, $F_{1,33} = 9.6$, $p = 0.004$, Fig. A.4), even when the species were grown together ($F_{1,42} = 29.5$, $p < 0.001$); *H. vernicosus* suppressed the growth of *C. cuspidata* more than vice versa. When *H. vernicosus* was grown for three weeks in solutions of moderately rich to calcareous fens, its biomass production was somewhat higher than that of any other species (even in Cultivation 2 where N and P were limiting, Fig. A.2). In our nutrient-poor solutions, both species were limited by N, since P addition did not increase growth or biomass accumulation.

Higher iron concentration had no clear effect on species survival or growth in calcareous solutions (Fig. A.3). In solutions of low pH and/or low [Ca^{2+}], high [Fe^{3+}] reduced brown moss survival ($p < 0.005$) but surprisingly not their growth or biomass production. Addition of K^+ to the solution of 2.4 mM [$Ca(HCO_3)_2$] compensated for calcium toxicity ($F_{1,2} = 29.5$, $p = 0.035$), as shown by the better survival of calcifuges (Fig. A.5).

Although growth and biomass production were correlated in most species, the correlation was not strong, particularly in brown mosses, which invested more into lateral growth ($R^2 \approx 0.2$ to 0.5 in *Sphagnum* species and 0.05 to 0.4 in brown mosses).

Effect of magnesium bicarbonate

Magnesium bicarbonate was more toxic and had a different effect on species survival and growth than calcium bicarbonate, (Fig. A.5, Tables B.1, B.2). Calcium and magnesium tolerance showed no common pattern among species. Only some *Sphagnum* species survived in 2.4 mM $Mg(HCO_3)_2$ (*S. squarrosum, S. teres, S. flexuosum* and *S. warnstorfii*), but all brown mosses were intolerant; *H. vernicosus* in particular did not survive even in 1.2 mM $Mg(HCO_3)_2$. When Mg^{2+} ions were combined with Ca^{2+} ions, each of the ions either ceased to be toxic or became generally less toxic than when applied alone (Fig. A.5).

Spore germination and growth of protonemata at contrasting pH and [Ca^{2+}]

Spore germination and the growth of protonemata were affected by [Ca^{2+}] and pH, similarly to adult shoots (i.e. gametophores) (Fig. A.6). Spores of all species could germinate and grow protonemata in acid solutions of high or low [$CaCl_2$]. *Sphagnum* species created large, healthy-looking leafy protonemata (Fig. A.7). The brown mosses produced healthy looking fibrous protonemata, yet their biomass was approximately more than 10 times smaller than in bicarbonate solutions. None of the species created buds with small shoots within the six weeks of incubation.

Except for the most calcifugous species *S. subnitens* and *S. russowii*, sphagna germinated even in solutions of medium and high [$Ca(HCO_3)_2$] (1.2 and 2.4 mM, respectively, pH 7.1). However, they produced only few-celled "protonemata" and ceased to grow under the higher concentration. Only the calcium-tolerant S. teres produced small growing protonemata deformed into a digitate or spherical shape (Fig. A.8), while it created larger deformed protonemata with buds of young gametophores under medium [$Ca(HCO_3)_2$]. *Sphagnum squarrosum* and *S. flexuosum* formed smaller and deformed protonemata with few buds under medium [$Ca(HCO_3)_2$].

The brown mosses germinated best in alkaline solutions, where they attained large biomass and produced small shoots (Fig. A.7). Spores of the best-growing species *C. cuspidata* germinated already after 8 days, i.e. 14 days before any other species in any of the solutions, and produced the largest protonemal biomass.

Sphagnum protonemata did not acclimatize to calcareous solutions when germinated on agar in solutions of high [Ca^{2+}] and pH. On the contrary, they tended to die out more quickly when transferred to solutions of 0.8, 1.5 and 2.4 mM $Ca(HCO_3)_2$, pH 7.1, than those germinated in 0.1 mM $CaCl_2$ ($F_{1,10} = 4.9$, $p = 0.052$).

Effect of flowing solutions of calcium bicarbonate and small pH changes

When compared with stagnant solutions, the survival of calcifugous sphagna in flowing solutions was notably poorer. While the calcifugous *S. russowii, S. subnitens* and *S. fimbriatum* (almost) died within 3 weeks of submersion in flowing solutions of 2.4 mM $Ca(HCO_3)_2$, pH 7.1, the same species survived well for 9 weeks in chemically identical stagnant solutions and were growing slightly throughout the cultivation period (Fig. A.9). The species were also able to endure 16 days of flooding by 5 mM $Ca(HCO_3)_2$, which had been preceded by 14 days of flooding by 2.4 mM $Ca(HCO_3)_2$. At the end of the cultivation, the survival was slightly affected by small pH differences around the neutral value; the mosses survived better in pH 6.7–7.1 than in pH 7.3–7.4, in which massive carbonate precipitation had already occurred.

Interactions between cell wall and Ca^{2+}

The response of cation exchange capacity (CEC) of cell walls for Ca^{2+} to external pH has a very similar pattern in the two *Sphagnum* and two brown-moss species (Fig. 2). In flowing solutions of 2.4 mM $Ca(HCO_3)_2$, pH=7.1, cation exchange sites of dead *Sphagnum* became saturated within two weeks. In stagnant solutions, the exchange sites of dead shoots were not fully saturated even after 3 weeks (Table 1).

Cell walls of living *Sphagnum* and brown mosses bound a noticeably lower amount of Ca^{2+} in calcareous solutions than those of dead shoots (Table 1). The CEC of living shoots equilibrated at pH 7.1 corresponded to the CEC of same dead shoots equilibrated at pH 3.5–4.5 or even less (tested for flowing and stagnant solutions; Table 1 values compared with Fig. 2).

Fen mosses increased the concentration of cell-wall cation-exchange sites when the pH of the solution decreased (Tables A.3, A.4). This pH-dependent (but not Ca^{2+}-dependent) change in CEC was observed in protonemata ($F_{1,4} = 24.7$, $p = 0.008$) and newly grown parts of adult shoots ($F_{1,2.7} = 26$, $p < 0.018$). The high nutrient availability slightly increased the CEC ($F_{1,2.4} = 12.2$, $p = 0.057$; Table A.4).

Intracellular effects of excess Ca^{2+}

All mosses accumulated Ca^{2+} in intracellular compartments when submerged in alkaline-fen water (2.4 mM $Ca(HCO_3)_2$, pH 7.1), in which they increased their intracellular [Ca^{2+}] around two to nine times within 9 days, without any clear difference between moss groups (Fig. 3). None of the species accumulated Ca^{2+} in acid solutions, even under high [Ca^{2+}] (1.2 or 2.4 mM $CaCl_2$, pH 5.0). On the contrary, in these solutions and in $Mg(HCO_3)_2$, brown mosses were loosing intracellular Ca^{2+} (Figs. A.10, A.11). In $Mg(HCO_3)_2$, the accumulated [Mg^{2+}] was two times higher in all species than [Ca^{2+}] accumulated in calcareous solutions.

Contrary to divalent cations, phosphorus uptake differed between brown mosses and *Sphagnum* species. In solutions of calcium bicarbonate, brown mosses assimilated more P than *Sphagnum* species ($F_{1,5} = 96$, $p < 0.001$), even when their growth rate equalled the growth of calcium-tolerant sphagna. In acid solutions, the uptake was similar in both species groups ($p = 0.8$). Species which were least affected by magnesium bicarbonate (*S.*

squarrosum, S. warnstorfii, S. flexuosum) had high biomass [P] in $Mg(HCO_3)_2$ solutions (Fig. A.12).

Increased intracellular Ca^{2+} accumulation reduced *Sphagnum* photosynthesis and the rate of dark respiration. Photosynthesis was lowered in calcicoles (*S. warnstorfii* and *S. squarrosum*) and almost ceased in calcifuges (*S. russowii, S. fimbriatum, S. subnitens*); dark respiration of calcifuges was reduced slightly (Table A.5). Based on the reduced maximum quantum efficiency of PS II photochemistry (F_v/F_m), Ca^{2+} partly impaired PSII in the least calcium-tolerant species *S. russowii* (Table A.6). By contrast, brown-mosses in calcium bicarbonate solutions showed increased rates of dark respiration and photosynthesis, possibly profiting from higher free $[CO_2]$.

The addition of 0.1 mM orthovanadate (an ATPase inhibitor) to distilled water only slightly reduced photosynthesis and respiration ($p > 0.1$ and > 0.5, respectively) in most species within nine days of cultivation, yet all samples looked healthy. Orthovanadate itself, added to 2.4 mM calcium bicarbonate (pH 6.8 and 7.2), reduced photosynthesis and respiration in all species (the decrease was not distinguishable in species whose photosynthesis ceased in calcium bicarbonate solutions). Apart from *T. nites* and some shoots of *S. warnstorfii*, PSII were damaged (lowered F_v/F_m, Table A.6). *Scorpidium cossonii* was the only species that died before the ninth cultivation day. Orthovanadate addition itself did not affect the accumulation of intracellular Ca^{2+} (Fig. 3).

Table 1. Concentration of cell-wall bound exchangeable Ca^{2+} in mosses cultivated in flowing or stagnant solutions of 2.4 mM $Ca(HCO_3)_2$ (*Cultivation*) compared with cation exchange capacity of the same shoots saturated in 30 mM $CaCl_2$, pH 7.0 (*CEC*). The species were grown submerged in solutions for either 2–3 weeks, the growth increment is denoted in the column *Growth*. *: *Calliergonella cuspidata* allocated resources to production of numerous side branches resulting in small apical growth increment.

Flowing solutions species	**Cultivation** (mg/g)	**CEC** (mg/g)	**Ratio** (Cult./CEC)	**Growth** (mm)
14 days				
Brown mosses	**13**	**16**	**0.80**	
Calliergonella cuspidata	14	16	0.87	1*
Hamatocaulis vernicosus	12	17	0.73	6
Sphagnum	**14**	**22**	**0.62**	
S. fimbriatum	18	22	0.80	0
S. flexuosum	13	20	0.65	0
S. subsecundum	13	19	0.68	2
S. contortum	15	20	0.73	1
S. squarrosum	10	28	0.34	4
21 days				
Brown mosses	**13**	**21**	**0.64**	
Tomentypnum nitens	12.3	21.6	0.57	5
Scorpidium cossonii	14.5	20.4	0.72	5
Sphagnum	**14.8**	**22.3**	**0.66**	
S. squarrosum	11.7	19.7	0.59	5
S. squarrosum	12.5	20.1	0.62	5
S. teres	15.1	21.9	0.69	2
S. warnstorfii	19.8	27.3	0.72	0
Dead shoots (16 days)				
Sphagnum	**25**	**25**	**1.00**	
S. flexuosum	26	27	0.97	
S. flexuosum	23	24	0.97	
S. flexuosum	24	24	1.01	

Stagnant solutions species	**Cultivation** (mg/g)	**CEC** (mg/g)	**Ratio** (Cult./CEC)	**Growth** (mm)
21 days				
Brown mosses	**13**	**18**	**0.71**	
Tomentypnum nitens	13	18	0.70	3
Tomentypnum nitens	13	19	0.68	4
Scorpidium cossonii	14	18	0.77	8
Scorpidium cossonii	11	17	0.68	8
Sphagnum	**17**	**21**	**0.81**	
S. russowii	19	23	0.82	0
S. russowii	18	23	0.78	0
S. fimbriatum	17	22	0.79	0
S. fimbriatum	22	23	0.93	0
S. flexuosum	14	18	0.78	0
S. flexuosum	15	19	0.80	1
S. squarrosum	14	18	0.77	4
S. squarrosum	14	18	0.80	2
Dead shoots				
S. squarrosum – 6 days	18	22	0.79	
S. squarrosum – 8 days	17	22	0.79	
S. squarrosum – 21 days	19	22	0.86	

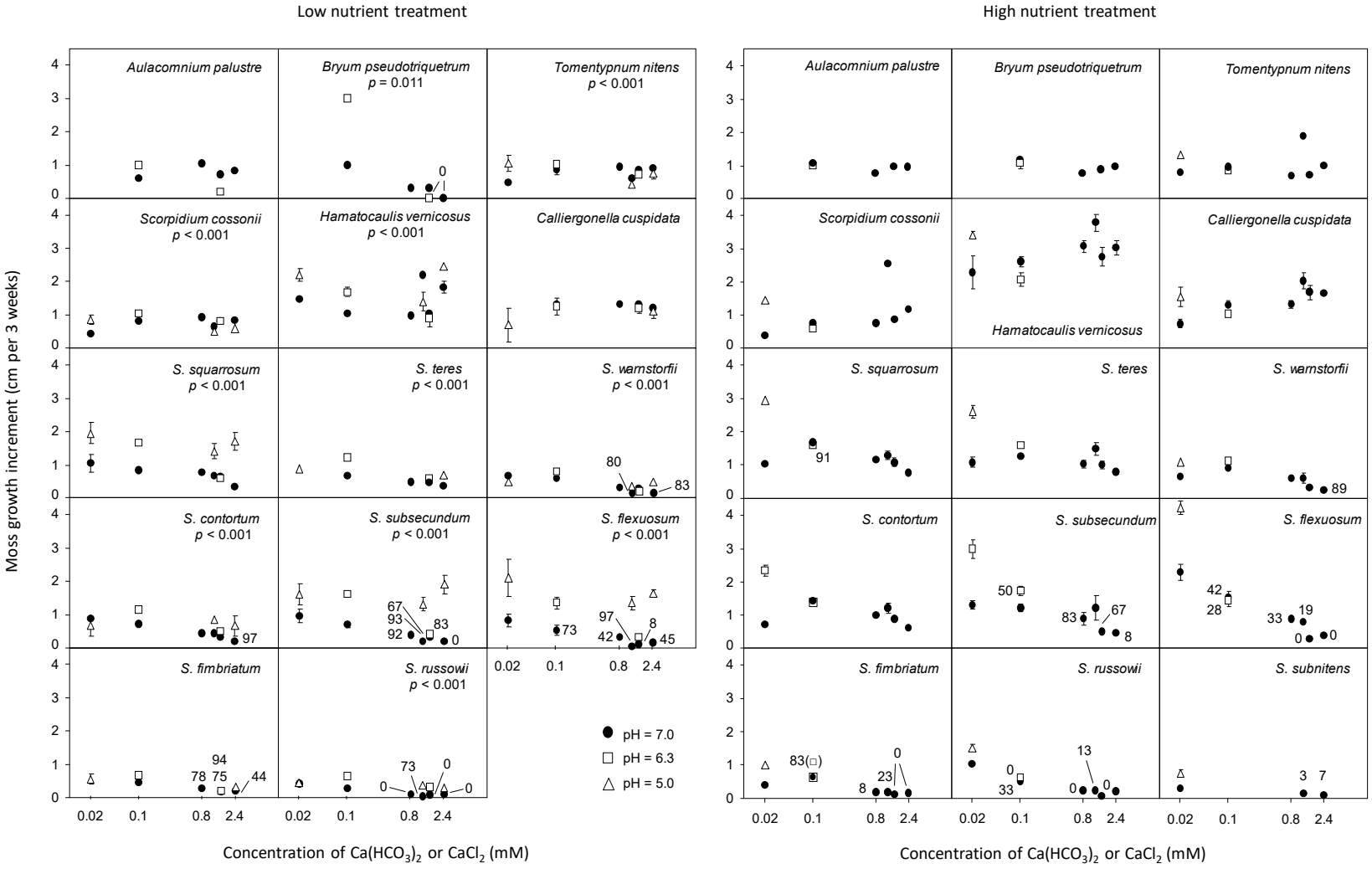
Low nutrient treatment
High nutrient treatment
Moss growth increment (cm per 3 weeks)
Aulacomnium palustre
Bryum pseudotriquetrum
p = 0.011
Tomentypnum nitens
p < 0.001
Scorpidium cossonii
p < 0.001
Hamatocaulis vernicosus
p < 0.001
Calliergonella cuspidata
S. squarrosum
p < 0.001
S. teres
p < 0.001
S. warnstorfii
p < 0.001
S. contortum
p < 0.001
S. subsecundum
p < 0.001
S. flexuosum
p < 0.001
S. fimbriatum
S. russowii
p < 0.001
S. subnitens
pH = 7.0
pH = 6.3
pH = 5.0
Concentration of Ca(HCO3)2 or CaCl2 (mM)

Fig. 1. Growth of moss shoots as a function of Ca^{2+} concentration. The shoots were grown submersed in flowing solutions of calcium bicarbonate (pH 7.0 and 6.3) or calcium chloride (pH 5.0) for three weeks. The *p*-values denote significant statistical difference (<0.05) between low and high nutrient treatment. The number shown for some points indicate percentage of shoot survival if it was lower than 100. (Low nutrient treatment shows results of cultivation 2, 4 and 5, high nutrient treatment the cultivation 1 and 3; the number of specimens used in cultivations denotes Table A2.)

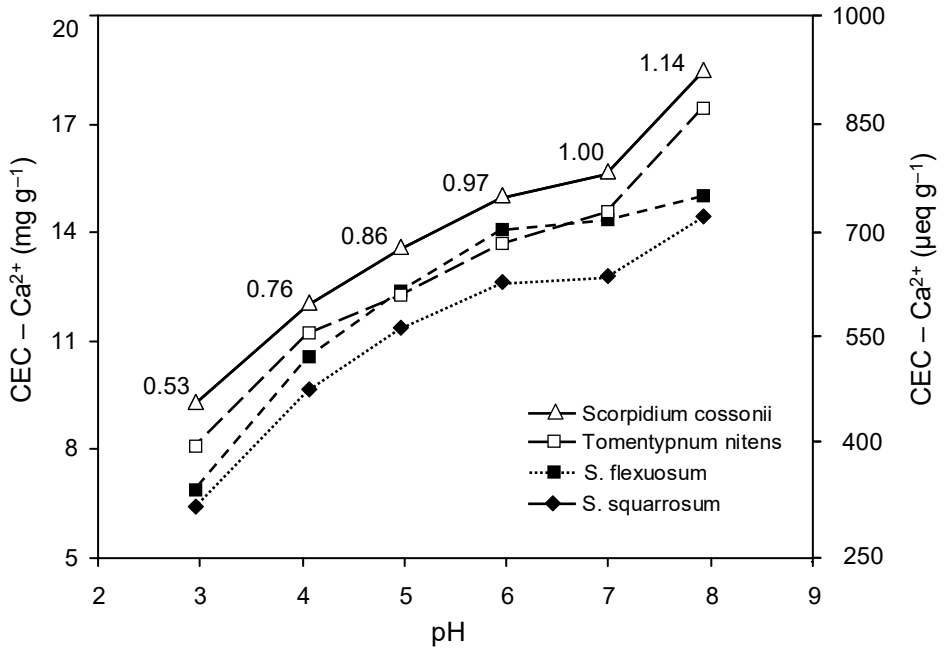

Fig. 2. Cation exchange capacity (CEC) of moss shoots (expressed as mg or µeq of Ca^{2+} per g of dry mass) as a function of pH. The numbers show ratio between CEC of moss material at a given pH and CEC in pH 7. Cell-wall cation-exchange sites were fully saturated in 100 mM $CaCl_2$ at given pH followed by elution with hydrochloric acid. The data present mean of three replicates per species.

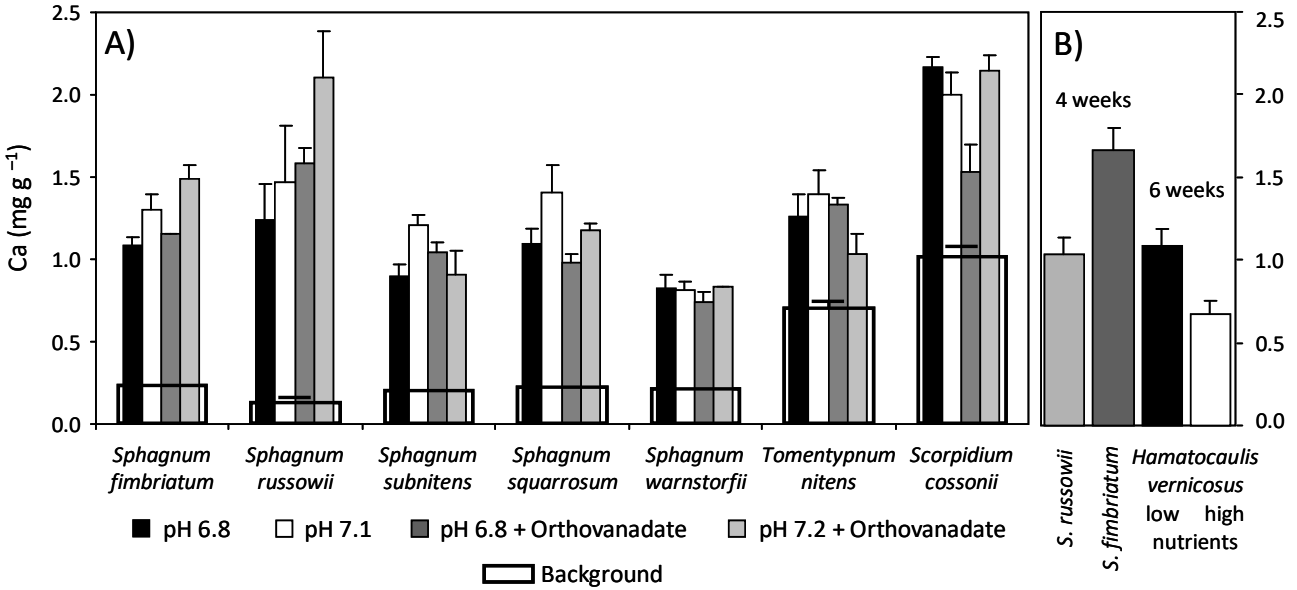

Fig. 3. Intracellular Ca^{2+} accumulation (mg g^{-1} of dry mass) in *Sphagnum* species and brown mosses grown in calcareous solutions (2.4 mM $Ca(HCO_3)_2$) for A) 9 days (wide empty columns indicate background, i.e. pre-treatment intracellular [Ca^{2+}]; gray columns indicate the addition of 0.1 mM sodium orthovanadate as ATPase inhibitor) and B) 4 and 6 weeks. The data present A) two replicates per species (background, *S. squarrosum* treatments) or three replicates (other species); B) two replicates for each *Sphagnum*, three replicates for *H. vernicosus* per treatment.

Discussion

The tolerance of fen mosses to calcium bicarbonate concentrations in artificial rich fen waters (fundamental niches) exhibited the same pattern as the general, field-based calcifuge–calcicole behaviour (realized niches). However, the fundamental niches of *Sphagnum* species (i.e., the conditions allowing moss survival) were much broader and overlapped with the realized niches of brown mosses (Hájková and Hájek 2004; Z. Plesková et al. (unpublished results)).

The combined toxicity of high Ca^{2+} and pH confirms the results of Clymo (1973). The role of bicarbonate in calcium toxicity obviously resides in its buffering properties maintaining high pH. The physiological mechanisms behind calcium toxicity in bryophytes were generally unknown. Our results indicate that the metabolism of calcifuges is disturbed by imbalance in cytosolic [Ca^{2+}], which may result in quick death. On the other hand, nutrient deficiency or processes at the cell-wall level related to cation exchange may affect long-term survival through changes in competitive relationships between brown mosses and sphagna.

Role of the cell wall in calcium tolerance

Under high pH and [Ca^{2+}], negatively charged carboxyl groups of pectic polysaccharides, one of the principal components of the cell wall, become saturated with Ca^{2+} (Richter and Dainty 1989). The saturation make the cell wall rigid (Fraeye et al. 2009), precluding its loosening, and consequently its growth and cell division (Proseus and Boyer 2006; Hepler and Winship 2010). We therefore expected a connection between growth inhibition of *Sphagnum* in calcium bicarbonate solutions and saturation of cell-wall cation-exchange sites. However, the exchange sites were not saturated with Ca^{2+} in either flowing or stagnant solutions in any of the species, even in shoots that were not actively growing. It therefore appears that Ca^{2+} does not "condense" on cell walls of living cells as suggested by Dainty and Richter (1993); they proposed that such oversaturation (in the context of Manning's counterion condensation theory) of cell-wall carboxyls with calcium may inhibit intracellular uptake of monovalent cations. Thus, cell-wall bound Ca^{2+} does not seem to affect shoot growth.

The apparent paradox between the low saturation of exchange sites by Ca^{2+} in living cells under high pH can be explained by extrusion of H^+ to the apoplast, a fundamental process in plant cells. Protons compete for cation exchange sites with Ca^{2+}, but may also react with bicarbonate, which buffers acidification. In contrast to flowing solutions of $Ca(HCO_3)_2$, the low level of stress in stagnant solutions indicates that long diffusion distance of the bicarbonate anion may limit its pH-buffering ability in the cell wall near the plasma membrane. While protons diffuse over a maximum distance of 1 µm across the cell wall, bicarbonate anions in stagnant solutions must diffuse over distances up to several orders of magnitude longer. According to Fick's second law, diffusion time is proportional to the square of distance. The contrasting diffusion distance may greatly suppress the concentration of bicarbonate in the cell wall, where it reacts with protons extruded by the cell; this mechanism may maintain lower pH and exchangeable $[Ca^{2+}]$ in the cell wall. Interestingly, despite the low saturation of cation exchange sites, the greyish–dark-purple colouration of cell-wall anthocyanins indicated a rather neutral pH in the cell wall; nevertheless, such an approach to cell-wall pH estimation has not been validated and may be affected by the complex cellular structure of the *Sphagnum* leaf.

The CEC (pH 7.0) of newly formed shoots (only brown mosses were tested) and of protonemata decreased with increasing pH of the solution, independently upon $[Ca^{2+}]$. This constitutes the first experimental evidence that water chemistry may control CEC, i.e. that CEC is partly inducible. Surprisingly, this result does not fit with the general pattern of CEC in bryophytes and their calcifuge–calcicole behaviour; species that thrive in calcareous soils have generally greater CEC than species in acidic soils (Bates 1982; Koedam and Büscher 1983; Büscher et al. 1990), or there is no pattern (Soudzilovskaia et al. 2010). In addition, CEC in our cultivations slightly increased with increasing nutrient concentration, i.e. with increased growth. The positive correlation between growth and CEC is in accordance with field observation of Clymo (1963). Dainty and Richter (1993) suggested that the proximity of membrane transporters to cation exchange sites may facilitate cation uptake. In consequence, mosses in our solutions could lower CEC in attempt to decrease the amount of Ca^{2+} entering the protoplast. Moreover or alternatively, reduced CEC would facilitate cell-wall loosening at higher pH. By

contrast, generally high CEC of calcicoles under field conditions might facilitate uptake of micronutrients, particularly iron acquisition and uptake in calcareous environments, where, contrary to our solutions, these metals are deficient (Boyer and Wheeler 1989; Rozbrojová and Hájek 2008).

Intracellular processes associated with calcium tolerance

The survival of *Sphagnum* species in calcareous solutions was linked with intracellular Ca^{2+} accumulation. Calcium commonly accumulates in plants exposed to high extracellular [Ca^{2+}] (Conn 2011; Graham 2014 and references therein), increasing the expression of calcium antiporters (Ca^{2+}/H^{+} exchangers) and pumps (Ca^{2+}-ATPases; Garciadeblas 2001; Kamiya et al. 2006). Calcium may accumulate in vacuoles of vascular plants in millimolar concentrations, being precipitated as oxalate. Intracellular [Ca^{2+}] in our mosses was lower (about 10–100 times), presumably because vacuolar precipitation of calcium oxalate has not been observed in bryophytes. As far as we know, Ca^{2+} accumulation has not been noted to be pH dependent in plants; in our cultivations, intracellular Ca^{2+} accumulated only at high extracellular pH. By contrast, vascular plants may accumulate Ca^{2+} even in solutions with low pH (3.8–4.4; Graham 2014).

The dependence of Ca^{2+} accumulation on pH may be explained by pH-controlled calcium efflux and/or influx to the cytosol. Its efflux in eukaryotic cells is provided by Ca-ATPases and Ca/H exchangers. These exchangers are crucial for survival under high external [Ca^{2+}] (Guttery at al. 2013), and because they are governed by the transmembrane proton gradient, their function is pH-dependent (Pittman et al. 2005). The observed selective efflux of Ca^{2+} at low pH corresponds with this mechanism. By contrast, Ca^{2+} influx in plants is not regulated by pH, contrary to animal cells (Reid and Smith 1992; Thuleau et al. 1994). The pH-dependent Ca^{2+} accumulation, connected with efflux regulation, may explain the tolerance of calcifuges to high [Ca^{2+}] at low pH, while the combination of high [Ca^{2+}] and high pH is toxic.

Since the intracellular [Ca^{2+}] at high pH did not differ between calcicolous and calcifugous *Sphagnum* and brown mosses, it seems that its toxicity lies rather in insufficient control over the balance between

intracellular Ca^{2+} uptake and efflux (to vacuoles or the apoplast). Excessive cytosolic Ca^{2+} interferes with the cell metabolism; this corresponds with the reduced photosynthesis rate in calcicolous sphagna as well as the rates of photosynthesis and respiration in calcifugous sphagna. Moreover, F_v/F_m decreased in calcifugous sphagna in calcareous solutions, indicating detrimental changes in photosystems in thylakoid membranes. Inhibition of Ca^{2+} efflux by orthovanadate probably also reduced intracellular Ca^{2+} influx, as none of the species exhibited increased intracellular Ca^{2+} accumulation; the mechanism is unknown. However, despite the possible influx reduction, the efflux inhibition markedly reduced photosynthesis and respiration rates as well as F_v/F_m of all species in alkaline solutions. We therefore assume that brown mosses are specifically adapted to preserve Ca^{2+} homoeostasis even under conditions causing higher Ca^{2+} uptake; regulation of Ca^{2+} efflux seems to be involved in the mechanism. The adaptation is ion-specific because it did not alleviate Mg^{2+} toxicity. By contrast, calcifugous sphagna could be limited by Ca^{2+} efflux (as their response to high external $[Ca^{2+}]$ corresponded to brown mosses when Ca^{2+} efflux was blocked) and/or by influx regulation, as high external $[K^+]$ compensated for calcium toxicity (K^+-driven plasma-membrane depolarization leads to activation of different Ca^{2+} channels, see Miedema et al. 2001).

In summary, it seems that *Sphagnum* species are not adapted to cope with long-term high input of Ca^{2+} because it may lead to cellular energy depletion and/or increased cytosolic $[Ca^{2+}]$. The latter may interfere with metabolic and/or signalization processes, inducing apoptosis.

Effect of iron and phosphorus in calcium tolerance

Low Fe or P availability in calcareous environments has been suggested to be crucial in the calcifugous behaviour of vascular plants (Zohlen and Tyler 2004) and lichens (Paul et al. 2009). Its significance in bryophytes is rather uncertain, however. We did not observe iron deficiency in calcareous solutions in calcifuges; the survival of *S. fimbriatum* even declided under increased $[Fe^{3+}]$. Brown mosses also exhibited lower survival under high $[Fe^{3+}]$ of poor-fen waters, indicating that iron toxicity may limit brown mosses in poor fens. This corresponds with the rarity of

rare brown mosses in iron-rich regions that otherwise provide sufficiently calcium-rich fen habitats (Peterka et al. 2014).

Iron precipitates in alkaline waters (Hájková and Hájek 2007), and it was precipitating also in all of our solutions, although it was provided in chelated form. The available iron concentration in fen waters can be elevated at sites fed by fresh spring water enriched with soluble reduced Fe^{2+}; otherwise it seems that the analysed fen water [Fe^{3+}] above about 1 mg L^{-1} may have included precipitated iron whose effective concentration for uptake is thus lower.

Less phosphorus could be available for calcifuges in calcareous solutions, as sphagna assimilated less P than calcicolous brown mosses. However, the growth or survival of calcifuges in solutions of low [P] did not differ from the P-rich solution. These results indicate that low iron or phosphorus concentrations do not affect the establishment of calcifuges in rich fens, as previously suggested (Hájek et al. 2002, 2014).

Competition and survival of fen mosses along the poor–rich gradient

The wide fundamental niches (with respect to pH and calcium) of the moss species tested suggest that bryophyte distribution in poor to calcareous fens is strongly influenced by competition. Competitive hierarchies (usually without competitive exclusion) have been repeatedly evidenced within various bryophyte communities (Rydin 1997; Zamfir and Goldberg 2000; Mälson and Rydin 2009; Spitale 2009). Nevertheless, our experimental study demonstrates that the competitive hierarchy changes along the pH/calcium gradient in fens, corresponding to species distribution patterns observed in field studies. Brown mosses grew best in alkaline fen waters ([$Ca(HCO_3)_2$] > 1.2–1.5 mM, pH > 7.0), while *Sphagnum* exhibited the highest growth in moderately rich and poor fen waters ([$Ca(HCO_3)_2$] < 0.1 mM, pH 6.3–7.1). The solution of 0.1 mM $Ca(HCO_3)_2$, pH 6.3 was suitable for the growth and survival of *S. flexuosum* (Figs. 1, A.2), whose competitive dominance in Ca^{2+}-poor waters (0.02 mM $Ca(HCO_3)_2$) is responsible for the very low species diversity of poor fens. In the central part of the pH/calcium gradient (1.2 mM $Ca(HCO_3)_2$, pH 7.1 to 0.1 mM $Ca(HCO_3)_2$, pH 6.3–7.1), calcium-tolerant sphagna could coexist with brown mosses. This coexistence enables the development of species-rich fen communities referred to as

Sphagno warnstorfii–Tomentypnion nitentis in European vegetation science (Peterka et al. 2014).

Alkaline fens are naturally N- and P-poor systems with pH-limited microbial turnover of these nutrients (Kooijman & Hedenäs 2009). Thus, increasing P and N availability, demonstrated also by our experiments, increases the probability of competitive exclusion. Consequently, *Sphagno–Tomentypnion* communities are disappearing from anthropogenically N- and P-enriched regions of Central and Western Europe, severely endangering many of their important species (Peterka et al. 2014), while being preserved in nutrient-poor mountain or boreal regions (Moen et al. 2012).

The questions remain why intensified competition in these mixed communities currently more often leads to the development of *Sphagnum*-dominated fens than to brown-moss fens and why sphagna have recently expanded even into purely brown-moss communities. One possible explanation is that sphagna establish when brown-moss competition is lowered by perturbation. That can be caused mechanically or by environmental factors such as water table decline, ammonium toxicity for brown mosses (Paulissen et al. 2004, 2005; Kooijman and Paulissen 2006; Verhoeven et al. 2011) or other water chemistry effects. The possible effect of water table decline is illustrated by our finding of decreasing growth of *H. vernicosus* above the water table, where the growth of *Sphagnum* species accelerated or did not change, corroborating empirical experience from restoration of brown-moss patches by digging shallow holes in fens with lowered water tables (Štechová and Kučera 2007). Moreover, the interaction between mechanical disturbances, water level or weather (Hájek et al. 2014; E. Vicherová et al. (unpublished results)) may be crucial. Once established, spreading *Sphagnum* shoots accumulate rain water, which gets continually acidified by the production of new cation exchange sites (Dainty and Richter 1993; Kooijman 2012). This *Sphagnum*-driven change of local chemistry enables the development of more or less ombrotrophic *Sphagnum* hummocks in alkaline fens and, under suitable hydrological conditions, may trigger gradual separation of the mire surface from mineral spring water.

The next phase of successional change within fens is the expansion of quickly-growing calcifugous sphagna either into stands of calcium-

tolerant sphagna or directly into brown moss stands (Kooijman 2012; Paulissen et al. 2014). Our experiments have demonstrated that potassium, rather than phosphorus or nitrogen stressed by previous studies (Hájek et al. 2002; Kooijman and Paulissen 2006), alleviates the toxic effect of calcium on calcifugous peatmosses, facilitating their survival in calcareous environment. This result is in compliance with recent analyses of large datasets from Central Europe concluding that potassium availability and its tissue concentration coincide with the expansion of certain fen mosses into more alkaline habitats (Hájek et al. 2014; M. Hájek et al. (unpublished results)).

Nevertheless, the difference between our experiment and previous experiments showing an effect of ammonium (Paulissen et al. 2004, 2005; Kooijman and Paulissen 2006; Verhoeven et al. 2011) is that the previous experiments supplied ammonium as the sole N source whereas we used NH_4NO_3 (a combination non-toxic to brown mosses). Nutrients in general therefore do not seem to affect *Sphagnum*–brown-moss competition; however, the increased [NH_4^+] may decrease the competitive abilities of brown mosses, creating a competitive advantage for *Sphagnum* species.

Contrary to the relationship between *Sphagnum* and brown mosses, nutrients had a considerable effect on competition between individual brown mosses. Surprisingly, increased N and P concentrations had a positive effect on the growth and biomass accumulation of the rare *Hamatocaulis vernicosus*, making it the strongest competitor in solutions representing moderately rich, rich and calcareous fens. As a red-listed species also included in the EU Habitat Directive (Council Directive 92/43/EEC 1992), its competitive abilities and wide tolerance to calcium bicarbonate were rather surprising. Rare plant species are generally considered to be weak competitors (Cleavitt 2002; Dawson et al. 2012) therefore negatively affected by high nutrient availability (Dawson et al. 2012), which clearly did not apply to *H. vernicosus* when grown flooded. However, increased nutrient availability creates a denser cover of vascular plants, to which *H. vernicosus* is particularly susceptible (Bauer et al. 2007; Štechová et al. 2012; Cusell et al. 2014). Thus, the resulting effect of increased nutrients in the field may be close to neutral (Štechová and Kučera 2007) or even negative (Bergamini et al. 2009). The uneven distribution pattern of *H. vernicosus* thus seems to be shaped not by recent competitive exclusion by *Calliergonella cuspidata* or other fen

bryophytes, but rather by natural rarity, which may result from specialization for uncommon niches Cleavitt 2002; Markham 2014). The species has attributes of an R-strategist (Hájek et al. 2014), including rapid growth under optimal conditions (Bauer et al. 2007), but the rare sporophyte production (Hedenäs et al. 2003, Pépin et al. 2013) may limit its current effective dispersal in Central Europe with temperate (i.e. less humid) climate. *Hamatocaulis vernicosus* has two cryptic species (Hedenäs and Eldenäs 2007); at least one species was common in late-glacial and early-Holocene flora of European temperate mires (e.g., Dudová et al. 2014; Gałka and Lamentowicz 2014; Hájková et al. 2015). As an early-successional element of wetlands on deglaciated land (Glime et al. 1982), its frequent occurrence in post-glacial Europe could be enabled by availability of suitable microhabitats, which fits with the proposed specialization for uncommon niche.

Calcium tolerance of protonemata and its consequences in species establishment

The broad tolerance of fen moss protonemata to pH and Ca^{2+} complements the theoretical niches of adult gametophores and enables bryophytes to invade fen biotopes under lowered competition; particularly spore dispersal – if sporophytes are frequently produced – is efficient way how to spread to new localities (Miller and McDaniel 2004). It should be however noted that the long-distance dispersal of species less frequently producing sporophytes may rely on vegetative reproduction through microfragments distributed by zoochory (particularly birds; Lewis et al. 2014). We found the broadest tolerance and the fastest protonema development in the common species *Calliergonella cuspidata,* indicating that the wide distribution of common species is related to the low sensitivity of their protonemata to the environment (Löbel and Rydin 2010). Similarly, the most common peat moss of rich fens, *Sphagnum teres*, was the only *Sphagnum* tested that germinated even in calcareous fen waters, creating small, deformed protonemata. The deformation of protonemata is a common reaction to metal toxicity (Kapur and Chopra 1989) and in itself has no negative effect on the species' viability.

Calcium tolerance of protonemata is particularly important for *Sphagnum* expansion to alkaline fens. Since *Sphagnum* protonemata lack physiological tolerance to desiccation or morphological means of

avoiding it (Hájek and Vicherová 2014), their development is restricted to water-saturated and consequently calcareous microhabitats. Thus the common strategy of species recently expanding to alkaline fens (*S. squarrosum, S. flexuosum*; Kooijman and Paulissen 2006; Kooijman 2012) as well as the most common species of rich fens (*S. teres*) is to create small protonemata with early formation of adult gametophores that facilitate their competitiveness with brown mosses. In addition, fast growth keeps newly formed shoots away from a direct influence of mineral-rich fen water and provides the necessary acidification.

Sphagnum species were unable to acclimate to alkaline conditions even during early developmental stages (germination, protonema formation). The wider tolerance of some individuals of calcifugous species to alkaline waters thus may be caused by long-term genetic adaptation. Our observation corresponds with the results of a molecular study of the calcium-tolerant peat moss *Sphagnum warnstorfii*; a significant part of this species' genetic variability reflected a pH gradient, even within individual localities (Mikulášková et al. 2014). The inability of calcifuges to acclimate to calcium-rich waters obviously prevents *Sphagnum* establishment and expansion in alkaline fens. A comparative genomic and transcriptomic study of calcicolous species with more and less calcium-tolerant calcifuges would constitute a good experimental approach to identifying genes responsible for calcium tolerance in bryophytes.

Conclusions

The ecophysiological background of calcium intolerance in fen bryophytes may be linked to insufficient control over the balance of Ca^{2+} influx/efflux in the cytosol in alkaline solutions. Especially Ca^{2+} efflux seems to be involved in calcium tolerance – when it was blocked, brown mosses became susceptible to calcium bicarbonate to a similar extent as sphagna. By contrast, Ca^{2+} accumulation itself is not a direct cause of calcium toxicity, as it did not differ among species. The cell wall compartment is not involved in calcium toxicity through the inhibition of cell-wall expansion or monovalent cation uptake as proposed by Dainty and Richter (1993), who probably neglected the role of living protoplast in apoplast acidification, which keeps cell-wall cation-exchange sites unsaturated by Ca^{2+} even in calcareous solutions. Contrary to vascular plants, neither phosphorus nor iron deficiency was found to limit the survival or growth of calcifugous mosses in alkaline waters. Calcicolous as well as calcifugous fen bryophytes have rather wide fundamental niches nearly covering the complete natural pH/calcium gradient found in fens, except for calcifugous sphagna that cannot survive in flowing calcium-rich water. The strong compositional turnover in fens along the pH/calcium gradient observed in nature is therefore a product of competitive hierarchy among the three major functional groups of fen bryophytes (brown mosses, calcium-tolerant sphagna, calcifugous sphagna) that restricts their realized niches, but changes along the pH/calcium gradient. High calcium bicarbonate concentrations in alkaline fens favour the growth and biomass accumulation of brown mosses. Relatively calcium-tolerant *Sphagnum* species (*S. teres, S. squarrosum*) can nevertheless germinate and survive in these conditions, yet their growth and biomass accumulation is low. In nitrogen- and phosphorus-enriched fens (e.g., in temperate agricultural landscapes), the competition is stronger. This may lead to the disappearance of mixed communities with co-existing brown mosses and sphagna. However, nutrient enrichment cannot explain the decline of all brown mosses in general; nitrogen and phosphorus enrichment surprisingly improved the competitive ability of rare *Hamatocaulis vernicosus*.

If the competitive equilibrium is disrupted by perturbation, short-term survival outside the realized niches may trigger a successional shift from an alkaline fen to a *Sphagnum* fen dominated by calcium-tolerant

sphagna. The expansion of calcifugous sphagna into either fens dominated by calcium-tolerant sphagna or brown-moss fens triggers the succession that substantially threatens fen biodiversity in agricultural landscapes. This successional process is conditioned by increased potassium availability, which alleviates the detrimental effect of calcium on calcifugous sphagna. Our laboratory experiments did not confirm previously acknowledged effects of phosphorus and nitrogen.

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Supplementary material

Supplementary material related to this article can be found in the online version as two separate files.

Table A.1: List of sampled localities with information about groundwater chemistry.
Table A.2: List of species used in cultivation experiments and a number of replicates.
Table A.3 and A.4: Cation exchange capacity of protonemata or moss gametophores cultivated in solutions of various [Ca^{2+}] and pH or nutrient concentration.
Table A.5: Net photosynthesis and dark respiration rates of fen mosses grown in calcareous solutions or distilled water with/without 0.1 mM sodium orthovanadate addition (ATPase inhibitor).
Table A.6: Measurement of maximum quantum efficiency of PS II photochemistry before and during moss cultivation in calcareous solutions or distilled water with/without sodium orthovanadate addition.
Figure A.1: Cultivation technology – flowing solutions.
Figure A.2 and A.3: Biomass increment and survival of fen mosses cultivated in solutions of various [Ca^{2+}] and pH with/without an addition of other cations.
Figure A.4: Biomass increment of *Calliergonella cuspidata* and *Hamatocaulis vernicosus* in stagnant calcareous solutions after 9-week cultivation in different [N, P].
Figure A.5: Survival of fen mosses in flowing solutions of calcium/magnesium bicarbonate.
Figure A.6: Spore germination of fen mosses in solutions of calcium bicarbonate or calcium/potassium chloride.

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Supplementary material

Table A.1. List of sampled localities and their groundwater chemistry.

Locality name	Mire type	Localization	GPS coordinates	Altitude (m a.s.l.)	pH	$[Ca^{2+}]$ (mg L^{-1})
Chvojnov	rich fen	Vysočina region, Czech Republic	49°24'19"N, 15°25'11"E	606	5.8	17
Rojkov	calcareous fen	Žilina region, SZ Slovakia	49°08'55"N, 19°09'19"E	940	6.9	90
Brezové	calcareous fen	Prešov region, N Slovakia	49°03'03"N, 20°01'42"E	850	7.5	80
Kubínska hoľa	mountain springs	Orava region, Slovakia	49°16'22"N, 19°15'38"E	1300	7.0	30
Jalovec	rich fen meadow	Žilina region, N Slovakia	49°07'59"N, 19°37'42"E	670	6.1	14
U Hada	moderately rich fen (alder carr)	S Bohemia, Czech Republic	48°58'41"N, 14°25'37"E	380		
Řeka	rich fen	Vysočina region, Czech Republic	49°39'59"N, 15°51'11"E	550	7.2	50
Torronsuo NP	raised bog	Southern Finland	60°44'N, 23°37'E	130		
Sliačské Travertíny	calcareous fen	Žilina region, Slovakia	49°03'23"N, 19°24'52"E	560	7.7	116
Dlouhá louka	moderately rich fen	Plzeň region, Czech Republic	49°54'44"N, 13°10'43"E	570	6.3	6.5
Jochy	moderately rich fen	Žilina region, Czech Republic	49°07'15"N, 19°46'23"E	885	5.5	4
Zlatá louka	rich fen	Vysočina region, Czech Republic	49°42'49"N, 15°46'23"E	470	7.0	50
V Rájích	rich fen	S Bohemia, Czech Republic	48°59'09"N, 14°42'31"E	445	6.2	36
Liptovská Teplička	rich fen	Prešov region, Slovakia	48°57'50"N, 20°06'24"E	900	7.0	35
Demänová	calcareous fen	Žilina region, Slovakia	49°03'06"N, 19°34'47"E	665	7.5	76
Mydlovary	wet spruce forest	S Bohemia, Czech Republic	49°04'35"N, 14°23'12"E	400		
Swamp	poor fen	N Bohemia, Czech Republic	50°34'38"N, 14°39'50"E	270		
Žemlička	rich fen meadow	S Bohemia, Czech Republic	48°53'31"N, 14°41'17"E	470		
Hovízna	moderately rich fen	S Bohemia, Czech Republic	49°08'34"N, 14°41'39"E	420	5.5	4

Locality name	Mire type	Localization	GPS coordinates	Altitude (m a.s.l.)	pH	$[Ca^{2+}]$ (mg L^{-1})
Loučeň	calcareous fen meadow	N Bohemia, Czech Republic	50°18'06"N, 15°01'05"E	240	7.5	180
Kuresoo	poor fen	Central Estonia	58°27'55"N, 25°10'45"E	40		
Ruda	moderately rich fen	S Bohemia, Czech Republic	49°08'43"N, 14°41'27"E	416	5.0	10
Liptovská Lúžna	rich fen	Žilina region, Slovakia	48°56'24"N, 19°21'09"E	695	6.5	5
Chrastě	moderately rich fen	Žilina region, Slovakia	49°02'27"N, 19°31'29"E	655	5.5	10
České Budějovice	grassland	S Bohemia, Czech Republic	48°58'40"N, 14°26'44"E	400		

Table A.2. List of species, source localities and replicate numbers used in cultivation experiments. The two or three numbers indicate (from left): (i) number of replicates per treatment used in growth analysis; (ii), in parentheses, number of cultivated individuals (each replicate includes mean of one or more individuals); (iii) number of replicates used in biomass production (only cultivations 1, 2 and 5). In the spore cultivation experiment, number of mesh-bags (replicates representing thousands of spores from different individuals) used per each treatment is indicated.

Species	**Locality**	**Cultivation experiment**							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**Spores**
Aulacomnium palustre	Chvojnov	4(9)1	2(8)						
	Liptovská Teplička	4(10)1	2(8)1						
	Řeka	4(10)1							
	Rojkov		2(8)1						
Bryum pseudotriquetrum	Brezové	3(15)1	2(8)1						
	Rojkov		2(8)1						
	Sliačské Travertíny	3(15)1	2(8)1						
Calliergonella cuspidata	Brezové	4(17)2	3(8)1						
	České Budějovice					2(10)2	3(18)		
	Chvojnov	4(10)1							
	Řeka	4(10)1							
	Loučeň	4(8)1	3(8)1						
	Demänová			2(10)					
	Hrádecká Bahna						3(18)		
	Rojkov		3(8)1	2(10)					
	Ruda								4
	Žemlička			2(10)					

Species	Locality	Cultivation experiment							
		1	2	3	4	5	6	7	Spores
Sphagnum contortum	Chrastě	4(4)2	4(4)2						
	Chvojnov	4(4)2	4(4)2	4(4)	2(2)	2(2)2			
	Řeka	4(4)2	4(4)2	4(4)	1(1)				
	V Rájích			4(4)	3(3)	4(4)4			
Sphagnum fimbriatum	Brezové		6(6)3						
	Dlouhá louka	6(6)3	6(6)3						
	Řeka			6(6)			3(3)	2(2)	
	Rojkov			6(6)		3(3)3	3(3)	2(2)	2
	Sliačské Travertíny	6(6)3	6(6)3						
	Swamp			6(6)					2
Sphagnum flexuosum	Brezové	3(3)3	3(3)3						
	Chvojnov	3(3)3	2(2)2						
	Dlouhá louka			6(6)	3(3)	3(3)3			2
	Hovízna			6(6)	3(3)				
	Jochy	3(3)3	3(3)3						
	Liptovská Teplička	3(3)3	3(3)3						
	Kuresoo								2
	V Rájích			6(6)					
Hamatocaulis vernicosus	Hrádecká Bahna				1(7)	2(12)2	8(48)		
	Liptovská Lúžna	4(10)1	3(7)1						
	Liptovská Teplička	4(10)1	3(7)1	3(15)	1(7)				
	Řeka	4(10)1	3(7)1	3(15)	1(7)				

Species	Locality	Cultivation experiment							
		1	2	3	4	5	6	7	Spores
Sphagnum russowii	Torronsuo	6(6)3	6(6)3						
	Hovízna			6(6)					
	Hůrky				3(3)	3(3)3	3(3)		
	Kubínská hoľa	6(6)3	4(4)2	6(6)	2(2)		3(3)	2(2)	4
	Rojkov	6(6)3	6(6)3	6(6)	1(1)		2(2)	2(2)	
Scorpidium cossonii	Brezové	4(10)1	3(7)1	3(15)	1(7)				4
	Demänová			6(15)	1(7)				
	Rojkov	3(10)1	2(7)1	3(15)	1(7)				
	Liptovská Teplička	4(10)1	2(7)1						
	Řeka	4(10)1	3(7)1						
	V Rájích					2(12)2			
	Sliačské Travertíny	4(10)1	2(7)1						
	Loučeň	4(10)1	3(7)1						
Sphagnum squarrosum	Had	6(6)6	4(4)4	6(6)	3(3)	3(3)3			4
	Mydlovary			6(6)	3(3)	2(2)2			4
	Sliačské Travertíny	6(6)6	4(4)4						
Sphagnum subnitens	Swamp			2(2)			3(3)		
	Žemlička			2(2)					
	Zlatá louka			2(2)			3(3)	4(4)	4
Sphagnum subsecundum	Dlouhá louka	4(4)2	4(4)2	6(6)	3(3)	3(3)3			4
	Jochy	4(4)2	4(4)2						
	Kubínská hoľa	4(4)2	4(4)2	6(6)	3(3)				

Species	Locality	Cultivation experiment							
		1	2	3	4	5	6	7	Spores
Sphagnum teres	Chvojnov	4(4)4	3(3)3						
	Dlouhá louka			6(6)		4(4)4			
	Kubínská hoľa	4(4)4	4(4)4						
	Řeka	4(4)4	3(3)3	6(6)					
	Swamp								4
	Zlatá louka			6(6)					
Tomentypnum nitens	Brezové	4(10)1	3(12)1	3(12)	1(7)				
	Kubínská hoľa	4(10)1	2(12)1						
	Demänová			6(18)					
	Hrádecká Bahna				1(7)	2(12)1			
	Řeka	4(10)1	3(12)1						4
	Rojkov			3(12)	1(7)				
Sphagnum warnstorfii	Chvojnov		6(6)3						
	Hrádecká Bahna				3(3)	3(3)3			
	Jalovec	6(6)3	6(6)3						
	Jochy	6(6)3	6(6)3						
	Liptovská Teplička	6(6)3	6(6)3	6(6)	1(1)				
	Řeka			6(6)	2(2)	2(2)2			
	V Rájích			6(6)		3(3)3			

Table A.3. Cation exchange capacity (CEC) of protonemata cultivated in solutions of $Ca(HCO_3)_2$ or $CaCl_2$ (*Solution*) for six weeks. The CEC was determined after full saturation of cation-exchange sites in 30 mM $CaCl_2$ at pH 7.0. One sample (thousands of protonemata) per species and treatment was measured.

Solution	**Species**	**CEC (mg g^{-1})** (Ca^{2+}, pH 7.0)
pH 4.9 ± 0.1		
1.2 mM $CaCl_2$	*Tomentypnum nitens*	27
1.2 mM $CaCl_2$	*Calliergonella cuspidata*	25
1.2 mM $CaCl_2$	*Sphagnum teres*	29
1.2 mM Ca^{2+}	**mosses**	**27**
pH 5.3 ± 0.3		
2.4 mM KCl	*Tomentypnum nitens*	32
2.4 mM KCl	*Calliergonella cuspidata*	30
2.4 mM KCl	*Sphagnum teres*	27
2.4 mM KCl	**mosses**	**30**
pH 5.8 ± 0.2		
0.02 mM $Ca(HCO_3)_2$	*Tomentypnum nitens*	21
0.02 mM $Ca(HCO_3)_2$	*Calliergonella cuspidata*	21
0.02 mM $CaCl_2$	*Sphagnum teres*	22
0.02 mM Ca^{2+}	**mosses**	**21**
pH 7.1 ± 0.1		
1.2mM $Ca(HCO_3)_2$	*Tomentypnum nitens*	17
1.2mM $Ca(HCO_3)_2$	*Calliergonella cuspidata*	19
1.2mM $Ca(HCO_3)_2$	*Sphagnum teres*	12
1.2mM $Ca(HCO_3)_2$	**mosses**	**16**

Table A.4. Cation exchange capacity (CEC) of moss gametophores cultivated in 2.4 mM $Ca(HCO_3)_2$ in contrasting nutrients (low/high N: 0.05/1.4 mg L^{-1}; low/high P: 0.1/0.5 mg L^{-1}) for nine weeks. The CEC of newly grown apical parts was determined after full saturation of cation exchange sites in 30 mM $CaCl_2$ at pH 7.0.

Nutrients	**Moss shoots - species**	**CEC (mg g^{-1})** (Ca^{2+}, pH 7.0)	***n***
pH 5.5 ± 0.5 (pre-experimental conditions)			
low (P, N)	*Hamatocaulis vernicosus*	13 ± 0.13	2
	Calliergonella cuspidata	14 ± 0.69	2
pH 7.0 ± 0.2			
high (P, N)	*Hamatocaulis vernicosus*	11 ± 0.15	3
	Calliergonella cuspidata	10 ± 0.17	3
high P, low N	*Hamatocaulis vernicosus*	11 ± 0.81	2
low (P, N)	*Hamatocaulis vernicosus*	9	1

Table A.5. Net photosynthesis (P_N) and dark respiration (R_D) rates (nmol mg^{-1} s^{-1}; dry-mass based) of *Sphagnum* and brown mosses grown in calcareous solutions or distilled water for nine days with/without 0.1 mM sodium orthovanadate addition (ATPase inhibitor). One sample per species (about 0.03 g of dry mass, composed of 4–10 individuals) was measured.

		Distilled water		**Distilled water, orthovanadate**		**$Ca(HCO_3)_2$, pH 6.8**		**$Ca(HCO_3)_2$, pH 6.8, orthovanadate**		**$Ca(HCO_3)_2$, pH 7.2**		**$Ca(HCO_3)_2$, pH 7.2, orthovanadate**	
		P_N	**R_D**	**P_N**	**R_D**	**P_N**	**R_D**	**P_N**	**R_D**	**P_N**	**R_D**	**P_N**	**R_D**
Sphagnum warnstorfii	Non-treated	10.8	–8.3	20.1	–10.8	25.0	–10.0	33.5	–15.0	37.5	–10.0	7.0	–4.0
	Nine days	13.5	–8.3	18.1	–10.8	15.7	–10.0	11.6	–5.0	21.9	–10.0	6.0	–2.0
	Ratio	**1.3**	**1.0**	**0.9**	**1.0**	**0.6**	**1.0**	**0.3**	**0.3**	**0.6**	**1.0**	**0.9**	**0.5**
Tomentypnum nitens	Non-treated	10.0	–15.0	24.5	–14.0	25.0	–8.0	20.0	–9.0	25.0	–11.0	13.5	–5.5
	Nine days	8.5	–15.0	20.0	–11.5	25.0	–14.5	13.5	–6.2	33.5	–11.0	10.5	–3.6
	Ratio	**0.9**	**1.0**	**0.8**	**0.8**	**1.0**	**1.8**	**0.7**	**0.7**	**1.3**	**1.0**	**0.8**	**0.7**
Sphagnum subnitens	Non-treated	16.5	–9.3	18.4	–7.5	22.0	–9.1	16.5	–10.0	12.8	–10.0	28.0	–11.0
	Nine days	18.5	–9.3	17.4	–7.5	4.0	–9.1	6.5	–5.0	7.0	–6.5	6.5	–4.0
	Ratio	**1.1**	**1.0**	**0.9**	**1.0**	**0.2**	**1.0**	**0.4**	**0.5**	**0.5**	**0.7**	**0.2**	**0.4**
Sphagnum squarrosum	Non-treated	20.0	–12.0	21.0	–13.5	31.0	–12.5	41.5	–24.0	34.0	–13.0	40.0	–19.0
	Nine days	27.0	–12.0	25.0	–13.5	18.0	–12.5	8.5	–12.5	23.5	–12.5	17.0	–13.0
	Ratio	**1.4**	**1.0**	**1.2**	**1.0**	**0.6**	**1.0**	**0.2**	**0.5**	**0.7**	**1.0**	**0.4**	**0.7**
Scorpidium cossonii	Non-treated	28.5	–11.0	22.5	–9.5	25.0	–6.0	3.5	–9.5	22.0	–7.0	4.0	–8.0
	Nine days	24.0	–7.5	13	–7.0	22.5	–6.0	–1.0	–3.0	21.5	–6.5	–1.0	–3.0
	Ratio	**0.8**	**0.7**	**0.6**	**0.7**	**0.9**	**1.0**	**–0.3**	**0.3**	**1.0**	**0.9**	**–0.3**	**0.4**
Sphagnum russowii	Non-treated	18.5	–21.0	9.0	–9.0	12.0	–10.0	10.0	–11.0	15.5	–10.0	12.0	–10.0
	Nine days	34.0	–21.0	18.0	–9.0	2.0	–8.0	3.0	–7.0	–1.0	–8.0	–1.0	–6.0
	Ratio	**1.8**	**1.0**	**2.0**	**1.0**	**0.2**	**0.8**	**0.3**	**0.6**	**–0.1**	**0.8**	**–0.1**	**0.6**
Sphagnum fimbriatum	Non-treated	31.0	–16.0	23.0	–15.0	23.0	–10.5	33.5	–18.0	25.0	–13.0	40.0	–17.5
	Nine days	31.0	–16.0	23.0	–15.0	6.0	–9.0	9.5	–8.5	7.0	–10.0	6.5	–10.0
	Ratio	**1.0**	**1.0**	**1.0**	**1.0**	**0.3**	**0.9**	**0.3**	**0.5**	**0.3**	**0.8**	**0.2**	**0.6**

Table A.6. Measurement of maximum quantum efficiency of PS II photochemistry (F_v/F_m) before and during *Sphagnum*/brown-moss cultivation in calcareous solutions or distilled water. The F_v/F_m values measured during the treatment are expressed in % pre-treated shoots value. 0.1 mM sodium orthovanadate (ATPase inhibitor) was added to each of the solution types. One sample per species (about 0.03 g of dry mass, composed of 4–10 individuals) was measured.

Species	F_v/F_m pre-treated shoots	F_v/F_m (% of pre-treated shoots) 2 days	5 days	9 days	F_v/F_m pre-treated shoots	F_v/F_m (% of pre-treated shoots) 2 days	5 days	9 days
		$Ca(HCO_3)_2$, pH 6.8, Orthovanadate				**$Ca(HCO_3)_2$, pH 6.8**		
Sphagnum russowii	0.72	94	79	70	0.72	91	83	80
S. subnitens	0.77	93	77	83	0.75	97	92	92
S. fimbriatum	0.75	98	85	83	0.71	102	96	97
S. squarrosum	0.77	97	70	76	0.75	97	96	96
S. warnstorfii	0.78	95	82	89	0.76	99	98	96
Scorpidium cossonii	0.74	88	75	84	0.74	101	100	103
Tomentypnum nitens	0.76	98	94	98	0.74	106	103	103
		$Ca(HCO_3)_2$, pH 7.2, Orthovanadate				**$Ca(HCO_3)_2$, pH 6.8**		
S. russowii	0.71	93	78	81	0.73	88	86	76
S. subnitens	0.78	94	87	80	0.74	98	96	97
S. fimbriatum	0.76	96	91	79	0.70	101	103	102
S. squarrosum	0.79	95	86	57	0.77	99	97	97
S. warnstorfii	0.76	100	99	102	0.76	102	104	100
Scorpidium cossonii	0.76	95	89	86	0.74	103	104	103
Tomentypnum nitens	0.74	100	102	102	0.70	109	109	108
		Distilled water, Orthovanadate				**Distilled water**		
S. russowii	0.71	105	106	108	0.71	107	109	109
S. subnitens	0.69	109	109	113	0.75	104	104	102
S. fimbriatum	0.69	109	112	112	0.66	114	116	117
S. squarrosum	0.70	108	109	112	0.73	107	108	107
S. warnstorfii	0.70	111	110	111	0.76	101	102	103
Scorpidium cossonii	0.74	98	98	101	0.73	102	104	103
Tomentypnum nitens	0.70	107	108	110	0.70	105	108	103

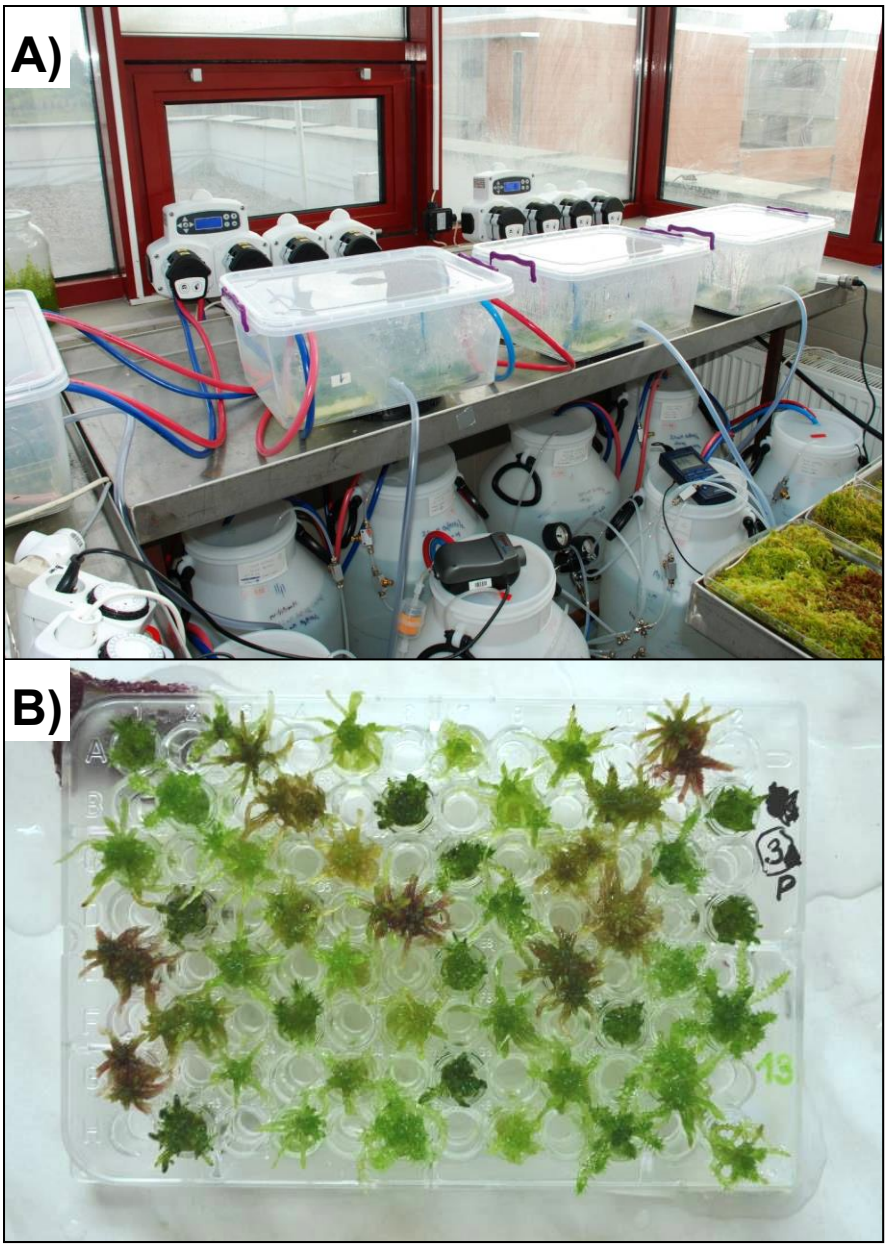

Fig. A.1. Cultivation technology. A) Flowing solutions, where the growth solution is pumped between stock solution (white barrels) and closed transparent boxes with moss shoots. B) Moss shoots placed in 96-well microplates that were attached to the bottom of the cultivation boxes and overflowed by the cultivation solution.

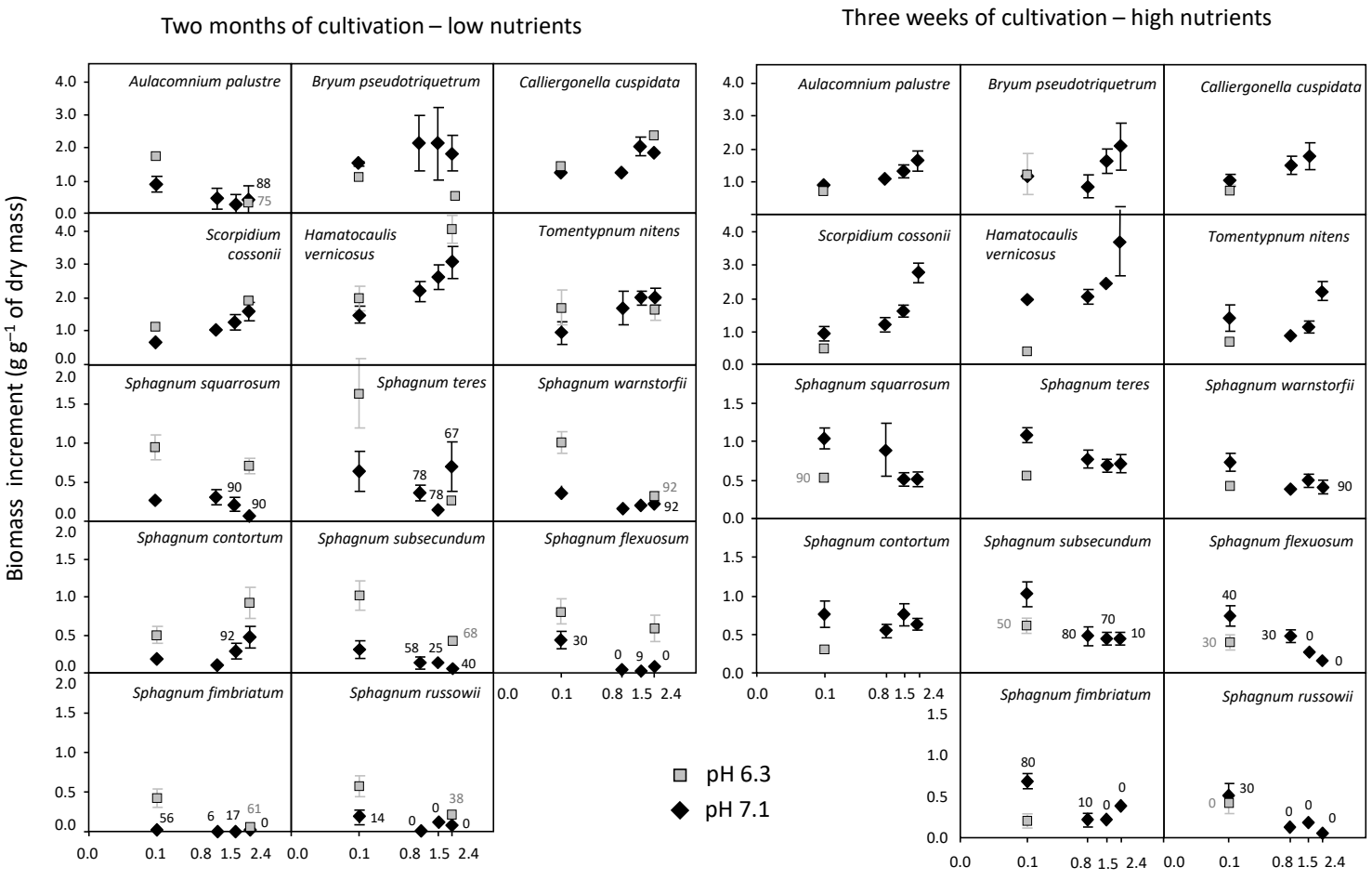
Two months of cultivation – low nutrients
Three weeks of cultivation – high nutrients
Biomass increment (g g−1 of dry mass)
Aulacomnium palustre
Bryum pseudotriquetrum
Calliergonella cuspidata
Scorpidium cossonii
Hamatocaulis vernicosus
Tomentypnum nitens
Sphagnum squarrosum
Sphagnum teres
Sphagnum warnstorfii
Sphagnum contortum
Sphagnum subsecundum
Sphagnum flexuosum
Sphagnum fimbriatum
Sphagnum russowii
pH 6.3
pH 7.1

Fig. A.2. Biomass increment of moss shoots as a function of Ca^{2+} concentration. The shoots were grown submersed in flowing solutions of calcium bicarbonate (pH 7.1 or 6.3). The biomass increment is given in g g^{-1} of initial dry-mass weight expressed for the whole cultivation period (three weeks – Cultivation 1 or two months – Cultivation 2; see methods for cultivation details and Table A2 for number of individuals). Initially, it was measured in fresh-mass weight (see methods for details). The calcifuge *Sphagnum* species and *S. teres* did not grow in second part of the two-months cultivation; therefore the growth was not expressed per unit of time. The number shown for some points indicate percentage of shoot survival if it was lower than 100.

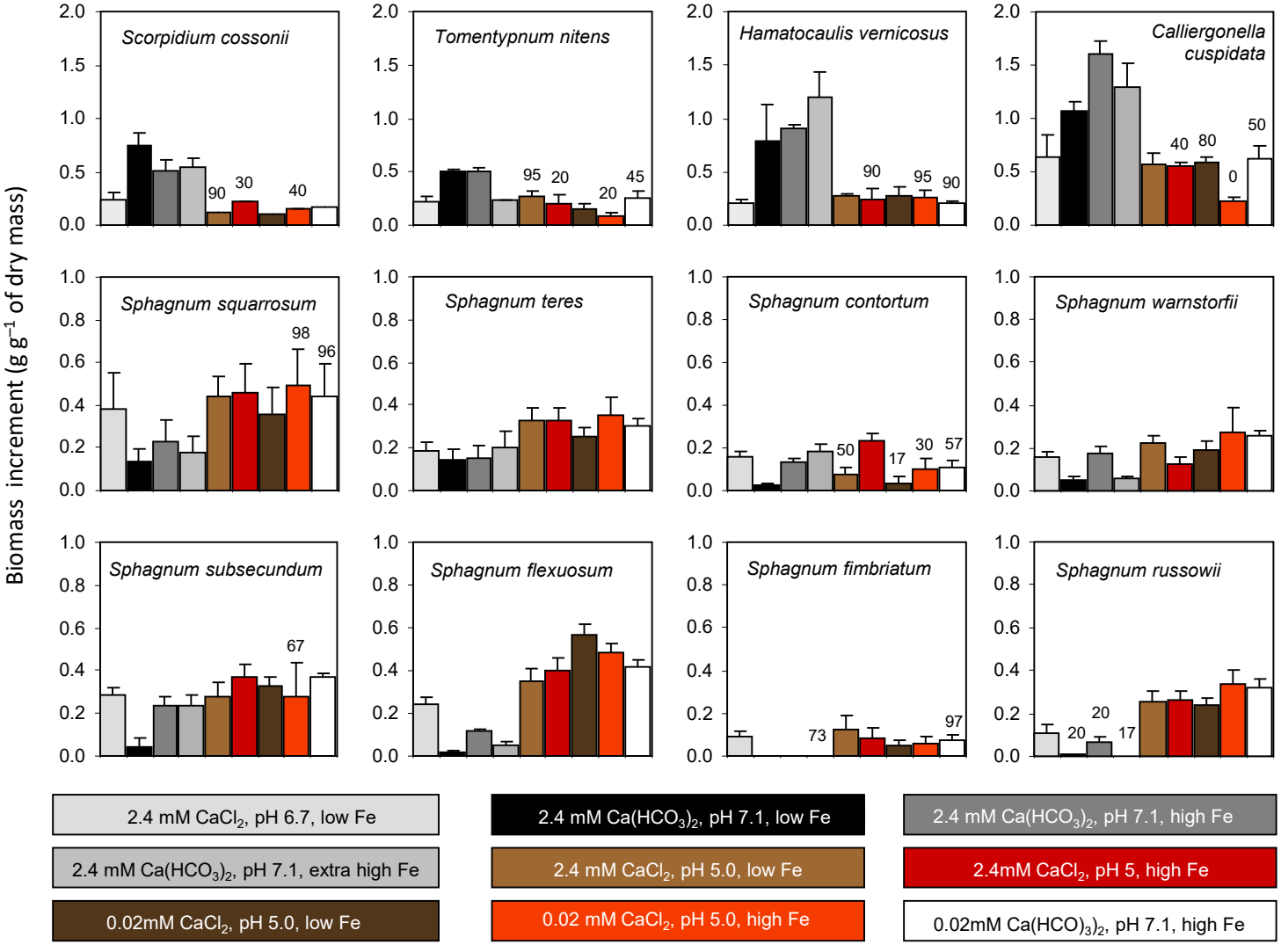
Biomass increment (g g^{-1} of dry mass)
Scorpidium cossonii
Tomentypnum nitens
Hamatocaulis vernicosus
Calliergonella cuspidata
Sphagnum squarrosum
Sphagnum teres
Sphagnum contortum
Sphagnum warnstorfii
Sphagnum subsecundum
Sphagnum flexuosum
Sphagnum fimbriatum
Sphagnum russowii
2.4 mM CaCl2, pH 6.7, low Fe
2.4 mM Ca(HCO3)2, pH 7.1, low Fe
2.4 mM Ca(HCO3)2, pH 7.1, high Fe
2.4 mM Ca(HCO3)2, pH 7.1, extra high Fe
2.4 mM CaCl2, pH 5.0, low Fe
2.4mM CaCl2, pH 5, high Fe
0.02mM CaCl2, pH 5.0, low Fe
0.02 mM CaCl2, pH 5.0, high Fe
0.02mM Ca(HCO)3)2, pH 7.1, high Fe

Fig. A.3. Biomass increment of fen mosses cultivated in solutions of calcium bicarbonate or calcium chloride (columns) of low nutrient concentration for three weeks (Cultivation 5, see methods for details). The number shown for some columns indicate percentage of shoot survival if it was lower than 100. The biomass increment is given in g g^{-1} of initial dry-mass weight for the whole cultivation period. The increment was initially measures in fresh-mass weight (see methods for cultivation details and Table A2 for number of individuals).

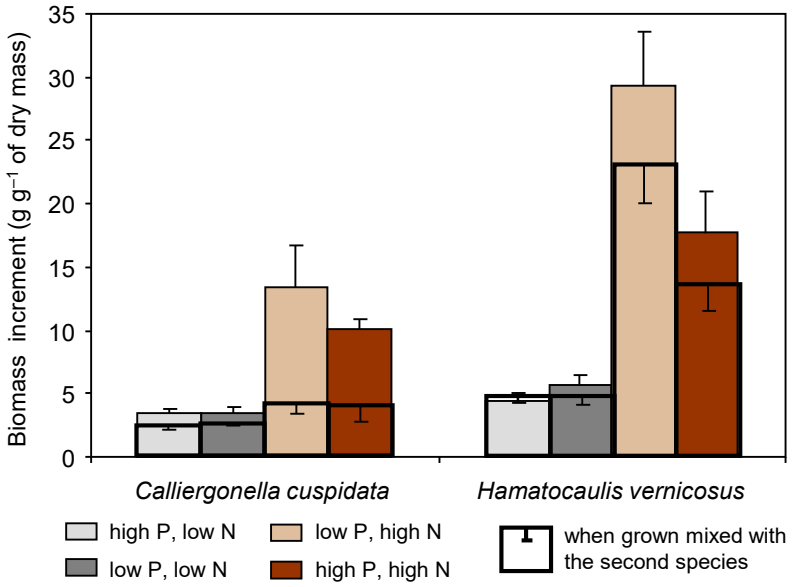

Fig. A.4. Biomass increment of *Calliergonella cuspidata* and *Hamatocaulis vernicosus* in stagnant calcareous solutions (2.4 mM $Ca(HCO_3)_2$, pH 7.1) after 9 weeks of cultivation in different [N] and [P] (see methods for cultivation details and Table A2 for number of individuals). The thin column line indicates solitary growth, the thick line the growth in mixture. The species' growth differed in nitrogen-rich solutions (grown individually or in mixture; $p<0.05$) but did not differ in nitrogen-poor solutions.

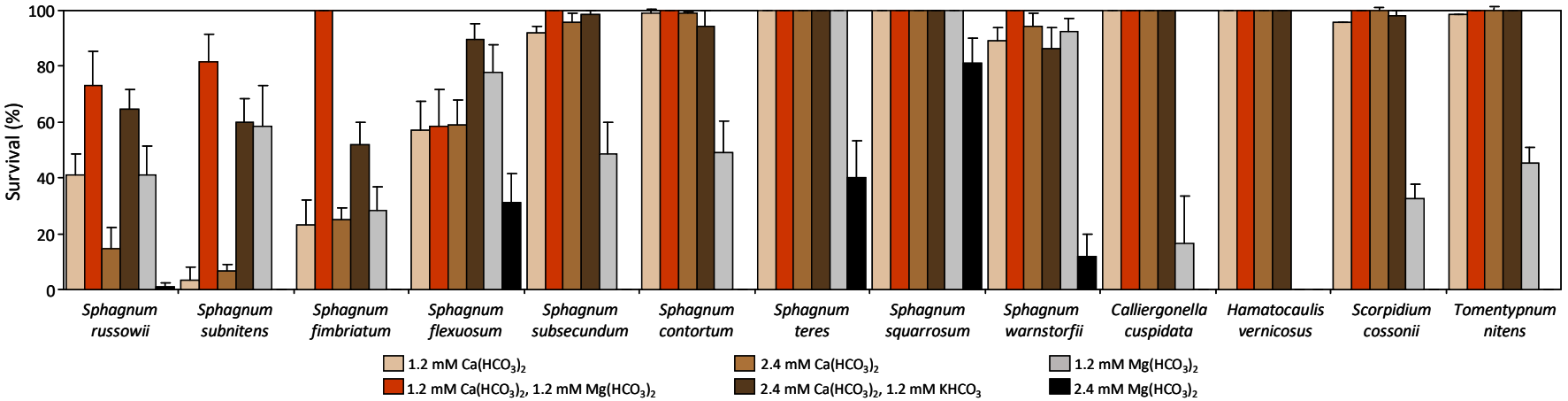

Fig. A.5. Survival of fen mosses submersed in flowing solutions of calcium bicarbonate and/or magnesium bicarbonate (pH 7.1) for three weeks (the data of Cultivation 3 and 4, see methods for cultivation details and Table A2 for number of individuals).

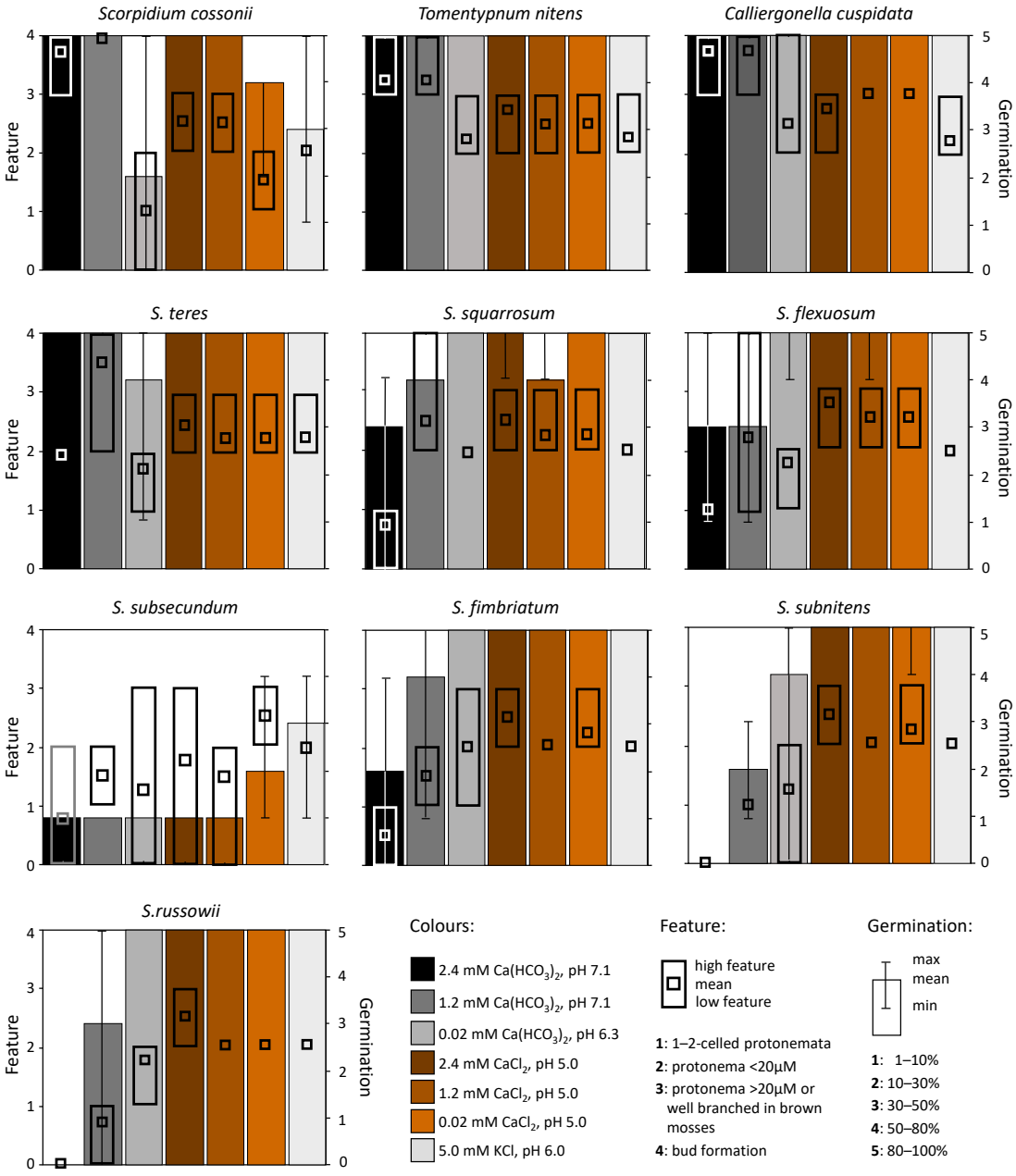
Scorpidium cossonii
Tomentypnum nitens
Calliergonella cuspidata
S. teres
S. squarrosum
S. flexuosum
S. subsecundum
S. fimbriatum
S. subnitens
S.russowii
Feature
Germination
Colours:
2.4 mM Ca(HCO3)2, pH 7.1
1.2 mM Ca(HCO3)2, pH 7.1
0.02 mM Ca(HCO3)2, pH 6.3
2.4 mM CaCl2, pH 5.0
1.2 mM CaCl2, pH 5.0
0.02 mM CaCl2, pH 5.0
5.0 mM KCl, pH 6.0
Feature:
high feature
mean
low feature
1: 1–2-celled protonemata
2: protonema <20µM
3: protonema >20µM or well branched in brown mosses
4: bud formation
Germination:
max
mean
min
1: 1–10%
2: 10–30%
3: 30–50%
4: 50–80%
5: 80–100%

Fig. A.6. Spore germination of fen mosses in solutions of calcium bicarbonate or calcium/potassium chloride (colours). The feature (left *y*-axis; box-plots) indicate the range of germination state features to which the spores germinated (concerning the protonema size and formation of gametophores, i.e. bud formation (see Fig. A.8.). Germination (right *y*-axis; empty columns with error bars) indicate the percentage estimate of germinated spores (maximum, mean, minimum) expressed in categories. Four replicates per species and solution were measured; for details, see methods and Table A2.

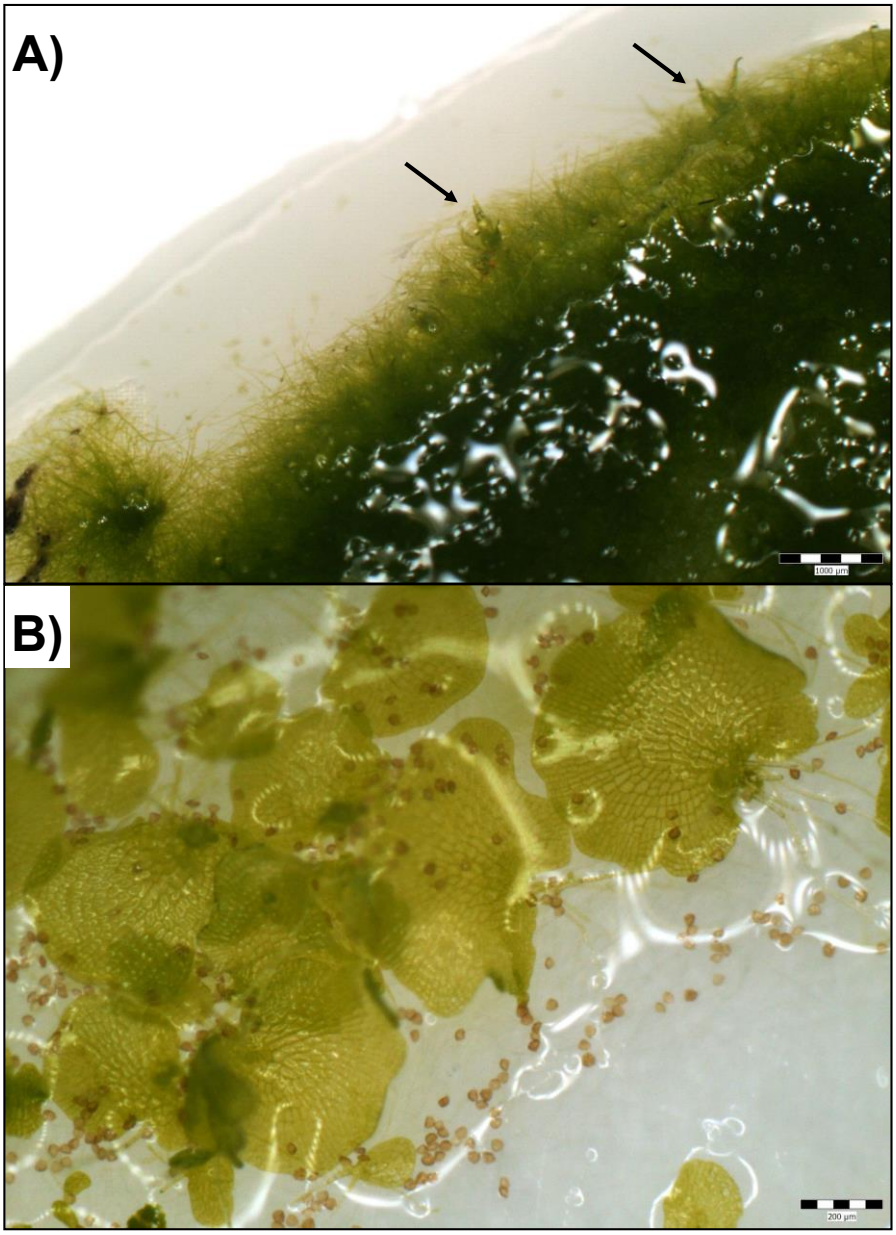

Fig. A.7. Protonemata of A) *Calliergonella cuspidata* germinated submersed in stagnant calcareous solutions (2.4 mM $Ca(HCO_3)_2$, pH 7.1) and B) *Sphagnum subsecundum* in 2.4 mM $CaCl_2$, pH 5. The arrows indicate buds with newly formed shoots. The photos were made six weeks after the start of the experiment.

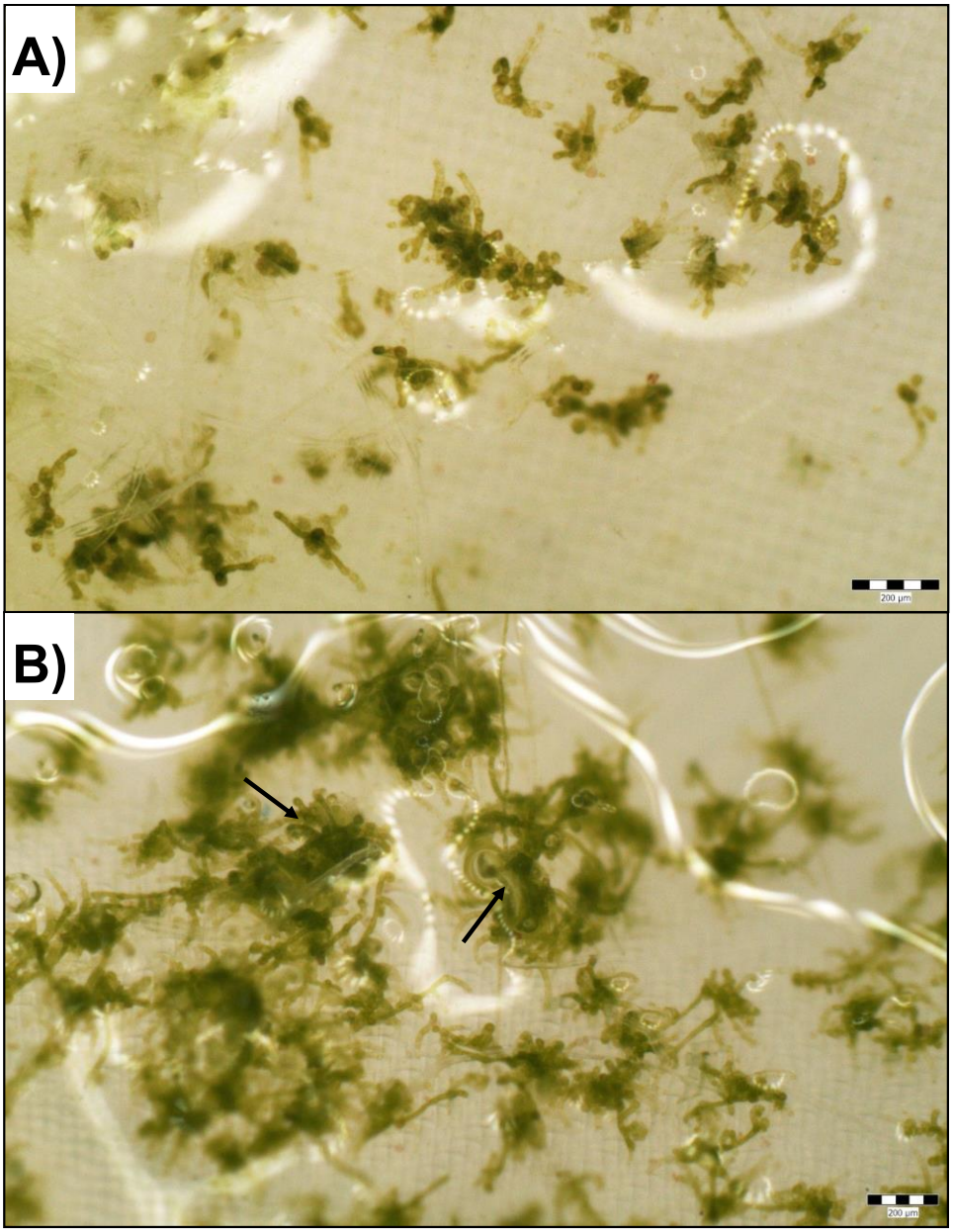

Fig. A.8. Protonemata of *Sphagnum teres* germinated submersed in A) stagnant calcareous solutions (2.4 mM $Ca(HCO_3)_2$, pH 7.1) or B) 1.2 mM $Ca(HCO_3)_2$, pH 7.1. Arrows indicate a formation of buds with new shoots. The photos were made 6 weeks after the start of the experiment.

Fig. A.9. Apical shoot segments of calcifuge species *Sphagnum subnitens* and *S. fimbriatum* grown for 9 weeks in stagnant calcareous solutions (2.4 mM $Ca(HCO_3)_2$, pH 7.1).

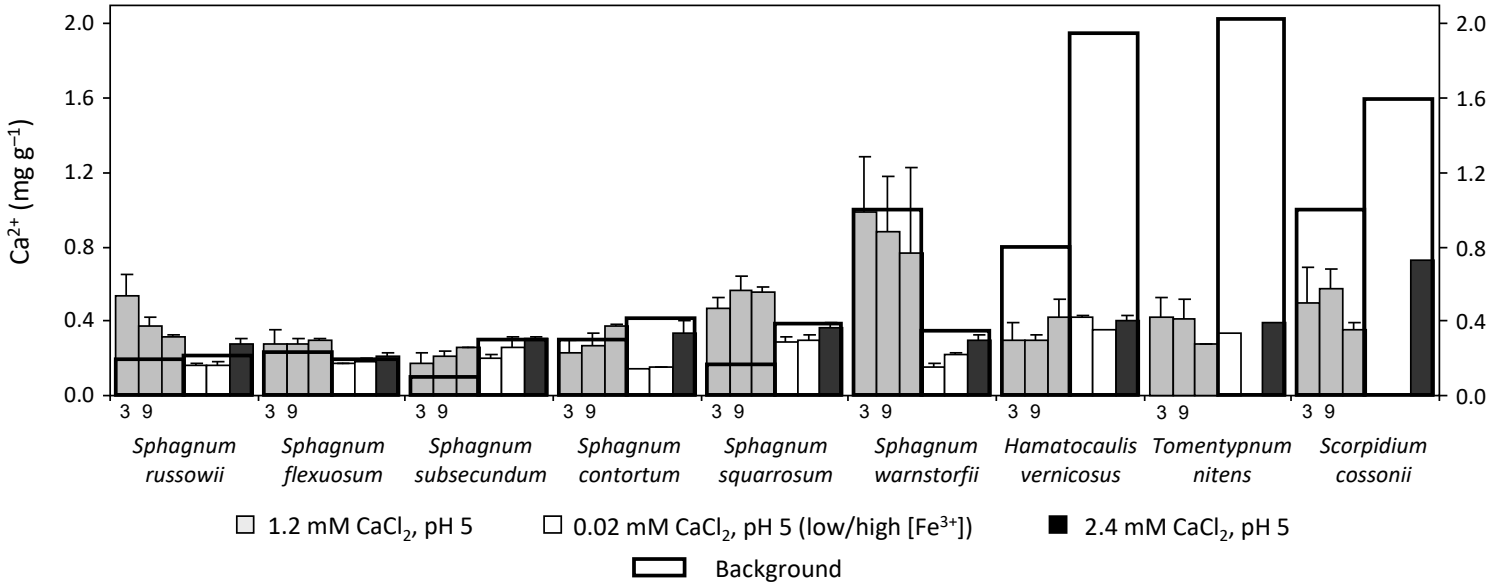

Fig. A.10. Intracellular [Ca^{2+}] (in mg g^{-1} of dry weight) of *Sphagnum* and brown mosses grown in chloride solutions of low pH for three weeks. The shortened cultivation period (3 or 9 days) is noted below relevant columns. The wide empty columns indicate the pre-treatment intracellular [Ca^{2+}]. Specimens of *T. nitens* grown in 0.02 mM $CaCl_2$, high [Fe^{3+}] and *S. cossonii* in 0.02 mM $CaCl_2$, low and high [Fe^{3+}] were not analysed; (1)2–3(4) replicates per species and solution were used.

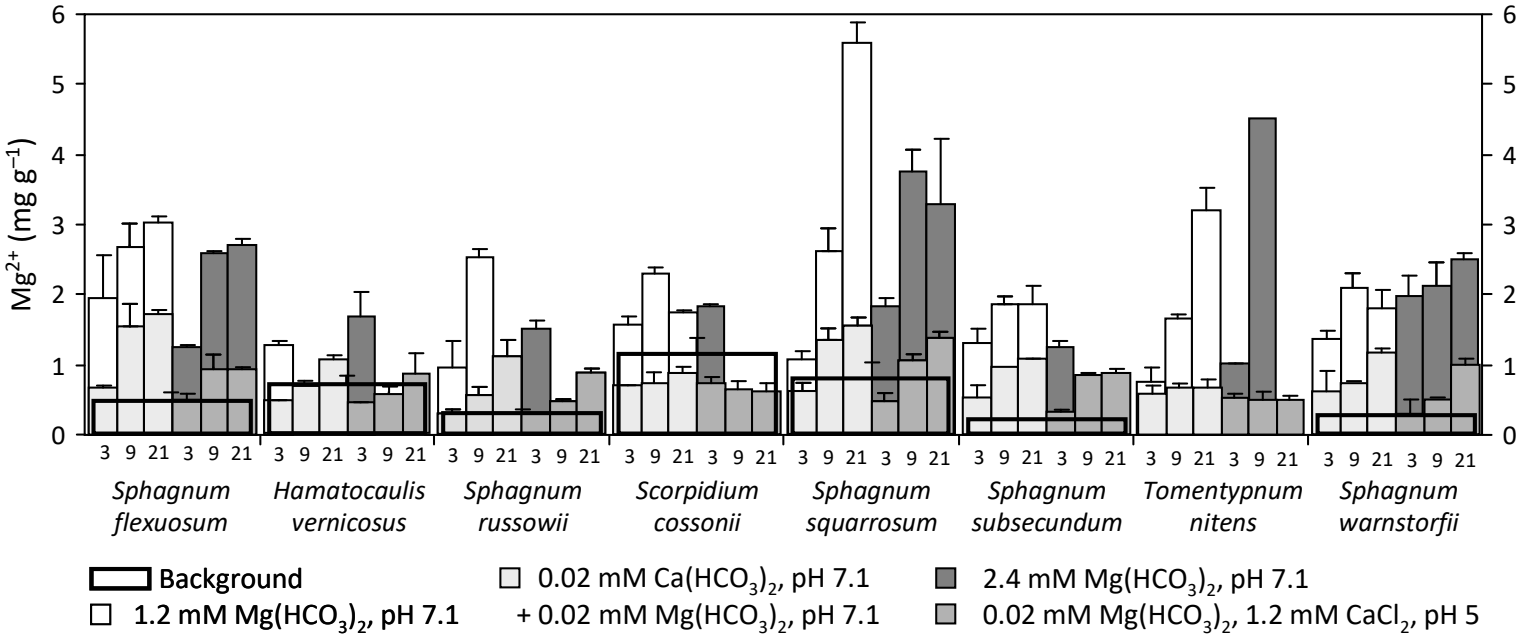

Fig. A.11. Intracellular Mg^{2+} accumulation (in mg g^{-1} of dry weight) in brown mosses and *Sphagnum* species after 3, 9 and 21 days (indicated by numbers below columns) in solutions of contrasting Ca^{2+} or Mg^{2+} concentrations. The wide empty columns indicate the pre-treatment intracellular [Mg^{2+}]. Specimens of *H. vernicosus, S. russowii, S. cossonii, S. subsecundum* grown in 2.4 mM $Mg(HCO_3)_2$ for 9 and 21 days, and *T. nitens* for 21 days were not analysed; (1)2–4 replicates per species and solution were used.

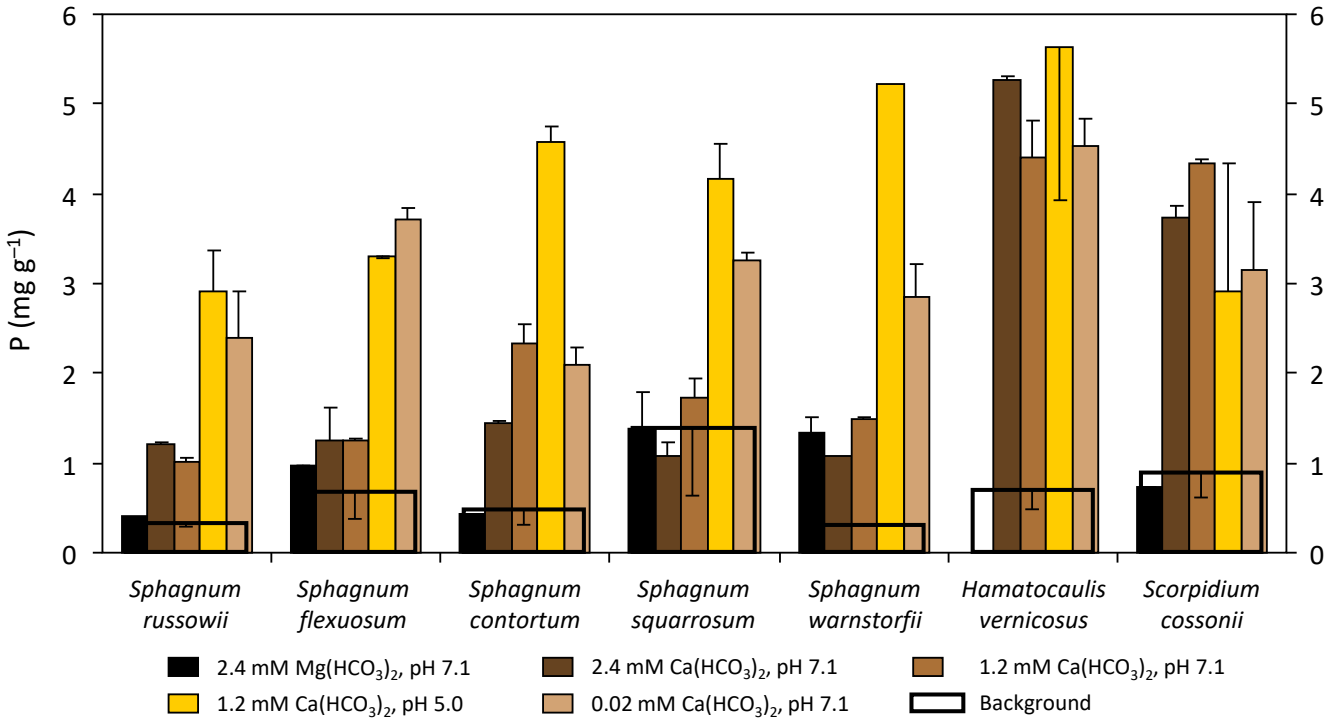

Fig. A.12. Intracellular concentration of phosphorus in *Sphagnum* and brown mosses grown for three weeks in solutions of $CaCl_2$ or $Ca(HCO_3)_2$ with contrasting pH. The wide empty columns indicate the pre-treatment intracellular [P]. The specimens of *H. vernicosus* grown in 2.4 mM $Mg(HCO_3)_2$ were not analysed; (1)2 replicates per species and solution were used.

Table B.1. Growth of moss shoots (means ± s.e.) cultivated in nutrient rich and poor solutions of $CaCl_2$, $Ca(HCO_3)_2$ (noted as Ca) and $Mg(HCO_3)_2$ (noted as Mg) of different pH (Solution). Some solutions were enriched with other cations (Fe, K). The data are presented separately for each cultivation (Cultivation #). See Table B.2 for shoot survival and Methods for details on nutrient concentration.

Solution	Cultivation #	*Aulacomnium palustre*	*Bryum pseudotriquetrum*	*Calliergonella cuspidata*	*Sphagnum contortum*	*Sphagnum fimbriatum*	*Sphagnum flexuosum*
High nutrient concetration				Growth increment (mm per 3 weeks)			
0.02 mM $CaCl_2$, pH 5	3			15 ± 2.8	23 ± 1.6	10 ± 0.7	42 ± 2.1
0.02 mM $CaCl_2$, pH 7.1	3			10 ± 1.4	7 ± 1.0	4 ± 0.6	23 ± 2.4
0.1 mM pH 6.3	1	10 ± 0.6	10 ± 0.9	7 ± 0.5	13 ± 1.1	6 ± 0.8	14 ± 1.7
0.1 mM Ca pH 7.1	1	10 ± 0.5	10 ± 0.6	12 ± 0.8	14 ± 0.7	6 ± 0.5	15 ± 1.8
0.8 mM Ca, pH 7.1	1	7 ± 0.7	8 ± 0.3	12 ± 0.7	10 ± 0.8	1 ± 0.5	8 ± 1.3
1.2 mM Ca, pH 7.1	3			19 ± 1.9	11 ± 1.4	2 ± 0.5	8 ± 1.1
1.5 mM Ca, pH 7.1	1	9 ± 0.6	9 ± 0.5	17 ± 1.4	8 ± 0.9	1 ± 0.3	3 ± 0.6
2.4 mM Ca, pH 7.1	1	9 ± 0.7	10 ± 0.6	14 ± 0.6	5 ± 0.7	1 ± 0.4	1 ± 0.3
2.4 mM Ca, pH 7.1	3			21 ± 1.4	6 ± 0.9	1 ± 0.6	5 ± 0.5
2.4 mM Ca, 1.2 mM K, pH 7.1	3			16 ± 1.2	8 ± 1.0	1 ± 0.3	5 ± 0.6
1.2mM Ca, 1.2mM Mg, pH 7.1	3			19 ± 1.4	7 ± 1.2	2 ± 0.5	6 ± 0.9
1.2mM Mg, pH 7.1	3			1 ± 0.5	3 ± 0.5	2 ± 0.3	6 ± 1.1
2.4mM Mg, pH 7.1	3			1 ± 0.8	3 ± 0.5	2 ± 0.3	4 ± 0.5
Low nutrient concetration				Growth increment (mm per 3 weeks)			
0.02 mM $CaCl_2$, pH 5	5			7 ± 5.0	2 ± 1.7	6 ± 1.5	21 ± 5.7
0.02 mM $CaCl_2$, pH 5, high Fe	5			3 ± 0.5	4 ± 2.0	8 ± 0.4	19 ± 4.2
0.02 mM $CaCl_2$, pH 7.1	4			11 ± 2.0	7 ± 3.1	3 ± 0.4	16 ± 1.2
0.02 mM $CaCl_2$, pH 7.1, high Fe	5			9 ± 0.0	7 ± 2.3	5 ± 0.9	14 ± 3.8
0.1 mM Ca, pH 6.3	2	10 ± 1.5	3 ± 0	12 ± 2.6	11 ± 1.0	7 ± 0.6	13 ± 1.8
0.1 mM Ca, pH7.1	2	6 ± 1.0	10 ± 0	13 ± 1.2	7 ± 0.5	4 ± 0.5	5 ± 1.5
0.8 mM Ca, pH 7.1	2	10 ± 1.2	3 ± 0	13 ± 1.3	4 ± 0.3	3 ± 0.3	3 ± 0.8
1.2 mM Ca, pH7.1	4				4 ± 0.7		0 ± 0.2
1.5 mM Ca, pH7.1	2	7 ± 1.0	3 ± 0	13 ± 1.5	3 ± 0.6	2 ± 0.3	1 ± 0.3
2.4 mM Ca pH7.1	2	8 ± 0.8	0 ± 0	14 ± 1.8	3 ± 0.4	2 ± 0.4	2 ± 0.9
2.4 mM Ca pH7.1	4				0 ± 0.2		0 ± 0.2
2.4 mM Ca pH7.1	5			7 ± 3.0	2 ± 0.2	0 ± 0.2	1 ± 0.0
2.4 mM Ca, pH 7.1, high Fe	5			6 ± 1.0	2 ± 0.4	1 ± 0.3	0 ± 0.3
2.4 mM Ca, pH 7.1, extra-high Fe	5			14 ± 0.5	6 ± 1.4	1 ± 0.2	1 ± 0.6
2.4 mM Ca a 1.2 mM K, pH 7.1	4				2 ± 0.8		0 ± 0.2
2.4 mM $CaCl_2$, pH 6.7	5			11 ± 2.5	13 ± 1.4	1 ± 0.2	11 ± 0.9
1.5 mM Ca, pH 6.3	2	2 ± 0.3	0 ± 0	12 ± 1.7	5 ± 0.6	2 ± 0.4	3 ± 0.9
1.2 mM $CaCl_2$, pH 5	4				9 ± 0.5		13 ± 2.0
2.4 mM $CaCl_2$, pH 5	5			11 ± 2.0	7 ± 3.1	3 ± 0.4	16 ± 1.2
2.4 $CaCl_2$, pH 5, high Fe	5			13 ± 1.0	11 ± 0.6	5 ± 0.6	14 ± 2.0
1.2 mM Ca, 1.2 mM Mg, pH 7.1	4				2 ± 0.6		0 ± 0.2
2.4 mM Mg, pH 7.1	4				1 ± 0.5		0 ± 0.2
1.2 mM Mg, pH 7.1	4				0 ± 0.2		2 ± 0.5

Table B.1 (continued).

Hamatocaulis vernicosus	*Sphagnum rusowii*	*Scorpidium cossonii*	*Sphagnum squarrosum*	*Sphagnum subnitens*	*Sphagnum subsecundum*	*Sphagnum teres*	*Tomentypnum nitens*	*Sphagnum warnstorfii*
Growth increment (mm per 3 weeks)								
34 ± 1.3	15 ± 1.2	14 ± 1.1	29 ± 0.7	7 ± 0.9	30 ± 2.7	26 ± 2.0	13 ± 0.9	11 ± 0.9
21 ± 4.9	10 ± 0.7	6 ± 0.7	10 ± 0.9	3 ± 0.4	13 ± 1.2	10 ± 1.5	9 ± 0.8	6 ± 0.5
22 ± 1.1	6 ± 0.4	4 ± 0.3	16 ± 1.1		17 ± 1.4	15 ± 0.9	8 ± 0.3	11 ± 0.7
25 ± 1.1	5 ± 0.6	7 ± 0.3	16 ± 0.6		12 ± 1.1	12 ± 0.8	9 ± 0.4	9 ± 0.5
30 ± 1.0	2 ± 0.4	7 ± 0.5	11 ± 1.0		9 ± 1.9	10 ± 1.2	7 ± 0.7	6 ± 0.5
39 ± 2.4	3 ± 0.4	23 ± 0.9	12 ± 0.7	1 ± 0.3	12 ± 2.0	14 ± 1.4	17 ± 1.4	5 ± 0.8
26 ± 1.7	0 ± 0.2	8 ± 0.3	10 ± 1.3		5 ± 0.5	10 ± 1.2	7 ± 0.5	3 ± 0.4
28 ± 1.3	2 ± 0.3	10 ± 0.4	7 ± 0.6		3 ± 0.5	6 ± 0.8	8 ± 0.6	2 ± 0.3
35 ± 7.1	2 ± 0.6	14 ± 1.8	9 ± 0.7	0 ± 0.1	6 ± 0.9	9 ± 0.8	12 ± 1.2	3 ± 0.4
36 ± 2.2	3 ± 0.3	16 ± 1.3	5 ± 0.9	1 ± 0.3	6 ± 1.3	7 ± 0.6	11 ± 0.7	2 ± 0.3
38 ± 4.3	2 ± 0.5	14 ± 1.5	10 ± 0.4	0 ± 0.1	6 ± 1.1	11 ± 0.8	15 ± 2.1	3 ± 0.5
3 ± 0.5	2 ± 0.2	4 ± 1.4	13 ± 0.9	1 ± 0.2	4 ± 0.4	5 ± 0.9	3 ± 0.2	4 ± 0.8
2 ± 0.6	2 ± 0.3	1 ± 0.3	2 ± 0.5	1 ± 0.2	4 ± 0.7	4 ± 0.3	2 ± 0.7	1 ± 0.4
Growth increment (mm per 3 weeks)								
22 ± 2.0	4 ± 0.9	9 ± 1..0	19 ± 3.1		16 ± 3.0	9 ± 0.3	11 ± 2.5	5 ± 0.7
19 ± 5.5	3 ± 0.2	8 ± 1.0	17 ± 2.4		15 ± 7.8	7 ± 0.5	5 ± 1.0	5 ± 1.0
25 ± 0.5	3 ± 0.8	6 ± 1.0	17 ± 2.5		19 ± 2.6	7 ± 1.3	8 ± 1.5	5 ± 0.7
30 ± 1.5	4 ± 2.1	11 ± 1.0	18 ± 3.4		14 ± 2.7	8 ± 2.0	11 ± 1.0	6 ± 0.7
17 ± 1.4	6 ± 0.7	10 ± 1.1	17 ± 1.4		16 ± 0.7	12 ± 0.7	10 ± 1.5	8 ± 0.4
10 ± 1.2	3 ± 0.4	8 ± 0.6	8 ± 0.7		7 ± 0.8	7 ± 0.7	9 ± 1.4	6 ± 0.3
10 ± 1.1	1 ± 0.3	9 ± 0.9	7 ± 0.6		4 ± 0.4	5 ± 0.6	10 ± 0.9	3 ± 0.3
22 ± 1.2	0 ± 0.2	6 ± 0.7	7 ± 0.5		2 ± 0.5		6 ± 0.6	1 ± 0.5
10 ± 1.2	1 ± 0.2	8 ± 0.7	6 ± 0.6		3 ± 0.6	5 ± 0.3	9 ± 1.3	3 ± 0.2
9 ± 1.0	2 ± 0.3	9 ± 1.0	3 ± 0.8		2 ± 0.5	5 ± 0.7	11 ± 1.4	2 ± 0.2
19 ± 2.8	0 ± 0.2	3 ± 1.2	1 ± 0.4		1 ± 0.2		5 ± 1.2	1 ± 0.3
20 ± 7.0	0 ± 0.3	11 ± 2.0	4 ± 0.7		1 ± 0.9	2 ± 0.3	8 ± 1.0	1 ± 0.2
29 ± 5.0	0 ± 0.0	11 ± 0.5	5 ± 1.2		2 ± 0.7	3 ± 0.3	10 ± 2.0	1 ± 0.3
50 ± 5.0	0 ± 0.3	17 ± 3.5	5 ± 0.6		3 ± 0.6	4 ± 0.7	11 ± 0.0	1 ± 0.2
15 ± 3.2	0 ± 0.2	3 ± 0.7	3 ± 0.7		1 ± 0.2		5 ± 0.9	0 ± 0.2
24 ± 1.0	2 ± 0.4	10 ± 1.5	13 ± 3.4		12 ± 0.7	6 ± 2.0	12 ± 1.0	4 ± 0.7
9 ± 2.6	3 ± 0.5	8 ± 1.0	6 ± 0.7		4 ± 0.6	6 ± 0.5	7 ± 0.8	2 ± 0.2
14 ± 2.6	3 ± 0.7	5 ± 0.6	14 ± 2.2		13 ± 2.0		4 ± 1.3	4 ± 0.4
25 ± 0.5	3 ± 0.8	6 ± 1.0	17 ± 2.5		19 ± 2.6	7 ± 1.3	8 ± 1.5	5 ± 0.7
27 ± 3.0	4 ± 0.6	9 ± 0.5	19 ± 3.0		17 ± 2.7	8 ± 0.6	7 ± 2.5	5 ± 0.9
13 ± 1.8	0 ± 0.0	2 ± 0.7	1 ± 0.3		0 ± 0.2		5 ± 0.7	1 ± 0.4
0 ± 0.3	0 ± 0.0	0 ± 0.0	1 ± 0.3		1 ± 0.4		0 ± 0.0	0 ± 0.2
0 ± 0.0	0 ± 0.2	1 ± 0.3	5 ± 0.9		1 ± 0.3		1 ± 0.3	3 ± 1.1

Table B.2. Survival of moss shoots (means ± s.e.) cultivated in nutrient rich and poor solutions of $CaCl_2$, $Ca(HCO_3)_2$ (noted as Ca) and $Mg(HCO_3)_2$ (noted as Mg) of different pH (Solution). Some solutions were enriched with other cations (Fe, K). The data are presented separately for each cultivation (Cultivation #). See Table B.1 for shoot growth increment and Methods for details on nutrient concentration.

Solution	Cultivation #	*Aulacomnium palustre*	*Bryum pseudotriquetrum*	*Calliergonella cuspidata*	*Sphagnum contortum*	*Sphagnum fimbriatum*	*Sphagnum flexuosum*
High nutrient concetration				Shoot survival after 3 weeks (%)			
0.02 mM $CaCl_2$, pH 5	3			100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.02 mM $CaCl_2$, pH 7.1	3			100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.1 mM pH 6.3	1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	28 ± 12.7
0.1 mM Ca pH 7.1	1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	83 ± 16.7	42 ± 14.9
0.8 mM Ca, pH 7.1	1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	8 ± 8.3	33 ± 14.2
1.2 mM Ca, pH 7.1	3			100 ± 0	100 ± 0	23 ± 4.1	19 ± 5
1.5 mM Ca, pH 7.1	1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	0 ± 0	0 ± 0
2.4 mM Ca, pH 7.1	1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	0 ± 0	0 ± 0
2.4 mM Ca, pH 7.1	3			100 ± 0	100 ± 0	25 ± 8.6	38 ± 11.9
2.4 mM Ca, 1.2 mM K, pH 7.1	3			100 ± 0	92 ± 8.3	52 ± 8	84 ± 8.5
1.2mM Ca, 1.2mM Mg, pH 7.1	3			100 ± 0	100 ± 0	100 ± 0	58 ± 13.1
1.2mM Mg, pH 7.1	3			17 ± 16.7	53 ± 14.4	28 ± 8.7	67 ± 14.2
2.4mM Mg, pH 7.1	3			0 ± 0	0 ± 0	0 ± 0	8 ± 8.3
Low nutrient concetration				Shoot survival after 3 weeks (%)			
0.02 mM $CaCl_2$, pH 5	5			80 ± 0	17 ± 16.7	100 ± 0	100 ± 0
0.02 mM $CaCl_2$, pH 5, high Fe	5			0 ± 0	30 ± 16.1	100 ± 0	100 ± 0
0.02 mM $CaCl_2$, pH 7.1	4				100 ± 0		100 ± 0
0.02 mM $CaCl_2$, pH 7.1, high Fe	5			50 ± 0	57 ± 20.3	97 ± 3.3	100 ± 0
0.1 mM Ca, pH 6.3	2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.1 mM Ca, pH7.1	2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	73 ± 14.1
0.8 mM Ca, pH 7.1	2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	78 ± 10.1	42 ± 14.9
1.2 mM Ca, pH7.1	4				100 ± 0		97 ± 3.3
1.5 mM Ca, pH7.1	2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	75 ± 11.2	8 ± 8.3
2.4 mM Ca pH7.1	2	100 ± 0	0 ± 0	100 ± 0	100 ± 0	44 ± 12.1	45 ± 15.7
2.4 mM Ca pH7.1	4				97 ± 3.3		100 ± 0
2.4 mM Ca pH7.1	5			100 ± 0	100 ± 0	100 ± 0	100 ± 0
2.4 mM Ca, pH 7.1, high Fe	5			100 ± 0	100 ± 0	100 ± 0	100 ± 0
2.4 mM Ca, pH 7.1, extra-high Fe	5			100 ± 0	100 ± 0	73 ± 13.3	100 ± 0
2.4 mM Ca a 1.2 mM K, pH 7.1	4				100 ± 0		100 ± 0
2.4 mM $CaCl_2$, pH 6.7	5			100 ± 0	100 ± 0	100 ± 0	100 ± 0
1.5 mM Ca, pH 6.3	2	100 ± 0	0 ± 0	100 ± 0	100 ± 0	94 ± 6.3	100 ± 0
1.2 mM $CaCl_2$, pH 5	4				100 ± 0		100 ± 0
2.4 mM $CaCl_2$, pH 5	5			100 ± 0	50 ± 22.4	100 ± 0	100 ± 0
2.4 $CaCl_2$, pH 5, high Fe	5			40 ± 0	100 ± 0	100 ± 0	100 ± 0
1.2 mM Ca, 1.2 mM Mg, pH 7.1	4				97 ± 3.3		100 ± 0
2.4 mM Mg, pH 7.1	4				0 ± 0		77 ± 15
1.2 mM Mg, pH 7.1	4				40 ± 20		100 ± 0

Table B.2 (continued).

Hamatocaulis vernicosus	*Sphagnum rusowii*	*Scorpidium cossonii*	*Sphagnum squarrosum*	*Sphagnum subnitens*	*Sphagnum subsecundum*	*Sphagnum teres*	*Tomentypnum nitens*	*Sphagnum warnstorfii*
Shoot survival after 3 weeks (%)								
100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	0 ± 0	100 ± 0	91 ± 9.1		50 ± 22.4	100 ± 0	100 ± 0	100 ± 0
100 ± 0	33 ± 14.4	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	0 ± 0	100 ± 0	100 ± 0		83 ± 16.7	100 ± 0	100 ± 0	100 ± 0
100 ± 0	13 ± 2.8	100 ± 0	100 ± 0	3 ± 2.2	100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	0 ± 0	100 ± 0	100 ± 0		67 ± 16.7	100 ± 0	100 ± 0	100 ± 0
100 ± 0	0 ± 0	100 ± 0	100 ± 0		8 ± 8.3	100 ± 0	100 ± 0	89 ± 11.1
100 ± 0	0 ± 0	100 ± 0	100 ± 0	7 ± 4.5	100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	70 ± 9	100 ± 0	100 ± 0	60 ± 8.5	100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	73 ± 11.9	100 ± 0	100 ± 0	82 ± 10	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0 ± 0	57 ± 13.7	35 ± 5	100 ± 0	58 ± 14.9	18 ± 7	100 ± 0	48 ± 7.5	100 ± 0
0 ± 0	0 ± 0	0 ± 0	85 ± 10.1	0 ± 0	0 ± 0	40 ± 13.5	0 ± 0	0 ± 0
Shoot survival after 3 weeks (%)								
100 ± 0	100 ± 0	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0	100 ± 0
95 ± 5	100 ± 0	40 ± 20	98 ± 2		67 ± 33.3	100 ± 0	20 ± 0	100 ± 0
100 ± 0	100 ± 0	100 ± 0	100 ± 0		100 ± 0		100 ± 0	100 ± 0
90 ± 10	100 ± 0	100 ± 0	96 ± 4		100 ± 0	100 ± 0	45 ± 5	100 ± 0
100 ± 0	100 ± 0	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	100 ± 0	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	0 ± 0	100 ± 0	100 ± 0		92 ± 8.3	100 ± 0	100 ± 0	100 ± 0
100 ± 0	73 ± 14.3	100 ± 0	100 ± 0		93 ± 4.2		100 ± 0	80 ± 12.6
100 ± 0	0 ± 0	100 ± 0	100 ± 0		67 ± 14.2	100 ± 0	100 ± 0	100 ± 0
100 ± 0	0 ± 0	100 ± 0	100 ± 0		0 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	43 ± 18.9	100 ± 0	100 ± 0		90 ± 4.5		100 ± 0	83 ± 13.1
100 ± 0	20 ± 0	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	20 ± 0	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	17 ± 3.3	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	53 ± 12.3	93 ± 6.7	100 ± 0		97 ± 3.3		100 ± 0	63 ± 16.7
100 ± 0	100 ± 0	100 ± 0	100 ± 0		100 ± 0	100 ± 0	100 ± 0	100 ± 0
100 ± 0	100 ± 0	100 ± 0	100 ± 0		83 ± 11.2	100 ± 0	100 ± 0	100 ± 0
100 ± 0	100 ± 0	80 ± 0	100 ± 0		100 ± 0		93 ± 6.7	100 ± 0
100 ± 0	100 ± 0	90 ± 10	100 ± 0		100 ± 0	100 ± 0	95 ± 5	100 ± 0
90 ± 0	100 ± 0	30 ± 10	100 ± 0		100 ± 0	100 ± 0	20 ± 0	100 ± 0
100 ± 0	63 ± 16.7	80 ± 0	100 ± 0		80 ± 8.9		93 ± 6.7	77 ± 12
0 ± 0	3 ± 3.3	0 ± 0	70 ± 19.1		0 ± 0		0 ± 0	50 ± 22.4
0 ± 0	10 ± 4.5	27 ± 13.3	100 ± 0		90 ± 10		40 ± 0	77 ± 12

Chapter 3.
Sphagnum establishment in alkaline fens: importance of weather and water chemistry

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Abstract

Sphagnum expansion to alkaline fens has accelerated during the last decades in Europe, leading to changes in diversity, habitat distributions and carbon storage. The causes are still not clearly understood and involve an interplay between climate change, hydrology, nutrient supply and *Sphagnum* physiology. We conducted a 4-year field experiment in eight fens in Central European highlands and assessed survival and establishment of individual apical shoot fragments of *S. flexuosum*, *S. warnstorfii* and *S. squarrosum* transplanted along the microtopographical gradient. In a laboratory experiment, we tested combined effects of desiccation and high calcium bicarbonate concentration on *Sphagnum* survival. We found that in unflooded positions, living shoots of *Sphagnum* and brown mosses lowered [Ca^{2+}] and pH in their capillary water, in contrast to dead fragments; yet without differences between species. Survival and expansion of *Sphagnum* fragments, which did not die of acute calcium toxicity during first weeks/months, was negatively affected by dry weather and alkaline water chemistry, reflecting *Sphagnum* intolerance to desiccation and to combined high [Ca^{2+}] and pH. Shoot fragments expanded to patches only when precipitation was high. Interestingly, non-toxic concentration of calcium bicarbonate reduced desiccation damage in *Sphagnum*, probably through protection of membranes or other cell components. This mechanism would facilitate *Sphagnum* survival in elevated, frequently desiccated microhabitats of calcareous fens such as brown-moss hummocks. However, since water-retaining capacity of few *Sphagnum* shoots is insufficient to change water chemistry in its surroundings, surface acidification may occur only once the environment (e.g. sufficient humidity) enabled expansion to larger mats. Then, the retained rainwater together with hardly decomposable *Sphagnum* litter would separate mire surface from groundwater, speeding up successional shift towards poor fens. *Sphagnum* expansion to alkaline fens is therefore more likely in humid regions.

Key-words: fen succession, *Sphagnum* transplants, desiccation, calcium tolerance, climate humidity, competition

1. **Introduction**

The last century brought severe changes to all terrestrial ecosystems. However, only few biotopes became as threatened as mineral-rich mires, and this threat merits their inclusion in EU Habitat Directive (Council Directive 92/43/EEC). Apart from detrimental changes connected with direct human activity and/or global change (i.e. nutrient enrichment, cessation of traditional management, forestation, agriculture, peat extraction), a less understood process of natural succession is turning these species- and rare-species-rich mires to species-poor, acidic fens (Kooijman 2012). The transition is naturally a slow process lasting hundreds or thousands of years (Kuhry et al. 1993, Swinehart and Parker 2000, Wehrli at al. 2010); however, as a consequence of a rapid expansion of *Sphagnum* mosses, it may take only decades or even years, either naturally (Lamentowicz et al. 2009, Gałka et al. 2013, Väliranta et al. 2016) or as a result of recent human activity (Tahvanainen 2011, Kooijman 2012, Paulissen et al. 2014, Hájek et al. 2015).

Species of the genus *Sphagnum* are generally acidophilous calcifuges, i.e. species intolerant to high [Ca^{2+}] combined with high pH (Clymo 1973, Vicherová et al. 2015). By contrast, high [Ca^{2+}] and pH suits calcium-tolerant, non-sphagnaceous mosses, referred to as brown mosses in mire ecology._Consequently, the species composition of fen communities is determined by a gradient in groundwater chemistry; calcium bicarbonate-rich mires (alkaline fens) are occupied by the sedge–brown-moss vegetation of the *Caricion davallianae* alliance (Jiménez-Alfaro et al. 2014), while acidic, calcium bicarbonate-poor fens are dominated by *Sphagnum* species (Hájek et al. 2006). Regardless of the toxicity of calcium, however, some sphagna may expand even to alkaline fens, creating hummocks that raise above the mat of dominant brown mosses (Rydin and Jeglum 2006, Šoltés & Školek 2012) or even carpets close to the water level (Kooijman 2012, Hájek et al. 2015). Accelerated *Sphagnum* expansion and rapid decline in rich-fen species has recently been observed throughout Europe (the Netherlands: Kooijman and Kanne 1993, Paulissen et al. 2004, Kooijman and Paulissen 2006; Slovakia (Western Carpathians): Hájek et al. 2002; Czech Republic: Hájek et al. 2015; southern Sweden: Hedenäs and Kooijman 1996, Gunnarsson et al. 2000). The environmental characteristics that enable *Sphagnum* mosses to overcome calcium toxicity and thus allow their expansion to calcium bicarbonate-rich mires are not fully understood. A water table decline is one of the possible reasons (van Diggelen et al. 1996, Soudzilovskaia et al. 2010, Kooijman 2012). Flooding of capitula by mineral-rich water naturally eliminates *Sphagnum* establishment in alkaline fens, either through direct calcium toxicity, especially in flowing water (Vicherová et al. 2015), or by lowering species' competitive abilities (Granath et al. 2010, Laine et al. 2014). When the water table

declines, sphagna are no longer stressed and may expand. Rainwater accumulates in the moss layer, facilitating their spread. Nutrient enrichment is considered an important catalyst of the observed change (Kooijman 2012, Hájek et al. 2015).

However, as the chemistry of capillary water cannot be easily measured, we have no information about pH and [Ca^{2+}] inside the bryophyte layer and their variation along the vertical hummock–hollow gradient of groundwater availability. We expect that brown-moss hummocks, which are partly isolated from the influence of groundwater, are more suitable for *Sphagnum* establishment than hollows. However, brown-moss hummocks lack the high water retention capacity of *Sphagnum* hummocks, preventing the establishment of sphagna if the climate is dry. As sphagna are highly sensitive to desiccation, in contrast to other mosses (Abel 1956, Hájek & Vicherová 2014), a dry climate may limit *Sphagnum* establishment in alkaline fens. Indeed, hummocks of calcium-tolerant sphagna are absent from more continental alkaline fens in SE Europe, where the risk of desiccation is higher (Hájek et al. 2014). They are, however, quite abundant in boreal-oceanic northwestern Europe (Kooijman 2012, Flatberg 2013). Kooijman (2012) even stresses the role of rainwater accumulation in the moss layer in the ongoing succession. We therefore conclude that climate humidity is an important cue affecting the succession from brown-moss to *Sphagnum* fens, but no study had tested experimentally its effect on a larger geographical scale.

Apart from direct damage to moss cells, desiccation might affect bryophytes indirectly, through changes in the chemistry of capillary water. High evaporation increases capillary transport of groundwater rich in calcium bicarbonate to moss apices (Brehm et al. 1971, Eppinga et al. 2010), which may result in calcium carbonate precipitation. This is accompanied by an increase of pH above 8, which is highly toxic to *Sphagnum* because of Ca^{2+} (Vicherová et al. 2015). In addition, we expect cells of *Sphagnum* shoots, which are harmed by desiccation, to be more affected by high [Ca^{2+}] and pH than undesiccated cells. Alternatively, because calcium is an important messenger in signalling pathways involved in maintaining membrane integrity during desiccation stress (Ramanjulu and Bartels 2002), its effect on desiccated *Sphagnum* shoots may not be necessarily negative.

Nutrient deficiency (specifically, low availability of P, N and/or K) may represent another environmental conditions limiting the expansion of *Sphagnum* in alkaline fens, as low nutrient availability generally slows down any succession. Calcium bicarbonate-rich fens are naturally P-limited, as phosphate precipitates with calcium or iron under high pH (Zak et al. 2010). Sphagnum species were indeed observed to invade calcium-rich biotopes after the concentration of phosphorus increased (*S.*

squarrosum in the Netherlands, Kooijman and Paulissen 2006; *S. teres* and *S. flexuosum* in Central European highlands, Hájek at al. 2002, 2015; Plesková et al. 2016). Moreover, increased [K^+] has been shown to alleviate calcium toxicity in calcifuge sphagna (Vicherová et al. 2015), which is mirrored by wider pH/calcium niches of calcium-tolerant sphagna in K-rich regions (Plesková et al. 2016) and by the recent spread of *S. teres* in calcium-rich fens enriched by [K^+] (Hájek et al. 2015). The effect of nitrogen on *Sphagnum* survival in alkaline fens could be indirect. Although the addition of nitrogen itself does not change the ability of *Sphagnum* to compete against brown mosses (Vicherová et al. 2015), brown moss competitiveness can be radically decreased by (i) toxicity of NH_4^+ if it is the dominant form of nitrogen (Paulissen et al. 2004, 2005) or by (ii) increased shading by vascular plants, to which some brown-moss species are particularly susceptible (Bauer et al. 2007; Štechová et al. 2012; Cusell et al. 2014). Once the competition from brown mosses is lowered, calcitolerant sphagna may spread in alkaline fens (Vicherová et al. 2015).

To disentangle the effects of weather (a proxy of climate humidity), local water chemistry and microtopography on the succession from brown-moss to *Sphagnum* fens, we conducted a four-year experimental field study supplemented by laboratory experiments. We studied the survival and expansion of *Sphagnum* fragments transplanted to various microhabitats of rich and alkaline fens (differing in calcium bicarbonate) and addressed the following questions and hypotheses:

(1) What is the chemistry of capillary water along the hummock–hollow gradient of rich and alkaline fens in Central Europe? Specifically, we asked whether differences in water chemistry between hummocks and hollows are negligible, such as in some alkaline fens in continental Canada (Karlin and Bliss 1983) or whether the hummocks are poor in calcium bicarbonate, such as in some rich fens in oceanic Western Europe (Bellamy and Rieley 1967) or subcontinental Central Europe (Hájková and Hájek 2004).

(2) What is the importance of capillary-water chemistry and weather conditions for the survival and expansion of *Sphagnum* transplants? We predicted water chemistry in hollows and desiccation in hummocks to limit the establishment of *Sphagnum* fragments, particularly under dry weather conditions.

(3) Is the acidifying ability of living *Sphagnum* and brown-moss fragments sufficient to alter the chemistry of capillary water in their vicinity? We predicted that the large water-holding capacity of *Sphagnum* shoots allows the species to retain a sufficient amount of rainwater that retards the upward flow of groundwater. Such capillary water is therefore expected to have a lower pH and [Ca^{2+}], which may be further maintained by proton exudation and cation exchange. Compared to brown mosses, *Sphagnum* is expected to have a

greater capacity to decrease extracellular pH via proton exudation, as lowered pH prevents the uptake of excess Ca^{2+} to the cytoplasm (Vicherová et al. 2015).

(4) Is desiccation tolerance of Sphagnum affected by calcium? In a laboratory experiment, we tested whether non-toxic concentration of calcium bicarbonate in capillary water of target Sphagnum species, which had been subjected to desiccation/rehydration cycles, has a positive or detrimental effect on their desiccation tolerance.

2. Materials and Methods

2.1. Field transplantation experiment

The experiment was conducted in eight base-rich spring-fed or percolation fens in Slovakia (Western Carpathians; *n*=5) and the Czech Republic (Bohemian Massif; *n*=3; for details Table S1). Study fens are developed on gentle slopes or on flat terrain surrounded by mountain slopes. We chose fens with (i) well-developed hummock–hollow microtopography, (ii) water table generally 0–5 cm above the lowest hollows and with (iii) vegetation that is characteristic to these habitats. Selected localities cover the range of drier and more humid regions of Central Europe. All of study fens have 1–2 m deep peat layer and if studied stratigraphically, they originated at the transition between Pleistocene and Holocene (Brezové, Liptovská Teplička, Rojkov, Zlatá louka), sometimes with a spruce-alder carr phase during the Middle Holocene (see Brezové fen as a typical example; Hájková et al. 2015). The fen terminology follows Hájek et al. (2006) with the exception that we apply the term 'alkaline fens' to cover both calcareous and extremely rich fens in our study.

Three *Sphagnum* species were studied: *S. warnstorfii* (the most common species forming hummocks in rich and extremely rich fens; Hájek et al. 2014), *S. flexuosum* (moderately calcium-tolerant species with an optimum in calcium-poor fens, expanding locally to rich fens; Plesková et al. 2016) and *S. squarrosum* (a common forest species that may expand to nutrient-polluted, extremely-rich fens; Kooijman and Paulissen 2006). *Sphagnum* shoots were sampled in April 2011 at seven localities (Table S2), stored wet in cold, dark conditions and transplanted to the target localities within three weeks.

To cover the entire gradient of water chemistry and availability, apical shoot fragments (1.5 cm) were planted along linear hummock–hollow transects (six per locality, usually 0.5–2.0 m long; Table S1). Fragments were arranged at vertical distances of 2 cm. Each vertical position contained a fragment of *S. warnstorfii, S. squarrosum* and *S. flexuosum* marked by a coloured wooden skewer (Fig. S1). Moreover, one apical branch of each fragment and its basal part was labelled by a non-toxic, ethyl acetate-based fluorescent forestry marker to ensure its traceability.

We evaluated fragment survival (live/dead) and expansion (changes in the number of surviving shoots) in five subsequent periods, in September 2011, May 2012, September 2012, June 2013, April 2014, and October 2014. Establishment is defined as the permanent survival of *Sphagnum* transplants

at a given locality, accompanied by the creation of larger patches (aggregation of more individuals).

At the end of the experiment, all transplants were removed from the experimental plots for conservation reasons.

2.2. Environmental characteristics

Sphagnum growth and survival was confronted with environmental characteristics expected to influence *Sphagnum* survival: humidity of weather and flooding.

Humidity (expressed as precipitation:temperature ratio; hereafter referred to as *weather*) was considered for the period when positions along the transect could desiccate. It was expressed as the sum of summer (July–September) precipitation in 2011–2014 divided by the sum of summer mean monthly temperatures in 2011–2014 (inspired by the Aridity index of Lang 1920). The meteorological data were provided by the Czech and Slovak Hydrometeorological Institutes.

Flooding height was estimated twice a year using 2–4 plastic (PP) tubes sealed at both ends and stuck into the soil. The above-ground part of each tube was perforated from the side at a given height, allowing water (but not rain) to fill the tube. We evaluated the height of flood in five subsequent periods, together with fragment survival (see above) and current water table in plastic wells.

2.3. Chemistry of capillary water

Apart from flooding and humidity of weather, survival of *Sphagnum* fragments was linked with capillary water chemistry on the hummock–hollow gradient.

The concentration of Ca^{2+} in capillary water was measured in August 2013 (after a dry period that lasted two months) and in April 2014 (after winter and spring flooding followed by rainless three weeks). Small mesh bags (2 × 1 cm; 1 mm openings) packed with dead apices of *Sphagnum squarrosum* or *Scorpidium cossonii* (around 0.15 mg of dry mass) were placed in their transect positions in between moss apices in June 2013 (for the August measurement) and September 2013 (for the April measurement). The dead material was prepared by several cycles of desiccation (≈1% RH) and elution

(0.02 M HCl). In August 2013 and April 2014, the mesh bags were sampled and immediately preserved with 2 mL of a 0.24% (v/v) chloroform solution. The mesh bags were taken to the laboratory within a week, and the capillary water was squeezed in a 25 mL syringe. The extracted solution was twice poured through the mesh bag. The final solution was centrifuged (10,000 ×*g*, 20 min), filtered through a 0.45 μm cellulose-acetate membrane syringe filter and stabilized by 0.6 mL of 9% HCl. [Ca^{2+}] was analysed by atomic absorption spectrometry (AAS). The pH of the capillary water in mesh bags was measured by a fine-resolution paper indicator (Merck, Germany) in August 2013 before the extraction of capillary water.

[Ca^{2+}] of capillary water represents the condition where capillary spaces are fully water-saturated but the ions have not been washed away (such as after mild rain or dew). Consequently, [Ca^{2+}] of a given mesh bag was calculated by multiplying [Ca^{2+}] of the extracted solution by the volume of the bag solution after addition of 2 mL of a chloroform solution divided by the capillary water-holding capacity of the moss mesh bag (around 2 mL). Since [Ca^{2+}] was not measured in all transect positions, the missing values were interpolated from linear regressions of [Ca^{2+}] plotted against the positions height above the water table. The immediate height above the water table was measured twice during the experimental period; both measurements were highly correlated ($R^2 = 0.89$).

Capillary-water [Ca^{2+}] of living *Sphagnum* and brown-moss shoots was measured in April 2014 in several individuals of *S. teres* transplanted to positions along the transect in September 2013 and in brown mosses present in the transects (*Hamatocaulis vernicosus, Calliergonella cuspidata, Scorpidium cossonii*). The apical parts (dry weight comparable to mesh bags) were used for the analysis. The process of capillary water extraction and evaluation of [Ca^{2+}] corresponded with that described for dead material, only the chloroform solution was replaced by distilled water and the extraction was performed directly in the field.

2.4. Analysis of cell wall-bound Ca^{2+}

The concentration of Ca^{2+} bound to cell-wall cation-exchange sites of *S. squarrosum* (as a reference species) was measured in September 2011 and May 2012 (fragments placed in their positions in April and September 2011). The sampled fragments were stored in humid conditions, organized in 96-well microplates and transported to the laboratory within one week. There

they were washed by distilled water to remove the unbound cation fraction and dried (60 °C, 24 h). The dry material was acid-digested (at 150 °C with 0.1 mL of concentrated HNO_3 per 3 mg of dry mass) and analysed by AAS. The intracellular Ca^{2+} fraction is very low, so it was neglected.

The capacity of *S. squarrosum* to bind Ca^{2+} on cell-wall cation-exchange sites was estimated at pH=7.0 following Hájek and Adamec (2009) using 0.5 M $CaCl_2$. Apical shoot segments (10 mm) were measured in 11 replicates.

2.5. Combined effect of desiccation and calcium – laboratory experiments

Four *Sphagnum* species of different calcifuge–calcicole behaviour and desiccation tolerance (*S. warnstorfii*, *S. flexuosum*, *S. squarrosum* and *S. contortum*) were subjected to a combination of two stress cues – desiccation and high concentration of calcium bicarbonate solution. Apical parts (2 cm) of *Sphagnum* shoots (source localities described in Table S3) were cultivated for a period of one week in distilled water and 2.4 mM $Ca(HCO_3)_2$ at pH 7 (referred to as cultivation 1). In cultivation 2, distilled water, 2.4 mM $Ca(HCO_3)_2$ (pH 7) and $CaCl_2$ (pH 4) was enriched with nutrients (K – 1.3 mg L^{-1}, P – 0.1 mg L^{-1}, S – 1.2 mg L^{-1}, N – 0.12 mg L^{-1}, Mg – 0.5 mg L^{-1}, Ca – 0.9 mg L^{-1}, Fe – 0.12 mg L^{-1}). Then, the samples were hardened (mild desiccation that causes *Sphagnum* to develop desiccation tolerance), following the methodology of Hájek and Vicherová (2014). Briefly, mosses were placed in an exsiccator above distilled water for five days. The relative air humidity (RH) of 98.5–100% in the exsiccator was regulated by altering the temperature. Then, the mosses were desiccated at 56% RH for 36 h (above a glycerol solution) and rewetted by spraying with (i) distilled water or 2.4 mM $Ca(HCO_3)_2$, pH 7 – cultivation 1 or (ii) the original cultivation solution – cultivation 2. The shoots were grown in these solutions for one week, after which the process of hardening, desiccation, rewetting and cultivation was repeated, twice in cultivation 1 and once in cultivation 2. The shoots were finally let to regenerate in distilled water enriched by nutrients (used in cultivation 2 above) for 25 days. Their survival and regeneration was scored on a five-point scale (0 – dead; 1 – slight regeneration; 2 – small regenerated shoots; 3 – large regenerated shoots; 4 – continued growth of the original capitulum, Fig. 1). The term ‘regeneration’ refers to the production of a new shoot from an adventitious bud. We used five and seven to eight replicates in cultivation 1 and 2, respectively.

Light-acclimated quantum yield of PSII photochemistry (*ΦPSII*) and maximum quantum efficiency of PSII photochemistry (F_V/F_M) in dark-acclimated (30 min) shoots from cultivation 2 was measured before desiccation and 24 h after rewetting. Further measurements would be valueless, as the fluorescence signal became low and heterogenous due to desiccation damage and regeneration. A modulated imaging fluorimeter FlorCam (PSI, Brno, Czech Republic) was used.

2.6. Statistical analysis

The effect of environmental characteristics on the survival and expansion of *Sphagnum* fragments was evaluated in the R statistical language (version 3.1.3; 2015-03-09) using (i) generalized linear mixed-effect models (GLMM) with assumed binomial distribution (package *glmer*; first growing season mortality evaluation), (ii) linear mixed-effect models (LMM) (package *nlme*; slope of *Sphagnum* expansion/retreat in time, maximal number of shoots per position that originated from a single transplanted shoot) and (iii) survival analysis with Cox proportional hazard models (package *coxme*; species survival for the whole experimental period without the first growing season). Random effects reflecting the experimental design, i.e. the effects of locality, transect and position, were included in the models. If binary data were not used, the number of *Sphagnum* shoots used in each analysis was transformed using the following logarithmic transformation: $y = \log(x+0.1)$.

The *Sphagnum* expansion rate was calculated individually for each position as the slope of linear interpolation of the log-transformed number of shoots present at the position during the experiment: slope = (log(number of shoots+0.1) – log(1+0.1))/time, where the intercept value of 1 denotes one initial single capitulum transplanted to each position.

The proportion of variation explained by environmental characteristic included in the GLMMs was estimated by comparing the deviance of a model with just the random effects with the deviance of model with the particular environmental predictor added. In the Cox proportional-hazard models and LMMs, reported sums of squares were used. When analysing the effect of the environmental characteristics on the rate of *Sphagnum* expansion and the maximal number of expanded shoots, the high correlation of the environmental characteristics with the experimental design did not enable us to estimate the explained variation for individual characteristics. Therefore,

the magnitude of the effect of each environmental characteristic was estimated from linear models not including any random effects.

The difference in capillary water chemistry between dead shoots of *S. squarrosum* and *S. cossonii,* living *S. teres* and brown mosses, and living and dead mosses was evaluated by a paired t-value test. The effect of desiccation and Ca^{2+} on *Sphagnum* survival was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The effect of the $Ca(HCO_3)_2$ application (prior and/or after desiccation) was tested separately for each species by one-way ANOVA followed by Tukey's test. The relationship between the chlorophyll fluorescence parameter F_V/F_M and regeneration was evaluated by a linear regression. Program Statistica, (StatSoft, USA) was used for ANOVA, t-test and regression analyses.

3. **Results**

3.1. Growth and survival of Sphagnum transplants

Some of the transplanted shoots of all species survived at all localities for four growing seasons, even in calcareous fens, where no sphagna were present prior to the experiment. However, the mortality of the transplanted shoot fragments was high; only few of them survived in calcareous fens (6% fragments of *S. warnstorfii* and *S. flexuosum* and 15% of *S. squarrosum*). Their survival in some rich fens where calcium-tolerant sphagna already locally occurred was higher (Řeka and Zlatá louka, >50% of all fragments).

Shoot mortality in the first growing season differed from the rest of experimental period; it was generally higher and strongly affected by capillary water chemistry, i.e. by high pH and $[Ca^{2+}]$. The effect was highest for the least calcium-tolerant species, *S. flexuosum* (χ^2_2=159, p<0.001, R^2 = 0.37). Consequently, most fragments transplanted to hollows and low hummocks of alkaline fens perished, and only those on higher hummocks, where $[Ca^{2+}]$ and pH values did not exceed 15 mg L^{-1} and 5.5, respectively, survived well (80% survival). The survival of the more calcitolerant species *S. warnstorfii* and *S. squarrosum* was better (χ^2_2=92, p<0.001) and less influenced by capillary water chemistry (χ^2_2=62, p<0.001, R^2 = 0.22; χ^2_2=82, p<0.001, R^2 = 0.20, respectively; the effect of pH was fully covered in the effect of $[Ca^{2+}]$ due to the correlation of these factors in *S. squarrosum*). Fragments of both species survived in more than 40% of hollow positions and 80% of hummock positions, even in alkaline fens. After subtracting the effect of water chemistry, the survival of *S. flexuosum* and partly also *S. squarrosum* depended on flooding by mineral-rich fen water (χ^2_1=10, p = 0.001 and χ^2_1=4, p = 0.04, respectively). The *weather* had no effect on the survival of *S. warnstorfii* and *S. squarrosum* and only slightly affected the survival of *S. flexuosum* (χ^2_1=5, p = 0.032).

In the following years, the mortality rate decreased. The long-term survival of *Sphagnum* transplants was influenced by the *weather* and depended less on capillary water chemistry (as compared with the first months). Thus, the transplants were dying along the entire hummock–hollow transects, although their survival in high hummock positions was generally better (Fig. 3, Fig. S2). Consistently, $[Ca^{2+}]$ was the best predictor of species survival (p<0.001, χ^2_1=116, 81, 130, R^2 = 0.13, 0.09, 0.08 for *S. warnstorfii, S. flexuosum* and *S. squarrosum*, respectively; pH had no additional effect to $[Ca^{2+}]$), yet the effect of the *weather* on species mortality was comparable with the effect of $[Ca^{2+}]$

($p<0.001$, χ^2_1=108, 76, 104, R^2 = 0.13, 0.08, 0.06 for *S. warnstorfii, S. flexuosum* and *S. squarrosum*, respectively). In general, transplants of all *Sphagnum* species survived better in colder, rainy regions. After subtracting the effect of [Ca^{2+}], the survival of *S. flexuosum* and partly also *S. squarrosum* was affected by flooding (χ^2_1=10, p=0.002 and χ^2_1=6, p=0.02, respectively).

S. warnstorfii and *S. flexuosum* fragments did not expand to more than 1–4 (8) shoots (i.e. capitula) per position during the whole experimental period; in *S. squarrosum*, it was 1–4 shoots. The exception were two localities in the Bohemian Massif (Řeka and Zlatá louka) where the transplants spread quickly during the last 1(–2) experimental years, creating patches composed of 10–50 shoots (*S. warnstorfii* or *S. flexuosum*) or 5–17 shoots (*S. squarrosum*) (Fig. 3, Fig. S2). The last experimental year was more humid, having 25 % more rainy days. *S. flexuosum* and *S. squarrosum* spread well also in a few positions in the Brezové calcareous fen in the Western Carpathians (10 shoots per position). Particularly on the Bohemian Massif, the transplants spread along the whole hummock–hollow gradient, so the maximal amount of shoots per position was only partly associated with water chemistry or flooding (Table 1).

However, the *weather* and capillary water chemistry had a notable effect on the speed of fragment expansion or retreat, particularly when considering long-term survival (i.e. positions where fragments survived at least the first growing season). The explained variation increased in the order *S. squarrosum* < *S. flexuosum* < *S. warnstorfii*, with the *weather* generally having a greater effect than water chemistry or flooding (Table 1).

Sphagnum fragments expanded (i.e., created new shoots) either by dividing their capitula or by regenerating from adventitious buds. Based on our field experience, the regeneration of transplanted shoots was induced by desiccation damage. *S. flexuosum* and *S. warnstorfii* usually created 1–2 shoots by regeneration whereas *S. squarrosum* usually produced 3–6 shoots.

Table 1. Results of linear mixed effect models (experimental design reflected in random effects) and linear models (experimental design not included in the analysis) testing the effect of environmental characteristics on the rate of *Sphagnum* fragment expansion/retreat (*Sphagnum* expansion rate) and the formation of the maximal number of shoots per position (during four experimental years; Maximal number of expanded shoots). The flooding characteristic was evaluated after accounting for the effect of water chemistry (i.e. pH and Ca). R^2 represents the amount of variation explained by the characteristics. The significance of the effects is based on a likelihood-ratio test; values of test statistics and corresponding degrees of freedom are given in the LR/df column.

		Maximal number of expanded shoots			***Sphagnum* expansion rate**		
		Linear mixed effect models (lme)		Linear models (lm)	Linear mixed effect models (lme)		Linear models (lm)
Species	**Environmental characteristics**	***p***	**LR/df**	***R²***	***p***	**LR/df**	***R²***
Sphagnum squarrosum	*Weather*	0.041	4.2/6	7.3	0.064	3.4/6	19
	Ca	0.008	7.1/6	2	<0.001	27.5/6	7.1
	pH	0.064	3.4/6	3.9	<0.001	22.2/6	14
	Flooding	0.277	1.2/8		<0.001	13.5/8	5
Sphagnum flexuosum	*Weather*	0.046	4.0/6	15	0.033	4.5/6	22.0
	Ca	0.115	2.5/6	4	0.006	7.5/6	5.5
	pH	0.010	6.7/6	9.4	0.002	9.2/6	8.0
	Flooding	0.037	4.3/8	4	0.002	9.5/8	5.5
Sphagnum warnstorfii	*Weather*	0.072	3.2/6	22	0.009	6.9/6	40
	Ca	<0,001	12.6/6	12	<0.001	20.5/6	19.0
	pH	<0,001	15.1/6	12.3	<0.001	16.5/6	20.0
	Flooding	0.640	0.2/8		0.439	0.6/8	1.5

3.2. Desiccation and Ca^{2+} effects on Sphagnum *survival*

In the laboratory experiment, the calcium bicarbonate solution (combined effects of high pH and [Ca^{2+}]), applied prior to the desiccation and during the rewetting, improved the survival (regeneration) of calcitolerant species *S. warnstorfii*, *S. squarrosum* and *S. contortum* ($F_{2,18}$=25, $F_{2,21}$=22, $F_{2,18}$=34, $p < 0.001$, Fig. 1). The effect of $Ca(HCO_3)_2$ was rather ambiguous in the calcifuge and desiccation-tolerant *S. flexuosum* (slight alleviation of desiccation damage, $F_{2,21}$=3, $p = 0.065$). The alleviation of desiccation damage occurred only when the calcium bicarbonate solution was applied prior to the experiment (Fig. 2). The sole increase of [Ca^{2+}] without pH increase had only a minor positive effect on survival and the sole application of $Ca(HCO_3)_2$ during rewetting had a negative effect (Fig. 2).

The survival (regeneration) of *Sphagnum* shoots correlated with the maximum and light-acclimated quantum yields of PSII photochemistry (F_V/F_M and *ΦPSII*), which were evaluated after the first desiccation/rehydration event ($p < 0.001$, R^2 = 0.27 and 0.62, respectively; Fig. S4). Correlation strength differed between species (*S. warnstorfii*: R^2 = 0.73 and 0.91; *S. contortum*: R^2 = 0.61 and 0.80; S. *squarrosum*: R^2 = 0.51 and 0.48; *S. flexuosum:* R^2 = 0.18 and 0.51).

3.3. Chemistry of capillary water, saturation of cell-wall cation-exchange sites

The chemistry of capillary water sampled in alkaline fens covered the full range of pH and [Ca^{2+}] of ground/pore water known for acidic to alkaline fens (Vitt et al. 1995; Wheeler and Proctor 2000; Sjörs and Gunnarsson 2002; Hájek et al. 2006). The chemistry of high hummocks was comparable with poor to moderately rich fens: 5 (10) mg Ca^{2+} L^{-1} and pH 5.0 whereas hollows represented the most extreme alkaline conditions: 50–160 mg Ca^{2+} L^{-1}, pH 7.5–8.0 (Fig. S3). Many of the hollow positions were flooded at least once between inspections.

We found no difference in capillary water chemistry between living *Sphagnum* and brown-moss shoots ($t_3 = 1.1$, $p = 0.3$ for Ca, $t_7 = 1.5$, $p = 0.2$ for pH), and not even between dead *Sphagnum* and brown-moss shoots in mesh bags ($t_{21} = 1.5$, $p = 0.14$ for Ca; $t_{26} = 0.4$, $p = 0.7$ for pH). By contrast, living shoots of both moss groups had lower [Ca^{2+}] ($t_{14} = 4.2$, $p < 0.001$) and

lower pH ($t_9 = 6.1$, $p < 0.001$). $[Ca^{2+}]$ in capillary water of living shoots was generally lower than 10 mg L^{-1}, but it varied between 1–70 mg L^{-1} in dead fragments in mesh bags. The pH of living shoots was generally of one unit lower as compared to dead fragments. Moreover, some of the living sphagna maintained low pH in cell walls even in alkaline fen hollows. This was revealed by lowered $[Ca^{2+}]$ as lower pH prevents (i) the precipitation of calcium carbonate in moss capillary spaces and (ii) the saturation of cell-wall cation-exchange sites with Ca^{2+}, which saturation was 20 ± 1.6 mg g^{-1} at pH=7.0 (mean ± s.d.; Fig. 4). By contrast, the exchange sites of other transplants were probably fully saturated by Ca^{2+} in alkaline environments, and calcium carbonate precipitated on the moss surface (observed with a hand lens) when the capillary water $[Ca^{2+}]$ reached around 50 mg L^{-1} (Fig. 4).

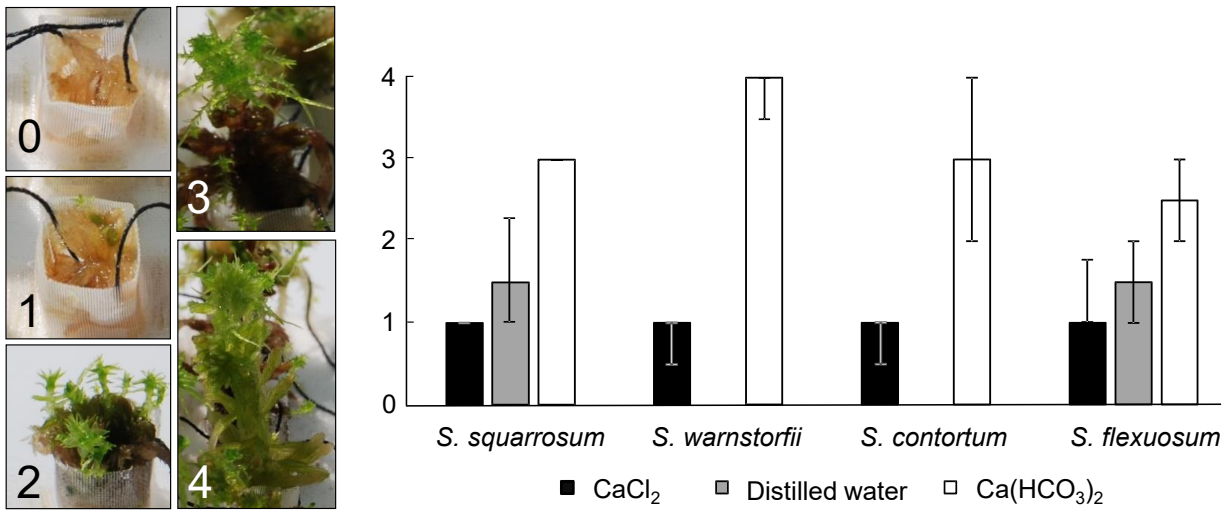

Fig. 1. Regeneration of *Sphagnum* shoots subjected to desiccation (Cultivation 2; see the Materials and Methods for details). The shoots were pre-treated and rewetted by (i) $CaCl_2$, (ii) distilled water or (iii) $Ca(HCO_3)_2$ (each solution was enriched by nutrients). The regeneration was estimated on an ordinal scale: 0 – dead, 1 – living cells, 2 – small regenerating shoots, 3 – large regenerating shoots, 4 – continued growth. The columns represent medians, and the error bars represent 25% and 75% quartiles.

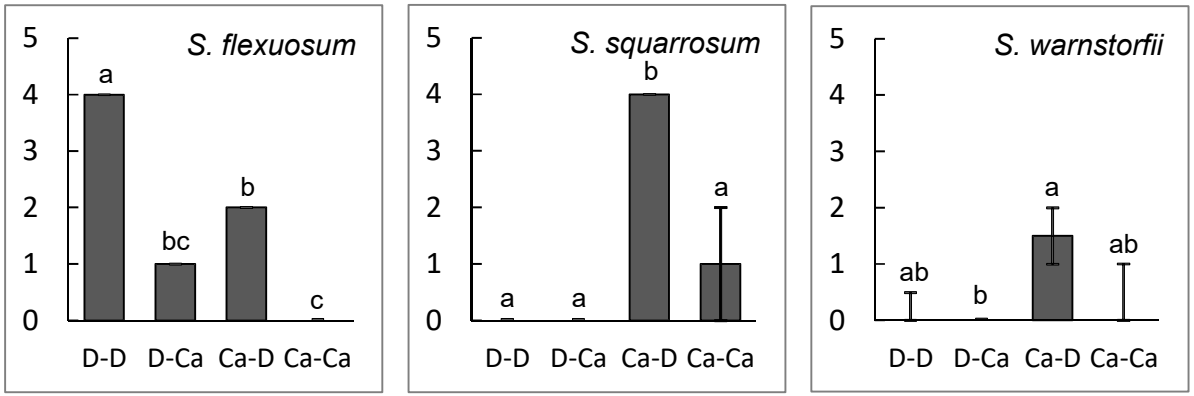

Fig. 2. Combined effect of the application of a calcium bicarbonate solution, pH 7 (Ca) before or after desiccation on regeneration of *Sphagnum* shoots hardened by mild desiccation (Cultivation 1; see methods for details). D-D – Ca^{2+} not applied during hardening or rewetting of desiccated shoots; D-Ca – Ca^{2+} applied only during rewetting; Ca-D – Ca^{2+} applied before hardening but not during rewetting; Ca-Ca – Ca^{2+} applied before hardening and during rewetting. The regeneration was assessed in categories: 0 – dead, 1 – living cells, 2 – small regenerating shoots, 3 – large regenerating shoots, 4 – continued growth (photos in Fig. 1). Treatments differences (Tukey-HSD) are denoted by letters. The error bars represent 25% and 75% quartiles, and the columns represent the median.

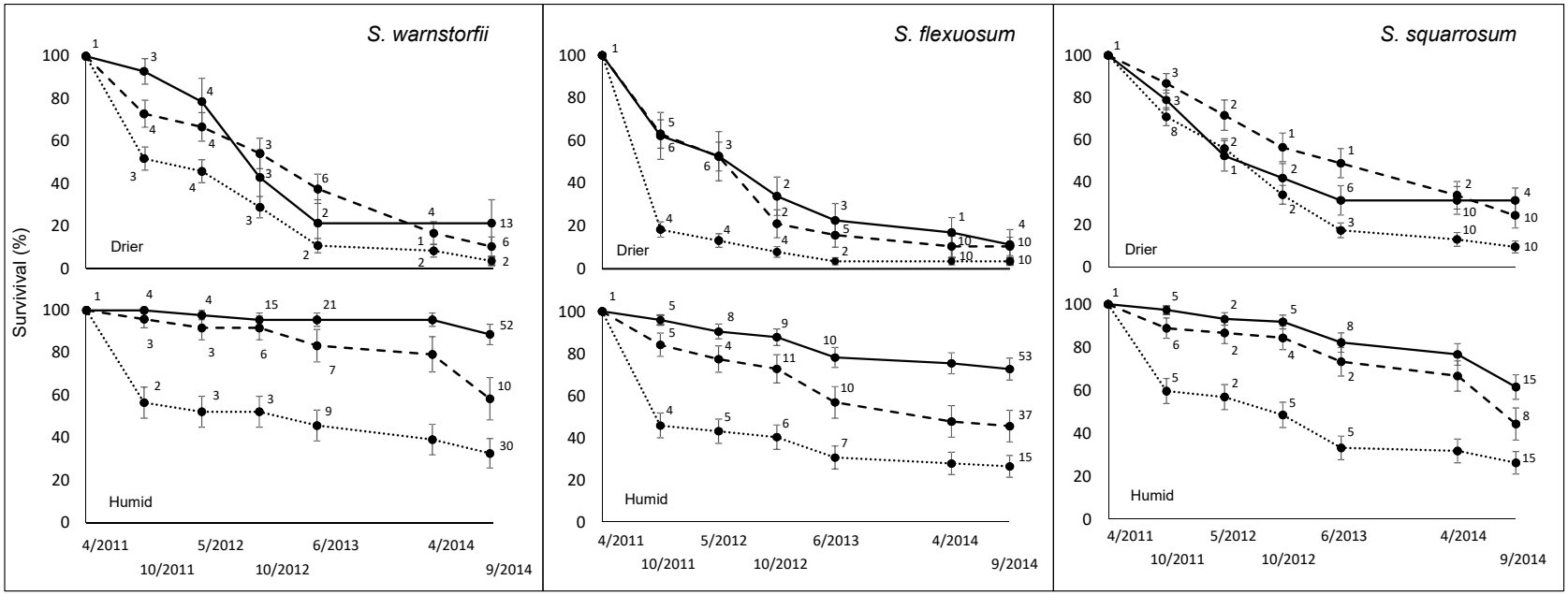
S. warnstorfii
S. flexuosum
S. squarrosum
Survivival (%)
Drier
Humid
4/2011
10/2011
5/2012
10/2012
6/2013
4/2014
9/2014

Fig. 3. Four-year survival rates of single *Sphagnum* shoots transplanted to positions of hummock–hollow transects of different capillary water chemistry (lines), in dry (Rojkov, Brezové, Demänová, Loučeň; upper diagrams) or humid (Řeka, Zlatá louka, Liptovská Teplička; lower diagrams) regions in terms of precipitation:temperature ratio in vegetation period. Black lines represent low pH positions with capillary water chemistry of pH 4.5–(5.0)–5.2, [Ca^{2+}] 3–(28)–53 mg L^{-1}, 2–(9)–19 cm above water table (dry regions) and pH 4.5–(4.8)–5.2, [Ca^{2+}] 2–(36)–61 mg L^{-1}, 7–(13)–24 cm above water table (humid regions); dashed lines denote positions of pH 5.5–(6.1)–6.5, [Ca^{2+}] 4–(43)–83 mg L^{-1} , 2–(11)–30 cm above water table (dry regions) and pH 5.5–(5.9)–6.5, [Ca^{2+}] 4–(29)–65 mg L^{-1}, 4–(10)–20 cm above water table (humid regions); dotted lines represent high pH positions of pH 6.7–(7.4)–8.0, [Ca^{2+}] 4–(68)–156 mg L^{-1} , 0–(6)–24 cm above water table (dry regions) and pH 6.7–(7.3)–8.0, [Ca^{2+}] 5–(61)–95 mg L^{-1}, 0–(4)–12 cm above water table (humid regions); values represent minimum–(average)–maximum. The number by each data point indicates the maximal number of shoots present in any position of any locality in a given region and year (initially, one shoot was present in each position at the start of the experiment, see the Materials and Methods for details). The height above water indicate average from values measured when visiting the localities (x axis).

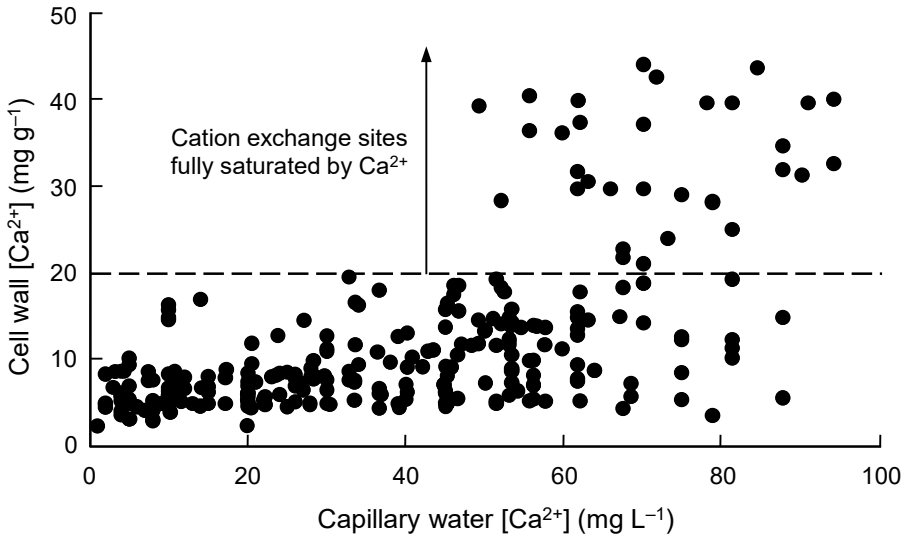

Fig. 4. Relationship of $[Ca^{2+}]$ in capillary water of *Sphagnum* shoots (*x* axis) and the amount of Ca^{2+} immobilized in cell walls of living *Sphagnum squarrosum* transplants (*y* axis) demonstrating the ability of the living moss to reduce extracellular pH. The $[Ca^{2+}]$ of moss capillary water was assessed using dead shoots placed in small mesh bags and exposed *in situ* among living shoots. The horizontal line at 20 mg g^{-1} indicates the concentration of Ca^{2+} in cell walls at which the cation-exchange sites were saturated by Ca^{2+} at pH=7.0 (at the maximum pH of 8.8 the calcium exchange capacity is 20% greater; Dainty and Richter 1993). Above this line, calcium carbonate precipitation was observed. The precipitation corresponded with alkaline pH of capillary water, in accordance with the carbonate–bicarbonate equilibrium in water. Unsaturated cation-exchange sites (below 20 mg g^{-1}) in positions with high $[Ca^{2+}]$ of capillary water (> 50 mg L^{-1}) indicate active acidification of the cell-wall–capillary-water interface due to proton extrusion by living cells; the lowered pH prevents calcium precipitation and reduce exchangeable Ca^{2+} due to competition with H^+.

4. Discussion

Our experimental study documents that *Sphagnum* fragments can survive and expand when transferred to various microhabitats in *Sphagnum*-free patches of alkaline fens or even in totally *Sphagnum*-free fens. However, their long-term survival and expansion is limited by water chemistry ([Ca^{2+}] and pH) and by weather humidity (expressed as the precipitation:temperature ratio), which obviously impacts the frequency and severity of desiccation and, in turn, also the chemistry of capillary water. Put simply, the greater the precipitation (relative to evaporation) the mosses experience, the smaller the risk of desiccation and the smaller the influence of mineral-rich groundwater, provided that the position does not become flooded.

4.1. Water chemistry, humidity and fen microtopography

Water chemistry governs the distribution of *Sphagnum* species in fens because of the toxicity of high [Ca^{2+}] and pH to *Sphagnum* mosses (Clymo 1973, Vicherová et al. 2015). Consequently, calcareous environment suppresses calcifuge species, especially when the water table overfloods their capitula (Granath et al. 2010). Individual *Sphagnum* species therefore have clearly diversified niches with respect to the pH/calcium gradient at the landscape scale (Plesková et al. 2016). At a small scale within individual mires with co-existing brown mosses and sphagna, an analogous gradient in water chemistry develops across hummock–hollow microtopography (Bellamy and Rieley 1967, Hájková and Hájek 2004). During our experiment, this even occurred in strongly calcareous brown-moss patches of alkaline fens, where individual *Sphagnum* plants were transplanted. The capillary water in the highest hummock positions maintained about 15 times lower [Ca^{2+}] and 100–1,000 times greater [H^{+}] than in hollows, allowing *Sphagnum* to grow (Vicherová et al. 2015). By contrast, only small differences in the chemistry of capillary water related to microtopography have been reported from continental alkaline fens in Canada (Karlin and Bliss 1984). This difference may be caused by different macroclimate conditions, as the drier (continental) climate provides lesser possibilities to modify the hummock microenvironment by rainwater accumulation (Kooijman 2012) and downward leaching of calcium (Eppinga et al. 2010, Hájek et al. 2014).

Fragments that did not die of acute calcium toxicity during the first weeks or months could survive in brown-moss carpets for several years, waiting for suitable conditions that would enable their expansion into larger mats. We documented such an expansion only during the relatively humid second half of the experimental period, indicating that conditions allowing fragments to expand had changed between the years and that humid weather facilitated *Sphagnum* establishment. In line with this observation, the precipitation:temperature ratio had a principal effect on *Sphagnum* expansion, highly exceeding the effect of water chemistry (Table 1). Consequently, small *Sphagnum* patches have developed only in the most humid fens on the Bohemian Massif that at the same time did not deviate in water chemistry and nutrient status except for lower [Mg^{2+}] (Table S4). However, the higher [Mg^{2+}] in the Western Carpathians should not have any detrimental effect on *Sphagnum* growth or survival, as sphagna are less affected by Mg^{2+} than brown mosses (Vicherová et al. 2015). The significance of weather or climate effects on *Sphagnum* establishment in alkaline fens, and on fen succession in general has never been highlighted before; yet, our results correspond with palaeoecological records of a climate-driven rich to poor fen transition (Kuhry et al. 1993) as well as with the documented absence of *Sphagnum* hummocks in more continental alkaline fens in SE Europe (Hájek et al. 2014).

Compared to the expansion of fragments, the weather and water chemistry had a rather small effect on the long-term survival of *Sphagnum* fragments; both environmental characteristics together explained only 5% of the variation. The remaining 95% of unexplained variation covers unaccounted cues such as the direct effect of desiccation stress. Competition does not seem to play an important role in the coexistence of bryophyte species (Slack 1990, Rydin 1993, Mälson and Rydin 2009; but cf. Udd et al. 2016) and obviously had a minor role in the survival of *Sphagnum* transplants in undisturbed brown-moss carpets of less alkaline microhabitats, where sphagna should be competitively superior over brown mosses (Mulligan and Gignac 2001; Udd et al. 2016). Accordingly, we frequently observed regeneration of previously desiccated fragments that were buried several cm deep below the moss surface.

4.2. Desiccation

Brown mosses in hummocks and other non-inundated microhabitats frequently desiccated during dry periods in our fens, because they lack efficient mechanisms of storing and conducting capillary water. Sphagna, by contrast, avoid desiccation by means of morphological adaptations and therefore require a long hardening period (slow dehydration) to develop desiccation tolerance. Interestingly, *S. teres* and *S. flexuosum* possess superior desiccation tolerance among *Sphagnum* mosses (Hájek and Vicherová 2014). This advantage may facilitate their establishment and/or expansion in calcium-rich fens, as has recently been observed (Hájek et al. 2015, Plesková et al. 2016). The risk of desiccation, however, decreases with plant size: Consistently, larger transplants of *S. squarrosum* dried out more slowly than smaller *S. warnstorfii* transplants in our study. The weather might hence affect species survival and expansion via species-specific size of desiccated fragments. In any case, in more humid oceanic Europe, alkaline fens are invaded by *S. squarrosum* (Kooijman 2012), which is less tolerant to desiccation (Hájek and Vicherová 2014).

Although desiccation and calcium uptake restricted the survival of *Sphagnum* fragments in fens, our laboratory experiment showed that their combined effect may not be always harmful. Indeed, the calcitolerant *S. warnstorfii* and *S. squarrosum* showed increased tolerance to desiccation if they grew in a calcium bicarbonate-rich solution prior to desiccation. The effect was negligible for the least calcium-tolerant species *S. flexuosum*, perhaps because the calcium concentration was already toxic. The positive effect of calcium bicarbonate may be connected with the signalling role of Ca^{2+} in pathways leading to a cellular response to stress. We have reported that cytosolic [Ca^{2+}] rises when the fragments are cultivated in a solution of high pH and [Ca^{2+}] (Vicherová et al. 2015). Elevated cytosolic [Ca^{2+}] may trigger a cellular mechanisms protecting against desiccation damage, possibly by the preservation of membrane integrity, for example (Ramanjulu and Bartels 2002) or synthesis of LEA proteins (Saijo et al. 2000). Consequently, the increase of [Ca^{2+}] without increasing pH, which does not result in elevated cytosolic [Ca^{2+}] (Vicherová et al. 2015), had no effect on the survival of any species. In addition, or alternatively, calcium ions forming coordination bonds with

membrane phospholipids (Inoue at al. 1992) might directly increase membrane stability during desiccation and rehydration.

Calcium-induced desiccation tolerance may help *Sphagnum* to establish in species-rich alkaline fens and to colonize drier but calcium-poorer microhabitats. The low ability of hummock sphagna to develop desiccation tolerance (Hájek and Vicherová 2014) can be compensated by a calcium-induced desiccation tolerance. This mechanism suggests a surprising ecological paradox: Calcium ions may participate in establishment of rich-fen sphagna among brown mosses, which may trigger acidification and rapid succession towards a species-poor fen.

4.3. Acidifying properties of living mosses

The amount of rain water retained by few *Sphagnum* shoots was not sufficient to counteract the mineral-rich fen water coming from the surroundings. However, living shoots of both *Sphagnum* and brown mosses exhibited similar capacity to decrease the pH and [Ca^{2+}] of capillary water. Such acidification can result from (i) new cell-wall cation-exchange sites that formed (and dissociated) during shoot growth (Dainty and Richter 1993) and (ii) active proton exudation from protoplast to apoplast (Vicherová et al. 2015). The first mechanism would also lower [Ca^{2+}] in capillary water by exchanging Ca^{2+} for protons. The cation exchange (CE) capacity of *Sphagnum* and brown-moss shoots is similar (Soudzilovskaya et al. 2010) even across a wide range of external pH (Vicherová et al. 2015). Because vertical growth rates of brown mosses and sphagna were similar (the production was therefore similar; Kooijman and Bakker, 1994), the observed acidification and [Ca^{2+}] decrease did not differ between species. The rate of proton exudation was probably similar, too, as this is a fundamental process that drives the nutrient economy of a plant cell. Nevertheless, proton exudation has never been experimentally studied in fen mosses.

The equal acidification capacity of *Sphagnum* and brown mosses observed in our field experiment contrasts with the lack of acidification ability of a brown moss, reported by Kooijman and Bakker (1994), as compared with a set of *Sphagnum* species. However, this experiment did not take into consideration that the CE sites of the brown moss had already been saturated by [Ca^{2+}] under the neutral pH of the environment where they

were collected (see also Vicherová et al. 2015); therefore, the brown moss lacked protons to be exchanged. Moreover, the slow production of biomass (and CE sites) of the brown moss did not compensate for the effect of saturation by [Ca^{2+}].

In summary, even a single *Sphagnum* shoot has a capacity to acidify its own microcosm of retained capillary water. It, however, cannot acidify the surrounding environment, because its acidifying capacity is limited, and ions get trapped by slow diffusion rates in the large volume of capillary water. The fine-scale heterogeneity in the volume and chemistry of capillary water in mixed brown-moss–*Sphagnum* communities may allow long-term coexistence of both species groups. However, once larger *Sphagnum* mats have formed, the rainwater retaining ability of the moss, as well as of the litter accumulated beneath, can result in surface acidification, speeding up the succession towards poor fens or bogs (as presumed by, e.g., Kooijman 2012).

5. Conceptual model of determinants of Sphagnum *establishment and expansion into brown-moss fens*

Our results suggest that humid conditions facilitate the expansion of *Sphagnum* in calcareous fens, confirming the increasingly frequent observations of recent rich-fen decline in the regions with rather high summer precipitation-to-evaporation ratio such as the Netherlands (Kooijman 2012), Bohemian-Moravian Highlands and Třeboň basin in the Czech Republic (Hájek et al. 2015) or boreal zone (Juutinen 2011, Kapfer et al. 2011, Tahvanainen 2011). Although the effect of weather overruled other effects in our study, the humid climate alone does not determine establishment of *Sphagnum* in a calcareous fen. Many brown-moss calcareous fens persist in precipitation-rich Atlantic or Alpine biogeographical regions or in the cold and humid Boreal region (Jiménez-Alfaro et al. 2014), some spring-fed brown-moss fens had resisted *Sphagnum* expansion for millennia (Hájková et al. 2015), and *Sphagnum* species often occurred in calcareous fens under the continental climate conditions of the Early Holocene (Hájková et al. 2012). Lastly, a certain effect of water table decline and/or nutrient enrichment on this succession or at least *Sphagnum* performance in calcium-rich environments seems to be well demonstrated (Tahvanainen 2011, Kooijman 2012, Hájek et al. 2015, Vicherová et al. 2015). It is therefore obvious that individual

environmental characteristics driving succession towards *Sphagnum* fens must interact. Furthermore, different conditions possibly affected the survival of adult plants that were transplanted in this study as compared to spore germination and survival of protonemata (Hájek and Vicherová 2014, Vicherová et al. 2015). Based on our experiments and existing evidence, we can suggest the following conceptual model explaining ongoing successional changes in alkaline fens:

(1) Because protonemata are more susceptible to desiccation than adult *Sphagnum* shoots (Hájek and Vicherová 2014), germination is possible only in sufficiently humid conditions. Increasing potassium supply to fens may lead to better survival of protonemata in calcium-rich conditions and improve the growth of sphagna by compensating for calcium intolerance (Vicherová et al. 2015).
(2) Higher nutrient supply may generally accelerate succession by favouring the biomass production and growth rate of competitively superior sphagna (Udd et al. 2016).
(3) Declining water levels caused by hydrological disturbances in the catchment (Tahvanainen 2011) or slight local drainage facilitate the long-term survival of established sphagna and prevent buffering of rainwater-fed capillary water in hummocks by groundwater rich in calcium bicarbonate.
(4) Humid climate conditions promote the spread of established sphagna by rainwater accumulation, which prevents upward calcium movement driven by high evaporation (Hájek et al. 2014) and especially, as our study demonstrates, hampers desiccation that would reverse the initiated process.

Fens in oceanic or boreal regions are therefore more prone to *Sphagnum* expansion, and only small changes in hydrology or nutrient enrichment may trigger a rapid ecosystem shift. On the other hand, drier continental or submediterranean climate conditions coupled with a water table decline bend the successional trajectory towards fen grasslands of the *Molinion caerulae* alliance with *Molinia caerulea* agg., *Schoenus nigricans, Sesleria uliginosa, Juncus subnodulosus, Ctenidium molluscum* and *Campylium stellatum,* when nutrient availability remains low, or into broadleaved fen grasslands of the *Calthion* alliance or tall-herb vegetation, when nutrient availability is high (Rozbrojová et Hájek 2008). The long-term stability of

brown-moss calcareous fens, which have persisted for millennia (Hájková et al. 2015), requires a permanently high water level (e.g., deep hydrological circulation) and a rather continental climate that prevents succession towards *Sphagnum* fens when the water level temporarily decreases. These contrasting, climate humidity-dependent successional pathways should be taken into account in predictions of future changes in diversity, habitat distribution or carbon storage under the ongoing global environmental changes.

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Supporting information:
Table S1. List of experimental localities
Table S2. List of source localities used for the transplantation experiment
Table S3. List of source localities used for laboratory experiments
Table S4. Water chemistry of the experimental localities
Fig. S1. Schema of positioning *Sphagnum* transplants to transects
Fig. S2. Four-year survival rates of *Sphagnum* transplants to hummock–hollow transects to positions of different capillary water chemistry (pH).
Fig. S3. Maximal and average number of transplanted *Sphagnum* shoots expanding in positions of hummock–hollow transects of a given locality during four experimental years
Fig. S4. Light-acclimated and maximum quantum yields of PSII photochemistry showing *Sphagnum* survival in reaction to combined effect of calcium and desiccation.

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Supporting information 1

Table S1. List of experimental localities indicating the type of mire (division based on vegetational composition following Hájek et al. 2006), localization (coordinates are in WGS 84), the range of groundwater pH and [Ca^{2+}] (based on 4 measurements), lists of species in transects surrounding the transplants at each locality and the number of undamaged positions and transects at each locality.

Locality name	Mire type — Bedrock	GPS coordinates	Altitude (m a.s.l.)	Ground water pH	Ground water [Ca^{2+}] (mg L^{-1})	Species in transects	Number of positions per locality/number of transects
Rojkov, Žilina region, SZ Slovakia	calcareous fen — limestone	49°08'55"N, 19°09'19"E	450	5.8–7.5	75–130	*Scorpidium cossonii* *Dicranum bonjeanii* *Aulacomnium palustre* *Campylium stellatum* *Calliergonella cuspidata*	50/6
Brezové, Prešov region, N Slovakia	calcareous fen — limestone	49°03'03"N, 20°01'42"E	850	7.3–7.7	60–85	*Scorpidium cossonii* *Campylium stellatum* *Tomentypnum nitens* *Fissidens adianthoides* *Bryum pseudotriquetrum*	50/5
Řeka, Vysočina region, Czech Republic	rich fen — lime claystone	49°39'59"N, 15°51'11"E	550	6.9–7.3	30–95	*Scorpidium cossonii* *Aulacomnium palustre* *Tomentypnum nitens* *Climacium dendroides* *Plagiomnium elatum* *Hamatocaulis vernicosus* *Calliergon giganteum*	46/6
Zlatá louka, Vysočina region, Czech Republic	rich fen — quartz sandstone	49°42'49"N, 15°46'23"E	470	7.3–7.6	30–80	*Scorpidium cossonii* *Bryum pseudotriquetrum* *Aulacomnium palustre* *Plagiomnium elatum* *Tomentypnum nitens* *Calliergonella cuspidata* *Campylium stellatum* *Climacium dendroides*	66/6

Locality name	Mire type — Bedrock	GPS coordinates	Altitude (m a.s.l.)	Ground water pH	Ground water [Ca^{2+}] (mg L^{-1})	Species in transects	Number of positions per locality/number of transects
Liptovská Teplička, Prešov region, Slovakia	rich fen — basalt + andesite, fluvial sediments on dolomite	48°57'50"N, 20°06'24"E	900	6.6–7.6	18–40	*Hamatocaulis vernicosus* *Tomentypnum nitens* *Aulacomnium palustre* *Sphagnum warnstorfii*	37/5
Demänová, Žilina region, Slovakia	calcareous fen — diluvial sediments on limestone and dolomite bedrock	49°03'06"N, 19°34'47"E	650	6.8–8.0	40–85	*Tomentypnum nitens* *Calliergonella cuspidata* *Scorpidium cossonii* *Campylium stellatum*	63/6
Loučeň, N Bohemia, Czech Republic	calcareous fen meadow — lime claystone	50°18'06"N, 15°01'05"E	240	6.9–7.4	100–180	*Calliergonella cuspidata* *Campylium stellatum* *Plagiomnium elatum* *Fissidens adianthoides* *Scorpidium cossonii*	23/3
Liptovská Teplička 2, Prešov region, Slovakia	rich fen — fluvial sediments on dolomite bedrock	48°57'08"N, 20°06'12"E	920	7.2–7.8	50–70	*Scorpidium cossonii* *Campylium stellatum* *Sphagnum warnstorfii* *Sphagnum flexuosum* *Paludella squarrosa* *Bryum pseudotriquetrum* *Tomentypnum nitens*	41/4

Table S2. List of source localities used for the transplantation experiment indicating the type of mire (division based on vegetational composition following Hájek et al. 2006), localization (coordinates are noted in WGS 84) and groundwater chemistry.

Locality name	Mire type	GPS coordinates	Altitude (m a.s.l.)	ground water pH	ground water $[Ca^{2+}]$ (mg L^{-1})	Species transplanted
Liptovská Teplička, Prešov region, Slovakia	rich fen	48°57'50"N, 20°06'24"E	900	6.6–7.6	18–40	*S. warnstorfii* *S. flexuosum*
Jochy, Žilina region, Slovakia	moderately rich fen	49°07'15"N, 19°46'23"E	885	5.5	4	*S. warnstorfii* *S. flexuosum*
U Hada, S Bohemia, Czech Republic	moderately rich fen (alder carr)	48°58'41"N, 14°25'37"E	380			*S. squarrosum*
Chvojnov, Vysočina region, Czech Republic	rich fen	49°24'19"N, 15°25'11"E	606	5.8	17	*S. flexuosum*

Table S3. List of source localities used for laboratory experiments combining the effect of desiccation and calcium. Mire type (division based on vegetational composition following Hájek et al. 2006), localization (coordinates are noted in WGS 84) and groundwater chemistry are indicated.

Locality name	Mire type	GPS coordinates	Altitude (m a.s.l.)	ground water pH	ground water $[Ca^{2+}]$ (mg L^{-1})	Species
Dlouhá louka, Plzeň region, Czech Republic	moderately rich fen	49°54'44"N, 13°10'43"E	570	6.3	6.5	*S. flexuosum*
V Rájích, S Bohemia, Czech Republic	rich fen	48°59'09"N, 14°42'31"E	445	6.2	36	*S. warnstorfii* *S. contortum*
U Hada, S Bohemia, Czech Republic	moderately rich fen (alder carr)	48°58'41"N, 14°25'37"E	380			*S. squarrosum*

Table S4. Concentration of mineral nutrients in groundwater in the experimental localities. The time of the sampling is noted as month/year. Ground water samples were filtered in field by glass microfibre filter (1 µm porosity), fixed by chloroform or HNO_3 and analysed by AAS. Detailed description of the methodology is in Hájek et al. 2014.

	NH_4^+–N (µg L^{-1})						NO_3^-–N (µg L^{-1})						PO_4^-–P (µg L^{-1})						total Fe (mg L^{-1})					
Month	6	9	4	5	10	6	6	9	4	5	10	6	6	9	4	5	10	6	6	9	4	5	10	6
Locality / Year	2010	2010	2011	2011	2011	2013	2010	2010	2011	2011	2011	2013	2010	2010	2011	2011	2011	2013	2010	2010	2011	2011	2011	2013
Řeka				23		23				0		0				125		89			1.8	0.0	0.4	
Zlatá louka				103		4				679		1100				69		18			1.9	0.0	0.1	
Rojkov	6	0	4		72		20	0	267		318		0	16	45		52		<0.08	0.1	0.8	4.9	18.1	
Liptovská Teplička	0		0			4	0		55			200	0		24			0	1.0		1.6	4.4	1.7	
Liptovská Teplička 2	346		43			27	69		258			800	28		84			7	1.6		2.3	10.9	0.5	
Brezové	5		0		62	10	0		37		502	0	0		149		116	8	0.2		0.7	1.5	4.5	
Demänová	21	17	0			22	31	0	95			0	0	0	74			6	0.1	0.6	1.6		0.3	
Loučeň				22						3053						98						0.1		

	K^+ (mg L^{-1})						Ca^{2+} (mg L^{-1})						Mg^{2+} (mg L^{-1})					
Month	6	9	4	5	10	6	6	9	4	5	10	6	6	9	4	5	10	6
Locality / Year	2010	2010	2011	2011	2011	2013	2010	2010	2011	2011	2011	2013	2010	2010	2011	2011	2011	2013
Řeka			8.2	0.1	<0.01	0.0			80	94	30	62			2	3	0	2
Zlatá louka			3.3	0.6	0.2	0.3			69	78	31	69			1	3	0	1
Rojkov	0.6	0.6	2.3		0.8		86	84	130	76	93		33	29	44	26	26	
Liptovská Teplička	0.1		0.3		1.6	0.2	35		17	33	40	24	13		7	14	9	7
Liptovská Teplička 2	4.9		2.5		0.6	0.1	45		52	59	69	54	21		23	28	27	25
Brezové	1.3		4.7		3.0	1.9	83		79	81	83	61	37		27	24	23	23
Demänová	1.4	1.0	1.2		8.9	0.3	76	69	85		43	53	39	27	24		23	24
Loučeň				0.6						184						37		

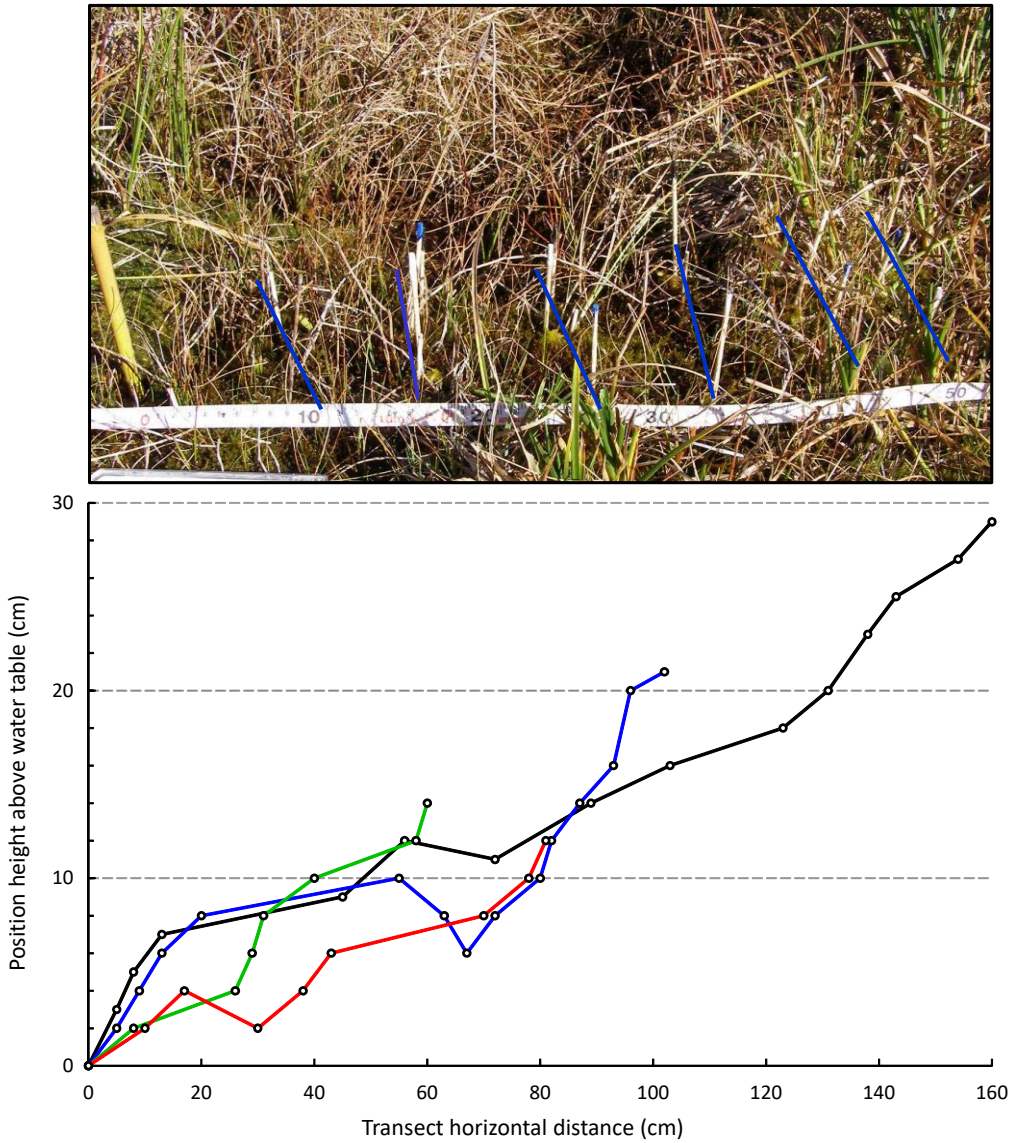

Fig. S1. Hummock–hollow transects: photographic example of single transect and graphical example of four transects. The localization of individual fragment transplants at each position is marked by a coloured wooden skewer and highlighted by blue lines in the photograph. The transect positions were set two cm apart vertically (dots in the diagram).

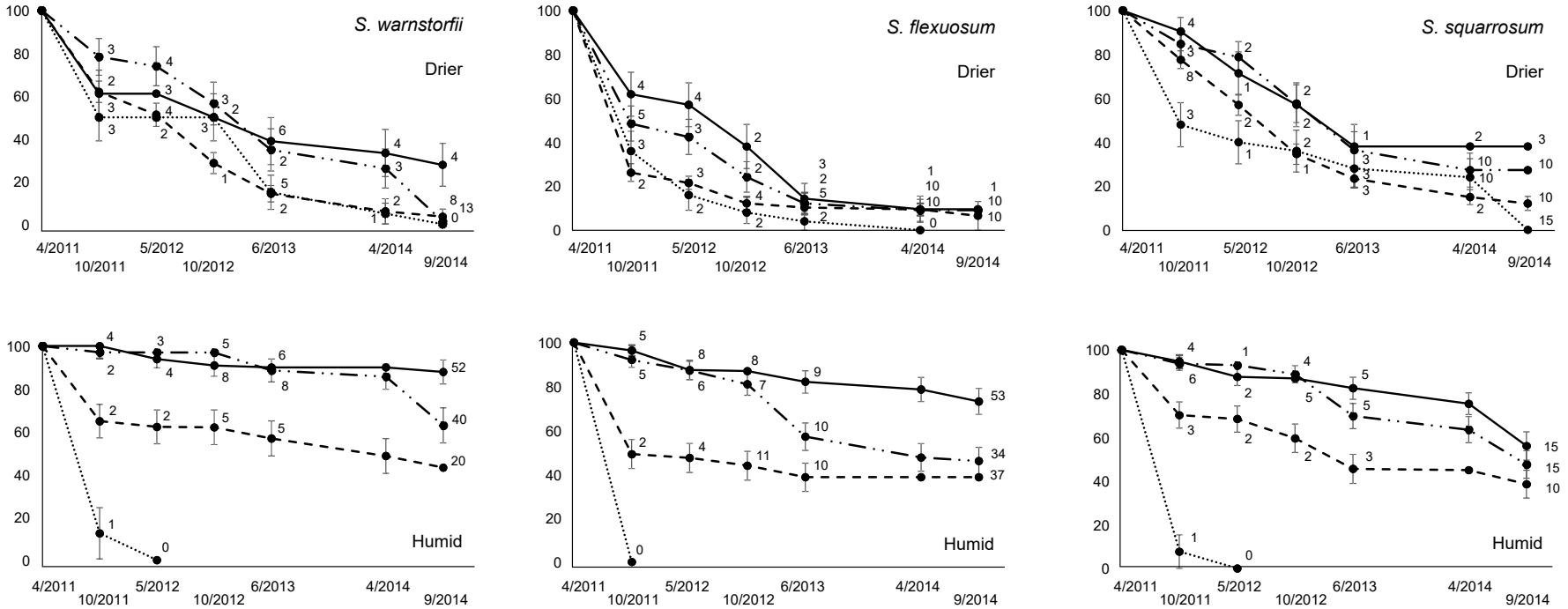

Fig. S2. Four-year survival rates of single *Sphagnum* shoots transplanted to positions of hummock–hollow transects of different capillary water chemistry (lines), in dry (Rojkov, Brezové, Demänová, Loučeň; upper diagrams) or humid (Řeka, Zlatá louka, Liptovká Teplička; lower diagrams) regions in terms of precipitation:temperature ration in vegetation period. Black lines represent mineral-poor positions with capillary water chemistry of $[Ca^{2+}]$ 1–(8.6) –15 mg L^{-1}, pH 4.5–(5.0)–7 (dry regions) and $[Ca^{2+}]$ 1–(7)–15 mg L^{-1}, pH 4.5–(4.8)–5.2 (humid regions); dot-dot-dashed lines denote positions of $[Ca^{2+}]$ 17–(30)–40 mg L^{-1}, pH 4.5–(6.3)–8, (dry regions) and $[Ca^{2+}]$ 17–(28)–40 mg L^{-1}, pH 4.5–(5.8)–8 (humid regions); dashed lines represent mineral-rich positions of $[Ca^{2+}]$ 41–(57)–84 mg L^{-1}, pH 5.0–(7.0)–8.0 (dry regions) and $[Ca^{2+}]$ 41–(63)–84 mg L^{-1}, pH 4.5–(6.9)–8.0, dotted lines represent calcareous positions of $[Ca^{2+}]$ above 88–(116) mg L^{-1}, pH 6.5–(7.2)–7.5, (dry regions) and $[Ca^{2+}]$ above 88–(95) mg L^{-1}, pH 7.0–(7.4)–7.5 (humid regions (representing minimum–(average)–maximum value). The number by each data point indicates the maximal number of shoots present in any position of any locality in a given region and year (initially, one shoot was present in each position at the start of the experiment, see the Materials and Methods for details).

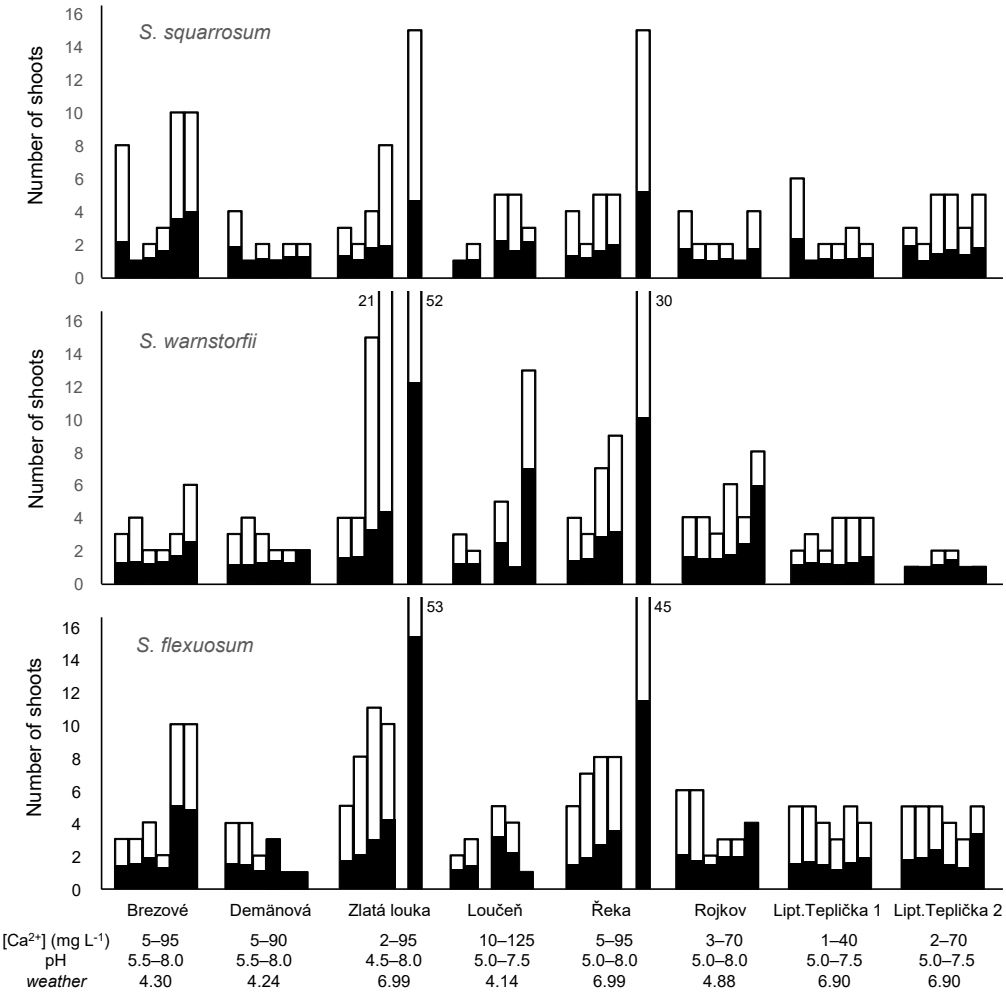

Fig. S3. Maximal (white columns) and average (black columns) number of transplanted *Sphagnum* shoots expanding in positions of hummock–hollow transects of a given locality during four experimental years (columns from the left: September 2011, May 2012, September 2012, June 2013, April 2014 and October 2014). The average number of shoot was calculated only from positions where the species stayed alive. Missing columns denote missing data. The environmental characteristics [Ca^{2+}] and pH describe the chemistry of capillary water in hummocks–hollows, and *Weather* denotes the precipitation:temperature ratio.

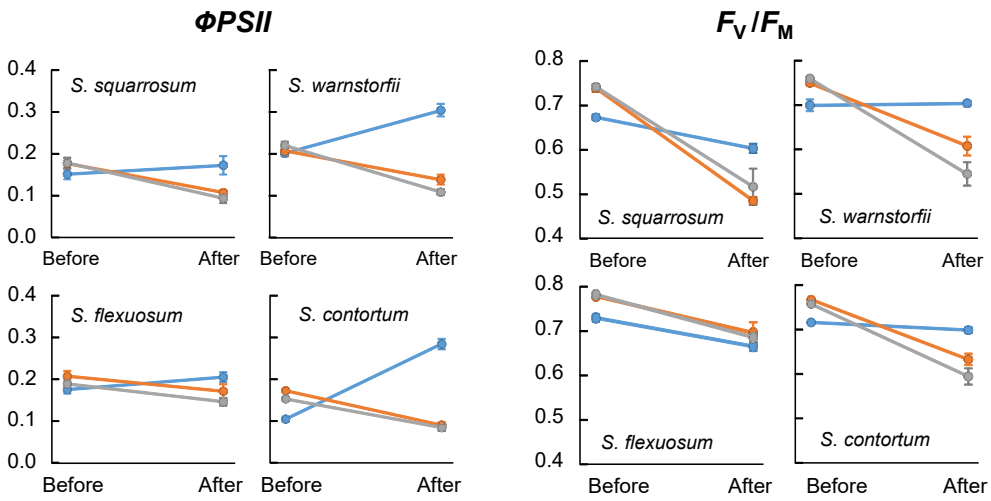

Fig. S4. Light-acclimated quantum yields of PSII photochemistry (*ΦPSII*) and maximum quantum yields of PSII photochemistry (F_V/F_M) showing survival of four *Sphagnum* species in reaction to combined effect of calcium and desiccation. The decrease in the parameters indicates cells damage. *ΦPSII* and F_V/F_M was measured after one week of cultivation (Before) in: calcium bicarbonate solution with nutrients (blue line), calcium chloride solution with nutrients (red line), nutrient solution (grey line). After the measurement, moss shoots were hardened, desiccated and the measurement was repeated 24 h after their rewetting with the solution they were pretreated with.

Chapter 4.
New insights to the mechanism of calcicole–calcifuge behavior in bryophytes

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Keywords: calcicole–calcifuge, calcium toxicity, bryophytes, glutathione, membrane potential

Highlights:

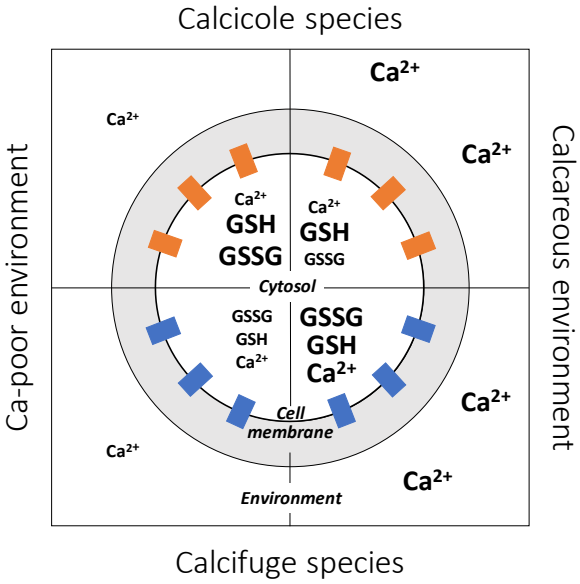
Calcicoles and calcifuges differ in composition of cation channels in plasma membrane.

Calcifuges in contrast to calcicoles rapidly accumulate Ca in cytosol when suddenly exposed to high extracellular [Ca^{2+}].

Calcicoles have constant high cytosolic GSH concentration.

Calcifuges increase GSH production under high extracellular [Ca^{2+}] and increase cytosolic GSSG concentration, presumably because of oxidative stress.

Graphical abstract



Abstract

Calcicole–calcifuge behaviour in bryophytes is controlled solely by a toxicity of Ca^{2+} in alkaline environment. The mechanism behind calcium toxicity seems intracellular, yet it is still not clear why the calcifuge species die in calcareous environment.

To understand the mechanisms behind calcium toxicity, we cultivated calcifuge and calcicole bryophytes submersed in solutions of artificial alkaline- and acidic-fen groundwater, measuring the rate of intracellular Ca^{2+} accumulation and GSH/GSSG production. We also blocked membrane ion channels and ATPases and measured changes in plasma membrane potential after a sudden exposure of cells to high Ca^{2+} concentration.

Our results showed that calcicole and calcifuge species differ in composition and/or regulation of plasma membrane cation channels responsible for Ca^{2+} uptake to the cytosol. Presumably, the difference indicates that calcicoles evolved a specific adaptation that controls Ca^{2+} influx/efflux and enables the plants to maintain stable cytosolic [Ca^{2+}] even in alkaline Ca^{2+}-rich water. In contrast, calcifuge species increased cytosolic [Ca^{2+}] in alkaline fen water, which is toxic for those species. The excessive Ca^{2+} uptake coincide with increase in GSH and GSSG concentration that could be possibly raised to quench ROS produced during oxidative stress.

Our results provide new insights into the mechanisms of calcium toxicity in plants and reveal cellular adaptations that calcicoles evolved to survive in calcareous environment.

Introduction

Calcifuge–calcicole behaviour, i.e. avoidance vs. preference of calcareous environment, is a widely distributed phenomenon across the whole eukaryotic domain. Generally, communities in calcareous, alkaline environment (calcicole communities) have entirely different biota than communities from calcium-poor, acidic environment (calcifuge communities) (Hájek et al. 2006). The universal response of organisms to the gradient of calcium bicarbonate availability involves direct response to calcium ions (since Ca^{2+} play crucial role in cell signalling, metabolism, gene expression, and membrane structure; White and Broadley 2003, Hepler and Winship 2010, Görlach et al. 2015) but can also involve factors indirectly related to Ca^{2+} concentration (e.g., nutrient availability). The direct effect of Ca^{2+} is particularly prominent in basal eukaryotes (e.g., algae and bryophytes as non-vascular land plants) that lack mechanical barriers separating their cells from direct effects of calcareous soil water.

The calcifuge–calcicole behaviour interested plant ecologists from the beginning of 20th century. In tracheophytes or lichens, the long experimental evidence indicates the calcifuge–calcicole behaviour is connected with toxicity of other metals or nutrient limitation that generally prevail over the effect of calcium: calcifuges are adapted to aluminium or iron toxicity of acidic soils having low metal ion uptake, while calcicoles deal with phosphorus and iron deficiency of calcareous soils by up-regulating uptake of these minerals (Snowden and Wheeler 1993, Zohlen and Tyler 2000, Paul at al. 2009). In contrast, calcicole–calcifuge behaviour in bryophytes does not seem to be caused by nutrient limitation or non-calcium metal toxicity. The substrate specificity seems to be connected primarily with Ca^{2+} availability and secondarily with competition. Calcifuge mosses die in calcareous environment from toxicity of high [Ca^{2+}] combined with high pH (Clymo1973, Vicherová et al. 2015). In contrast, calcicoles could survive along the whole gradient of natural [$Ca(HCO_3)_2$] but are excluded from acidic biotopes by competition (Vicherová et al. 2015). Direct connection of calcium toxicity with [Ca^{2+}] and pH makes bryophytes ideal organisms to study mechanisms behind calcium toxicity and adaptations evolved by calcicoles to survive in calcareous environment.

Although the mechanisms behind calcium toxicity in bryophytes remain unclear, two basic hypotheses can be formulated based on literature evidence: (i) calcicole–calcifuge behavior may be directly connected with cell wall

properties, where the excess of Ca^{2+} would prevent cell wall growth and uptake of monovalent cations (through saturation of abundant cell-wall cation-exchange sites, Dainty and Richter 1993, Proseus and Boyer 2006). Alternatively, (ii) calcicole–calcifuge behavior may be connected with Ca^{2+} influx/efflux mechanisms and/or regulation of intracellular calcium homeostasis in calcareous environment (White and Broadley 2003).

Comparing cell wall properties of calcicole and calcifuge peatland mosses did not validate the first hypothesis mentioned above (Vicherová et al. 2015). Cation exchange sites of neither species became saturated by Ca^{2+} in calcareous solutions and no species showed signs of nutrient deficiency. That indicates the cell wall-bound calcium ions cannot have inhibitory effect on growth or nutrient uptake (*sensu* Dainty and Richter 1993). The unsaturation of cell wall by Ca^{2+} was interpreted as a result of physiological acidification by proton exudation and hence Ca^{2+} elution.

Violation of calcium homeostasis is thus a presumable cause of calcium toxicity; bryophyte adaptations to calcareous environment may be targeted to that. The best clue for this argument might be the death of calcifuge mosses after excessive accumulation of intracellular Ca^{2+} (Vicherová et al. 2015). Consequently, the calcicole–calcifuge behaviour could be connected with regulation of influx/efflux of calcium ions in/out the cells, as suggested by Lee (1998): "*Calcicole species would minimalize calcium influx through plasma membrane ion channels to cytosol and maximize efflux and vacuolar sequestration through membrane Ca^{2+}-ATPases and Ca^{2+}/H^{+} antiporters. In contrast, calcifuges adapted to soils of low calcium supply would maximize calcium influx and have lower efflux capacity*". However, calcicole and calcifuge mosses do not seem to differ in cytosolic Ca^{2+} accumulation (Vicherová et al. 2015). Consequently, though the efflux mechanism is important for species survival in calcareous environment (Hirschi 2001, Guttery at al. 2013), calcium toxicity and adaptation of calcicole species might lie in processes different from sole influx/efflux regulation. They could be directly connected with preservation of steady-state $[Ca^{2+}]$ in cytosol, possibly by chelation of assimilated Ca^{2+} and its quick sequestration to vacuole. Alternatively, preservation of steady-state $[Ca^{2+}]$ in cytosol might concur with fine influx/efflux regulation that could not be observed by methods used in Vicherová et al. (2015).

Ligands able to chelate metals are present in cytoplasm of plant cells, including bryophytes. The most common are methallothioneins and

phytochelatins, yet their synthesis upon high external [Ca^{2+}] is improbable (He et al. 2005). Nevertheless, Ca^{2+} might be chelated also by glutathione, whose synthesis is up-regulated under high external [Ca^{2+}] (López-Climent 2014). Glutathione is synthesised in bryophytes (Choudhury and Panda 2005, Petraglia 2014); however, the studies used only calcifuge species with no intention to connect glutathione synthesis with calcium tolerance. In addition, glutathione is an important antioxidant, protecting cells against reactive oxygen species (ROS), whose high intracellular accumulation in stress conditions cause cell death (Noctor et al. 2012). ROS and glutathione production, and cytoplasmic [Ca^{2+}] are interlinked: decreased extracellular [Ca^{2+}] was found to lower the ROS production (Tasduq et al. 2008). The calcicole bryophytes thus could use glutathione as a ligand integrated in regulation of cytoplasmic [Ca^{2+}], besides protecting cells against oxidative damage.

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In summary, mechanisms of calcium tolerance/toxicity seem to be intracellular, probably directly connected with preservation of steady-state concentration of free Ca^{2+} maintained by regulation of Ca^{2+} influx/efflux and/or by production of glutathione as a ROS and/or Ca^{2+} scavenger. In order to disentangle the proposed mechanism, we cultivated calcifuge and calcicole peatland bryophytes and searched for differences in fine-scale cytosolic Ca^{2+}

accumulation, Ca^{2+} influx/efflux mechanism and glutathione production. In detail, we hypothesize that:

1) Calcicoles and calcifuges differ in composition, quantity or regulation mechanisms of membrane protein complexes responsible for Ca^{2+} influx/efflux.
2) Calcifuges accumulate high amount of Ca^{2+} in cells when exposed suddenly to high extracellular [Ca^{2+}] and pH, since their influx/efflux regulation is not adapted to high external [Ca^{2+}].
3) Calcicoles produce higher concentration of glutathione that could (i) chelate free Ca^{2+} before their sequestration or extrusion and (ii) protect cells against oxidative stress.

Methods

Moss material

Calcium-tolerant 'brown mosses' (*Hamatocaulis vernicosus, Calliergonella cuspidata, Scorpidium scorpioides*) and Ca^{2+}-intolerant bog bryophytes (liverwort *Cladopodiella fluitans* and mosses *Sphagnum magellanicum* and *S. cuspidatum*) were collected in peatlands in the Czech Republic and Poland (Table S1; species nomenclature follows Kučera et al. 2012). At least three bryophyte patches per locality were sampled and kept in a growth chamber (20 °C, 12 h day with PPFD of about 200 µmol $m^{-2}s^{-1}$) for a maximum of three months being watered by artificial rainwater alternated with distilled water (Vicherová et al. 2015).

Cultivation experiment and analysis of intracellular [Ca^{2+}]

To lower the intracellular [Ca^{2+}] potentially accumulated in the field, bryophyte shoots were immersed in a mixture of nitric, phosphoric and sulphuric acid (14 µL of 21.6 %, 10 µL of 8.5 % and 20 µL of 9.8 % in 1 L) at pH 3.9 for 16 h. The acid solution was changed several times at the beginning of the acid treatment to stabilize the pH at 4.0–4.5. The acid treatment was followed by a repetitive washing in weak N–P–K solution (1.08 g L^{-1} K_2HPO_4 and 0.29 g L^{-1} NH_4NO_3) for 36 h.

The bryophytes were then grown for five days in stagnant solutions of either: (1) distilled water enriched by mineral nutrients (K – 1.4 mg L^{-1}, Ca – 0.8 mg L^{-1}, Mg – 0.4 mg L^{-1}, Fe – 0.1 mg L^{-1}, N – 0.05 mg L^{-1}, P – 0.1 mg L^{-1}, Cl – 0.3 mg L^{-1}, Mn – 5.4 µg L^{-1}, S – 1.3 mg L^{-1}, Na – 1 mg L^{-1}, I – 1 µg L^{-1}, Zn – 1 µg L^{-1}, Cu – 0.7 µg L^{-1}, pH 6.8), or (2) solutions of 2.4 mM $Ca(HCO_3)_2$, pH 6.8, enriched by mineral nutrients (artificial groundwater of alkaline fens with slightly lowered pH). Both solutions were prepared in three treatments: (A) without any additional chemicals, (B) with Ca^{2+} efflux inhibitor (0.1 mM sodium orthovanadate, non-toxic under low [Ca^{2+}], Vicherová et al. 2015) and (C) with Ca^{2+} influx inhibitor (25 µM verapamil, inhibitor of voltage-dependent/independent calcium channels, Awasthi and Yadav 2007, Gómez et al. 2015). Consequently, we had six solution types in total. Before the cultivation, the bryophytes cultivated in solutions of calcium bicarbonate with inhibitor (orthovanadate or verapamil) were pre-treated in

distilled water with 0.1 mM sodium orthovanadate or 25 µM verapamil for 1 h. Sodium orthovanadate (Na_3VO_4, 0.1 mM, pH 7.0; Sigma-Aldrich) was prepared from an activated (depolymerized) 100 mM stock solution stored at –20 °C and the solution of calcium bicarbonate was prepared by dissolving suspended $CaCO_3$ by CO_2 (Vicherová et al. 2015).

During the cultivation, bryophytes were grown for 33 h or 5 days submerged in 20 L of stagnant solution in air-tight HDPE transparent containers (1 container per a solution type) in dark to limit potential negative effect of orthovanadate or verapamil on photosynthesis. The pH of the bicarbonate solutions was adjusted by bubbling with CO_2 every 12 hours. We used submerged cultivation because of the direct and well-controlled effect of water chemistry on moss physiology. Large volume of the solution and small weight of mosses (about 1 g of dry mass per 10 L of solution) allowed precise pH regulation and secured stable conditions during the whole experiment.

Before the cultivation (and orthovanadate/verapamil pre-treatment), and after the cultivation, some of the bryophyte material was eluted by 30 mM $NiCl_2$ (pH 7.0) for 3×30 min (while shaking) to extract the extracellular (cell wall-bound) exchangeable cations. We used about 2 L of solution per 1 g of shoot dry mass which ensured an excess amount of Ni^{2+} over the cation exchange capacity of the cell walls (Vicherová et al. 2015). The elution process did not cause leakage of intracellular Ca^{2+} (Vicherová et al. 2015). As neither of the species grew in calcareous fens with precipitating calcium carbonate, the eluted shoots are free of extracellular Ca (Vicherová et al. 2015). Eluted shoots were acid-digested in HNO_3 (following Vicherová et al. 2015) and analysed by FAAS. The rest of bryophyte material was carefully blotted by cellulose filter paper (following Vicherová et al. 2015), deep frozen in liquid nitrogen, stored at –80 °C and analysed on HPLC for glutathione content.

LC-MS/MS analysis of reduced L-glutathione (GSH) and oxidized L-glutathione (GSSG)

Approximately 100 mg of a frozen sample was weighted to the mortar precooled to –15 °C, 100 µL of internal standard (isotope-labelled GSH; 100 mg L^{-1}) was added, few millilitres of liquid nitrogen were added and the sample was left for 5 s to become fragile and ground with a pestle. The dry powder was homogenized with 900 µL of extraction solution (5 % meta-

phosphoric acid, 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate in 0.1 % formic acid) supplemented with 1 % (w/v) polyvinyl-polypyrrolidone just before use. The homogenate was transferred into 1.5-mL Eppendorf tube and centrifugated at 20 000 × *g* for 15 min at 6 °C. The supernatant was transferred into 1.5-mL glass vial and 10× diluted with the mobile phase A (100 µL of the supernatant and 900 µL of the mobile phase A). The vial was immediately placed in LC/MS autosampler precooled to 6 °C and analysed on the same day in triplicates.

Liquid chromatography was performed by a Thermo Scientific Dionex Ultimate 3000 Quaternary Analytical system equipped with a heated electrospray HESI II probe and mass detector Velos Pro. Twenty-five microliters of a sample maintained at 6 ± 1 °C in the autosampler were injected into the LC-MS/MS system and chromatographic separation was achieved at a flow rate 0.5 mL min^{-1} using a gradient of mobile phase A and B as follows. (I) 100 % mobile phase A, 0–5 min; (II) linear gradient increase to 90 % B, 5–9 min; (III) hold at 90 % B, 9–13 min and (IV) equilibration at starting conditions (100 % A), 13–24 min. Mass spectrometry analysis was completed in ESI negative mode using the following tune parameters: capillary voltage = –4 kV, desolvation temperature = 350 °C, sheath gas flow rate = 60 arb., auxiliary gas flow rate = 20 arb., transfer capillary temperature = 350 °C, S-lens RF level = 60 %, Front lens = 7.5 V, Ion time = 100 ms, number of microscans = 2. The data acquisition was carried out in target full scan MS/MS analysis (m/z 85–350 for GSH and IS and m/z 165–650 for GSSG) selecting the precursor ions at m/z 306.1 $[M–H]^-$ for GSH, at m/z 309.1 $[M–H]^-$ for IS and at m/z 611.2 $[M–H]^-$ for GSSG. Product ions at m/z 272.1 for GSH, at m/z 275.1 for IS and at m/z 482.1 for GSSG were used for quantitative analysis. Product ions at m/z 288.1 for GSH, at m/z 291.1 for IS and at m/z 338.1 for GSSG were used for confirmation purposes.

Linearity of both calibration curves (range 10–1000 µg L^{-1}; seven concentration levels) shown in Fig. S2 was assessed using correlation coefficient (R) and quality coefficient (QC): GSH (R = 0.9999, QC = 1.34 %) and GSSG (R = 0.9999, QC = 1.86). Satisfactory detector response of the lowest calibration solution (10 µg L^{-1}) is demonstrated in Fig. S3. Accuracy expressed as percent recovery and precision expressed as relative standard deviation were assessed using measurements of five replicates of real sample spiked at 5 mg L^{-1} of each analyte: GSH (accuracy 98.4 %, precision 1.7 %) and GSSG (accuracy 104.8 %, precision 3.2 %). Limit of detection (LOD)

and limit of quantitation (LOQ) were estimated based on signal-to-noise ratio at S/N = 3 and S/N = 10, respectively: GSH (LOD = 2.1 ng g^{-1}, LOQ = 7.1 ng g^{-1}) and GSSG (LOD = 5.0 ng g^{-1}, LOQ = 17 ng g^{-1}). One-day stability expressed as percentage deviation from initial amount of analytes was assessed in calibration solutions at three concentration levels and in one real moos sample: 10 µg L^{-1} (GSH +1.6 %, GSSG –6.1 %), 100 µg L^{-1} (GSH +0.1 %, GSSG –10.3 %), 1000 µg L^{-1} (GSH +3.7 %, GSSG –2.4 %) and real moss sample (GSH +1.5 %, GSSG –2.6 %). Matrix effect of GSH and GSSG was 92 % and 102 %, respectively.

Membrane potential measurement

Before the membrane potential measurement, an individual bryophyte shoot was glued by a tape to a Petri dish filled with 8 mL of bathing solution (0.1 mM KCl, 0.1 mM $CaCl_2$, 60 mM sorbitol, 2 mM MES, pH 7.0). The shoot was fully submersed in the solution for 10 h and then put for 1 h under the illumination of the measuring chamber (60 µmol m^{-2} s^{-1}).

The membrane potential was measured by a standard microelectrode technique described previously (Trebacz et al. 1996). Micropipettes were made from borosilicate glass capillaries containing an internal filament (Hilgenberg, Germany) using a vertical pipette puller (P-30 Shutter Instruments, USA). The micropipettes were filled with 3 M KCI directly before use and connected with micromanipulator (DC-3K, Märzhäuser Wetzlar, Germany), whose precise regulation enabled penetration of micropipette through cell wall to cytoplasm. The reference electrode was an Ag/AgCl wire surrounded by a jacket filled with 3 M KCI which was submerged in bathing solution. The electric potential difference between the reference electrode and the microelectrode was recorded by a high-input resistance amplifier (FD 223, World Precision Instruments, USA). Data acquisition was made by a Lab-Trax-4 analogue-to-digital converter running under LabScribe3 software (World Precision Instruments, USA).

The cell wall and plasma membrane penetration was followed by a measurement of resting potential after which 6 mL of bathing solution in Petri dish was carefully exchanged for $CaCl_2$ solution (20 mM $CaCl_2$, 2 mM MES, 0.1 mM KCl, pH 7.0; pH and osmolarity remaining unchanged). The tip of the microelectrode remained in cytosol during the solution exchange. The

changes in membrane potential between cytosol and cell wall created by the addition of $CaCl_2$ were measured until a changed level of resting potential was reached. To separate an effect of Ca^{2+} from Cl^-, the $CaCl_2$ was substituted for Ca-gluconate in some bryophyte specimens. We also verified that the manual process of solution changes had no effect on the resting potential level. In addition, cation channels or plasma membrane/vacuolar membrane ATPases were inhibited by an addition of either 3 mM $GdCl_3$, 1 μM Erythrosin B, 0.1 mM vanadate or 50 μM verapamil. The chemicals were added to bathing solution 5 h, 14 h, 16 h and 2 h (respectively) before the start of the measurement and were also present in bathing and $CaCl_2$ solution during the measurement. The number of replicates per specimens and solution is given in table S2.

Statistical analyses

Difference in intracellular concentration of calcium ions $[Ca^{2+}]^{int}$ between calcicole and calcifuge species cultivated for 33 h or 5 days in Ca^{2+}-rich/poor solutions (factor "treatment") was analyzed by nested-design ANOVA (random factor "species" nested in factor "calcicole/calcifuge" crossed with factor "treatment"). The difference in Ca^{2+} accumulation between different treatments was than evaluated by one-way ANOVA followed by Fisher LSD, separately for calcicoles and calcifuges. Difference in intracellular [GSH] and [GSSG] between calcicoles and calcifuges cultivated for 5 days in Ca^{2+}-rich/poor solutions (factor "treatment") was analyzed by nested-design ANOVA (random factor "species" nested in factor "calcicole/calcifuge" crossed with factor "treatment") followed by Tuckey HSD to test the difference between calcicoles and calcifuges for each solution separately. The software package Statistica ver. 8 (Statsoft, USA) was used for all data analyses.

Results

Intracellular Ca^{2+} accumulation

Calcifuge and calcicole species differed in cytosolic Ca^{2+} accumulation when grown submerged in artificial solution of alkaline fens ($F_{4,3} = 11,7$; $p < 0.001$; Fig. 1). While calcifuge species rapidly accumulated Ca^{2+} in cytosol (intracellular $[Ca^{2+}]$ almost doubled within 33 hours of cultivation), calcicole species had stable intracellular $[Ca^{2+}]$ in all treatments.

Membrane potential changes in reaction to $[Ca^{2+}]$

Sudden exposure of living cells to high extracellular $[CaCl_2]$ evoked few seconds long hyperpolarization continued by minutes long depolarization, probably connected with flow of Ca^{2+} to the cell (Fig. 2). The response did not differ between calcifuge and calcicole species and was observed also when $CaCl_2$ was exchanged for Ca-gluconate (although, in contrast to $CaCl_2$, Ca-gluconate failed to evoke the response in about 30 % of measurements). Inhibition of cation channels by Gd^{3+} decreased the extent of depolarization in all species; however, there was clear difference between calcicoles and calcifuges. While the depolarization was almost entirely blocked in calcicole species (4 % of former value in *H. vernicosus*, 8 % in *C. cuspidata*), the calcifuge species only lowered the extend of depolarization to 30–70 % (Fig. 2). Similar results were observed after an inhibition of plasma membrane ATPases (and potentially also tonoplast ATPases) by erythrosin B (Fig. 2). Surprisingly, in contrast to Gd^{3+} and erythrosin B, orthovanadate (Ca-ATPase inhibitor) and verapamil (inhibitor of cation channels) had no effect on the measured response of membrane potential to high extracellular $[Ca^{2+}]$.

Effect of Ca^{2+} on cytosolic glutathione accumulation

Calcifuge and calcicole species differed in cytosolic concentration of reduced (GSH) and oxidized (GSSG) form of glutathione after being cultivated submerged in artificial solution of alkaline fens ($F_{1,4} = 33$, $p < 0.001$ for GSH, $F_{1,4} = 44$, $p < 0.001$ for GSSG). While almost all calcifuge species markedly increased intracellular concentration of GSH and GSSG, calcicole species had permanently high [GSH] in all treatments and cytosolic concentration of

GSSG decreased in Ca^{2+}-rich solution (Fig. 3). The increased production of GSH and GSSG in calcifuges was substantial in species from wet bog hollows (*Sphagnum cuspidatum*, *Cladopodiella fluitans*; the concentrations were >2× greater than after the incubation in Ca^{2+}-poor solution) while in the bog-hummock species *Sphagnum magellanicum* the increase was not significant ($p = 0.17$).

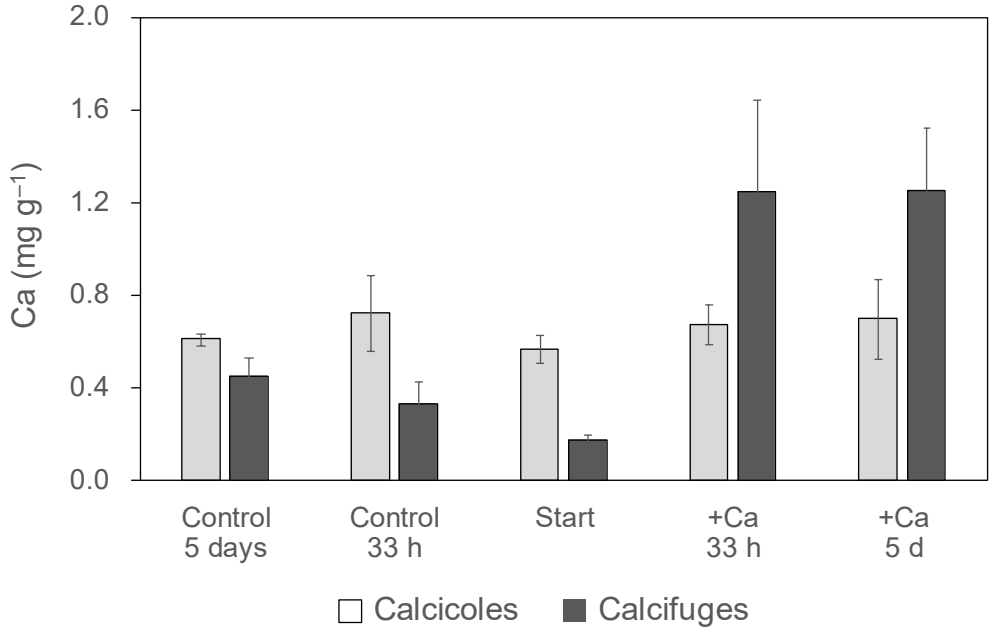

Fig. 1. Intracellular Ca^{2+} concentration (mg g^{-1} of dry mass) of calcicole and calcifuge species (gray and black columns, respectively) grown for 33 hours or 5 days in solution of distilled water enriched by nutrients (*Control*) or solution of 2.4 mM $Ca(HCO_3)_2$, pH 6.8, enriched by nutrients (*+Ca*). The columns *Start* depict [Ca^{2+}] before the *+Ca* incubation after acid treatment (pH 3.9, 16 h). Fisher LSD indicates statistical difference between different treatments in calcifuge species. Means ± s.e. are shown.

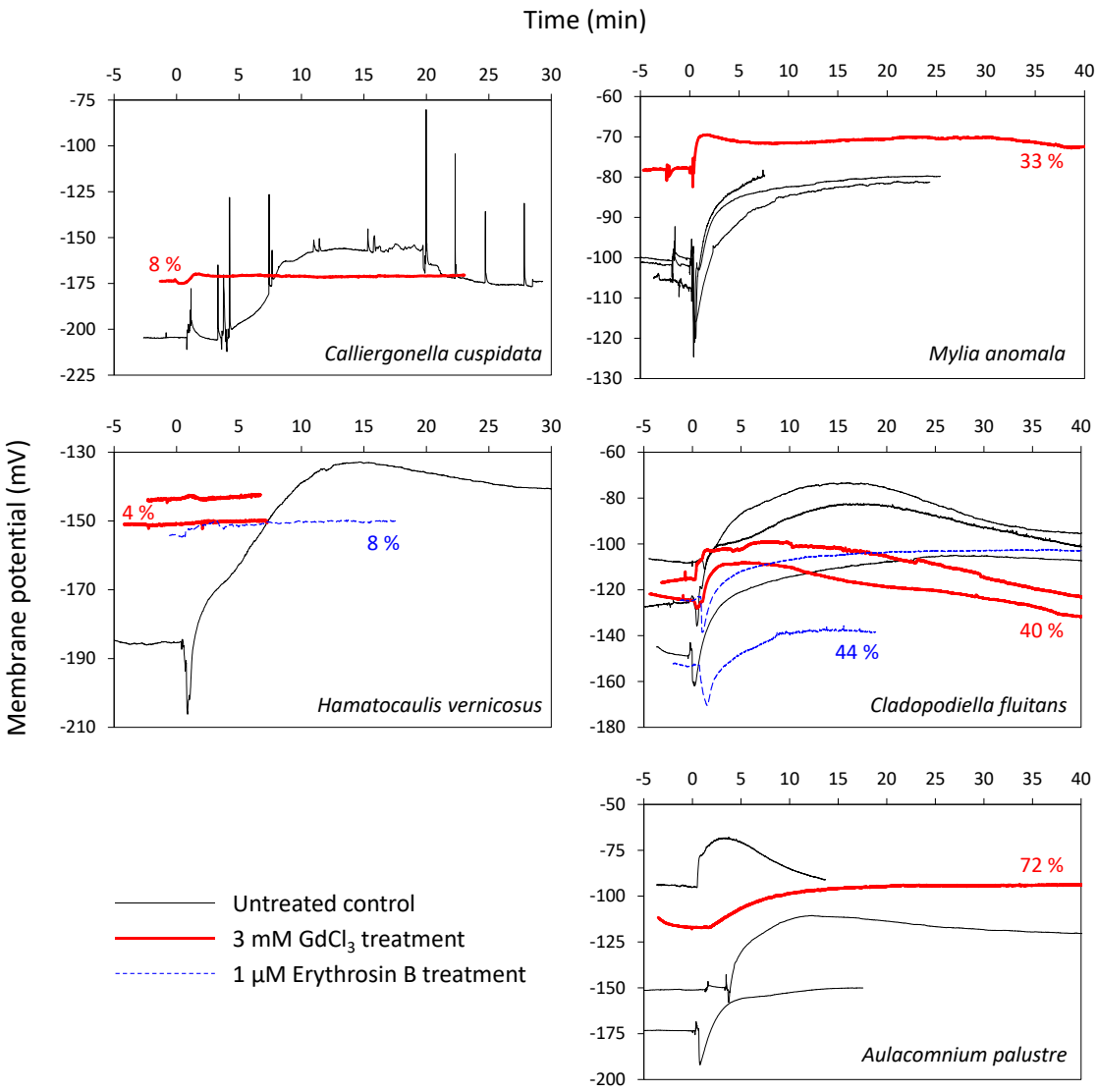

Fig. 2. Membrane potential (mV) changes in reaction to an addition of $CaCl_2$ in presence/absence (thin black / thick red line) of cation channels inhibitor (Gd^{3+}) or ATPase inhibitor (Erythrosin B; blue dotted line). After the initial measurement of resting potential, bathing solution (0.1 mM KCl, 0.1 mM $CaCl_2$, 60 mM sorbitol, 2 mM MES, pH 7.0) was exchanged for $CaCl_2$ solution (20 mM $CaCl_2$, 2 mM MES, 0.1 mM KCl, pH 7.0; pH and osmolarity remaining unchanged). The *x*-axis shows time (min) before (negative values) and after solution exchange (at time = 0).

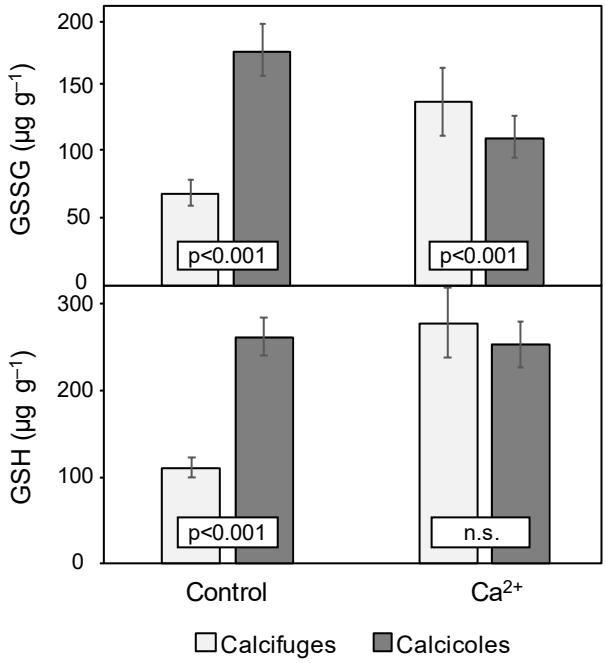

Fig. 3. Intracellular concentration of reduced (GSH) or oxidized (GSSG) glutathione (µg $g^{-1}$of dry mass) after 5 days of cultivation in solution of distilled water enriched by nutrients (*Control*) or solution of 2.4 mM $Ca(HCO_3)_2$, pH 6.8, enriched by nutrients (Ca^{2+}). The *p*-values denote significant statistical difference (<0.001) between calcifuge (grey columns) and calcicole (black columns) bryophytes. Means ± s.e. are shown.

Discussion

Our results confirm the hypothesis that calcicole and calcifuge species differ in composition and/or regulation of plasma membrane cation channels responsible for Ca^{2+} uptake to the cytosol. Presumably, the difference indicates that calcicoles evolved a specific adaptation that controls Ca^{2+} influx/efflux and enables the plants to maintain stable cytosolic [Ca^{2+}] even in alkaline, Ca^{2+}-rich fen groundwater, as observed in our experiment. In contrast, calcifuge species increase cytosolic [Ca^{2+}] in the alkaline fen water, which is toxic for those species (Clymo 1973, Vicherová et al. 2015). The excessive Ca^{2+} uptake coincide with increase in GSH and GSSG concentration that could be possibly raised to quench reactive oxygen species (ROS) produced during oxidative stress (Jozefczak et al. 2012).

How the calcicole and calcifuge species differ in composition (or regulation) of membrane complexes responsible for Ca^{2+} transport has never been studied, even though a significance of Ca^{2+} influx/efflux mechanism for species survival in Ca^{2+}-rich environment is well known (Hirschi 2001, Guttery at al. 2013). Our study indicates that while calcicole species use almost exclusively Gd^{3+}-sensitive cation channels to transport Ca^{2+} to cytosol in calcareous environment of high pH, the calcifuge species use both Gd^{3+}-sensitive and Gd^{3+}-insensitive channels. Since the Gd^{3+} is a non-specific inhibitor of various types of cation channels (mechanosensitive, voltage-dependent, voltage-independent; Demidchik 2002, Marshall et al. 1994, Miedema et al. 2008, Ding and Pickard 1993), it is not possible to specify what type of channels transports Ca^{2+} in calcicoles in contrast to calcifuges.

Contrary to the reaction to Gd^{3+} inhibitor, changes in membrane potential evoked by high extracellular [$CaCl_2$] did not differ between calcicoles and calcifuges; the short hyperpolarization followed by long depolarization seems to be universal, well-regulated reaction to high extracellular [Ca^{2+}] shared between bryophytes. Similar results were observed under exposure of green algae (Characeae) to high extracellular [$CaCl_2$] (Lunevsky et al. 1983). Because of the complexity of cellular metabolism and ion fluxes through membranes, our results cannot give reliable information about the individual ion fluxes. However, since the changes in membrane potential were evoked also by Ca-gluconate (although with lower intensity and probability), we assume the extracellular Ca^{2+} might trigger the hyperpolarization through increasing cytosolic [Ca^{2+}] (as observed in Characeae, Lunevsky et al. 1983),

which regulates opening of various anion channels in cell membranes (including Cl^-; Lunevsky et al. 1983, Fromm and Lautner 2007). By entering cytosol, Ca^{2+} could be directly involved also in membrane depolarization (as presumed by Qi and Spalding 2006); alternatively, the depolarization would be caused by K^+ fluxes.

Dissimilarity of Ca^{2+} influx/efflux between calcicole and calcifuge species could be implied also from a different rate of Ca^{2+} accumulation in cytosol. While calcicole species maintained stable intracellular [Ca^{2+}] in Ca^{2+}-rich solutions of high pH, calcifuges rapidly accumulated Ca^{2+} in cytosol. The rapid cytosolic Ca^{2+} accumulation occurs only under high pH in bryophytes (Vicherová et al. 2015), which is the direct cause of the toxicity of calcareous environment for calcifuge species (Clymo 1973). Moreover, rapid intracellular Ca^{2+} accumulation correlated with reduced photosynthesis rate due to a damage of photosystem II in calcifuge sphagna (Vicherová et al. 2015). Consequently, the regulation of intracellular Ca^{2+} homeostasis (via Ca^{2+} influx/efflux mechanisms) seems to be a crucial adaptation of calcicoles for survival in calcareous environment.

The pH dependence of cytosolic Ca^{2+} accumulation in bryophytes, together with the rapid decrease in cytosolic [Ca^{2+}] in acidic environment (Vicherová et al. 2015), suggests the involvement of Ca^{2+} efflux in maintaining the cytosolic [Ca^{2+}]. Ca^{2+}/H^+ antiporters were, indeed, found crucial for survival in calcareous environment (Hirschi 2001, Kamiya et al. 2006 – vascular plants; Guttery at al. 2013 – apicomplexan parasites); their partial elimination led to a direct dependence of survival on external [Ca^{2+}]. The calcium-tolerant *H. vernicosus* was much more sensitive to membrane ATPase inhibitor Erythrosin B than calcifuge hepatics *Cladopodiella fluitans*, indicating that calcifuge and calcicole species might differ in composition/regulation of Ca^{2+}/H^+ antiporters and ATPases involved in Ca^{2+} efflux. However, we would need to survey more species with more replicates to support this hypothesis.

Apart from Ca^{2+} accumulation, calcifuge species increased cytosolic glutathione concentration when grown in the calcareous solutions. In vascular plants, increased intracellular glutathione production is generally associated with protection against heavy metal toxicity (glutathione being phytochelatin precursor) and oxidative stress (Jozefczak et al. 2012). However, high external [Ca^{2+}] also leads to raised cytosolic glutathione concentration (López-Climent et al. 2014), the feature we observed in calcifuge bryophytes.

High cytosolic [Ca^{2+}] is directly linked with ROS production in eukaryotic cells as high [Ca^{2+}] increases (i) production of ROS (while ROS modulate Ca^{2+} signaling and its cytosolic concentration; Görlach et al. 2015) and (ii) glutathione content in cytosol (Price 1990, Tasduq et al. 2008). Therefore, we can presume the increased [GSH] in calcifuges grown in calcareous solutions is a direct reaction to oxidative stress. The reaction to oxidative stress is also indicated by a raised concentration of oxidized glutathione (GSSG) that arises from reaction of GSH with ROS.

In contrast to calcifuge species, calcicoles had constitutively high cytosolic [GSH] with lower [GSSG], indicating the glutathione is required for survival of calcifuge species in calcareous environment; however, only small amount is used for GSH reduction during oxidative stress. Glutathione is a metal chelator involved in maintaing metal homeostasis (Jozefczak et al. 2012). It exceeds the cytosolic [Ca^{2+}] about $10^6\times$ and can be thus involved in maintaining cytosolic [Ca^{2+}]; this hypothesis can be addressed by future studies of calcium tolerance.

Conclusion

Our results provide new insights into the mechanisms of calcium toxicity. They show that calcicole species have adapted to calcareous environment by a specific regulation of Ca^{2+} influx/efflux. Calcifuges exposed to alkaline Ca^{2+}-rich environment in are not able to maintain cytosolic homeostasis of Ca^{2+} due to composition and/or regulation of (i) cation channels that can transport Ca^{2+} and perhaps also (ii) ATPases and Ca/H antiporters involved in Ca^{2+} efflux. Moreover, they have permanently increased [GSH] that probably maintain low concentration of ROS in cytosol and possibly influence the regulation of calcium homeostasis.

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Author Contributions

EV: designed and conducted the experiments, measured membrane potential and Ca^{2+} accumulation, analysed data, KT: supervised membrane potential measurement, DK, PF: designed methodology for GSH/GSSG measurements and conducted the measurements, TH: designed and supervised the experiments. All authors contributed to writing of the manuscript and gave final approval for publication.

Supplementary material

Additional supporting information may be found in the online version of this article:

Fig. S1. Intracellular concentration of reduced (GSH) or oxidized (GSSG) glutathione in individual bryophytes after 5 days of cultivation.

Fig. S2 Calibration curves for GSH and GSSG.

Fig. S3. Detector response of the lowest calibration solution of GSH and GSSG.

Table S1: Detailed description of source localities for the bryophytes used in the experiments.

Table S2: Number of replicates used for a measurement of membrane potential.

Competing interests

The authors declare no competing interests.

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Supplementary material

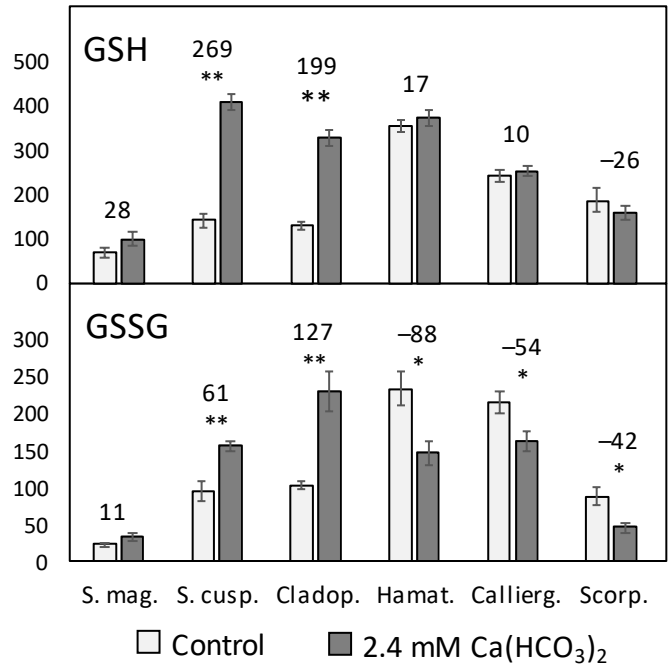

Fig. S1. Intracellular concentration of reduced (GSH) or oxidized (GSSG) glutathione (μg g^{-1} of dry mass) after 5 days of cultivation in solution of distilled water enriched by nutrients (*Control*) or solution of *2.4 mM* $Ca(HCO_3)_2$, pH 6.8, enriched by nutrients in calcifuge species *Sphagnum magellanicum* (S. mag.), *S. cuspidatum* (S. cusp.), *Cladopodiella fluitans* (Cladop.) and calcicole species *Hamatocaulis vernicosus* (Hamat.), *Calliergonella cuspidata* (Callierg.), *Scorpidium scorpioides* (Scorp). The asterisks denote significant statistical difference (<0.001, double asterisk; <0.05 single asterisk) between calcifuge (grey columns) and calcicole (black columns) bryophytes. The numbers indicate difference in glutathione concentration between solution of distilled water and $Ca(HCO_3)_2$ solution. Means ± s.e. are shown.

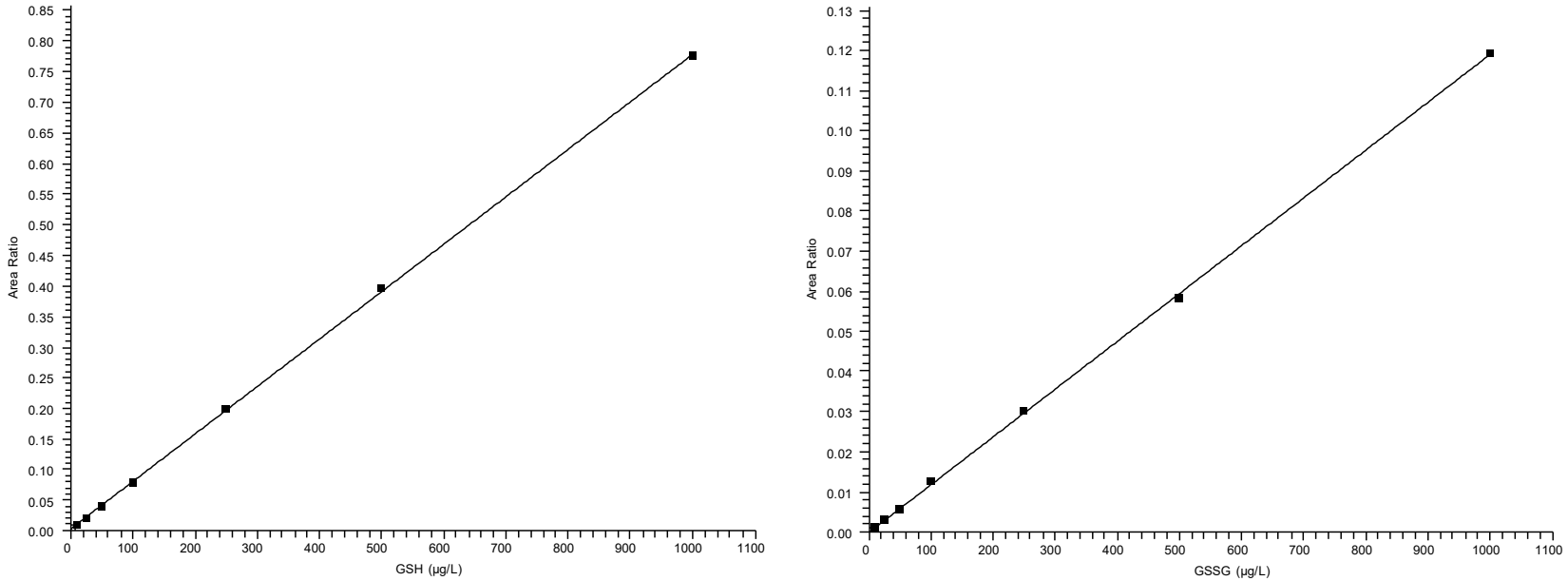

Fig. S2. Calibration curves of GSH (left) and GSSG (right) constructed by the internal standard method.

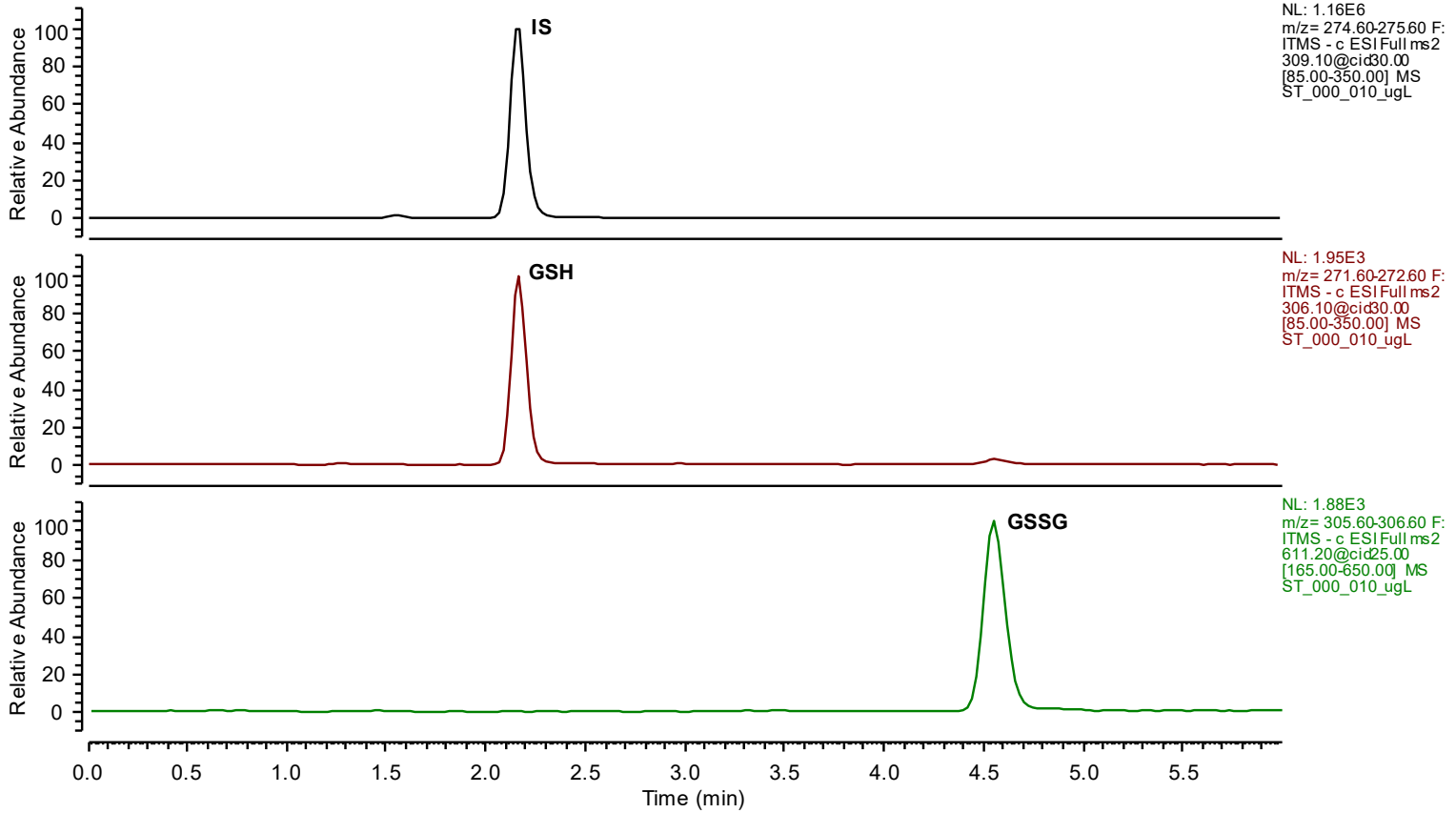

Fig. S3. Detector response at the lowest calibration point (10 µg L^{-1}) – extracted ion chromatograms at chosen m/z values of product ions of target analytes (GSH and GSSG) and internal standard (IS) providing the greatest method sensitivity.

Table S1: Detailed description of source localities for the bryophytes used in the experiments.

Locality name	Mire type	Localization	GPS coordinates	Altitude (m a.s.l.)	pH	$[Ca^{2+}]$ (mg L^{-1}) / κ (µS cm^{-1})	Sampled species
Poleski Park Narodowy	rich fen	Lublin region, Poland	51°22'23"N 23°16'02"E	170			*Scorpidium scorpioides*
Poleski Park Narodowy	rich fen	Lublin region, Poland	51°20'58"N 23°19'38"E	180			*Scorpidium scorpioides*
Staw Kosciuszko	bog	Lublin region, Poland	51°31'27"N 23°1'12"E	170			*Cladopodiella fluitans*
NPR Kladské rašeliny - Tajga	bog	Karlovy Vary region, Czech Republic	50°1'50"N, 12°41'11"E	800			*Sphagnum cuspidatum, Sphagnum magellanicum Mylia anomala, Aulacomnium palustre*
NPR Rolavská vrchoviště	bog	Karlovy Vary region, Czech Republic	50°24'20"N, 12°35'17"E	940			*Sphagnum cuspidatum, Sphagnum magellanicum Mylia anomala, Aulacomnium palustre*
PR Řeka	rich fen	Vysočina region, Czech Republic	49°39'59"N 15°51'11"E	550	7.2	50/—	*Hamatocaulis vernicosus*
PR Petrovka	moderately rich fen meadow	Plzeň region, Czech Republic	49°47'6"N, 13°22'19"E	350			*Calliergonella cuspidata*
NPR Brouskův mlýn	moderately rich fen meadow	České Budějovice region, Czech Republic	48°52'59"N 14°40'58"E	450	6.5	—/159	*Hamatocaulis vernicosus, Calliergonella cuspidata*

Table S2: Number of replicates used for a measurement of membrane potential in calcicole (*Hamatocaulis vernicosus*, *Calliergonella cuspidata*) and calcifuge (*Cladopodiella fluitans*, *Aulacomnium palustre*, *Mylia anomala*) bryophytes. The changes in membrane potential between cytosol and cell wall were created by an addition of $CaCl_2$ or Ca-gluconate. Cation channels or plasma membrane/vacuolar membrane ATPases were inhibited in some measurements by an addition of either 3 mM $GdCl_3$, 1 µM Erythrosin B, 0.1 mM orthovanadate or 50 µM verapamil. The chemicals were added to bathing solution 5 h, 14 h, 16 h and 2 h (respectively) before the start of the measurement and were also present in bathing and $CaCl_2$ solution during the measurement.

Treatment	**Calcicole species**		**Calcifugee species**		
	Hamatocaulis vernicosus	*Calliergonella cuspidata*	*Cladopodiella fluitans*	*Aulacomnium palustre*	*Mylia anomala*
No inhibitor, $CaCl_2$	1	1	3	3	3
No inhibitor, Ca-gluconate			3		
3 mM Gd^{3+}, $CaCl_2$	1	1	2	1	1
1 µM Erythrosin B, $CaCl_2$	1		2		
50 µM verapamil, $CaCl_2$	1	1	2		
0.1 mM orthovanadate, $CaCl_2$	2		4		

Chapter 5.
Bryophytes can recognize their neighbours through volatile organic compounds

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Vicherová E., Glinwood R., Hájek T., Šmilauer P. Ninkovic V. (in. prep). Bryophytes can recognize their neighbours through volatile organic compounds (accepted to Scientific Reports, IF = 4.1)

Abstract:

- Communication between vascular plants through volatile organic compounds (VOCs) impacts on ecosystem functioning. However, nothing is known about that between non-vascular plants.
- To investigate plant–plant VOCs interaction in bryophytes we exposed rare peatland moss *Hamatocaulis vernicosus* to VOCs of its common competitor *Sphagnum flexuosum* in an air-flow system of connected containers under artificial light, supplemented or unsupplemented by far-red (FR) light.
- When exposed to VOCs of *S. flexuosum*, shoots of *H. vernicosus* elongated and emitted six times higher amounts of a compound chemically related to β-cyclocitral, which is employed in stress signalling and allelopathy in vascular plants. The VOCs emission was affected similarly by FR light addition, possibly simulating competition stress.
- This is the first evidence of plant–plant VOCs interaction in non-vascular plants, analogous to that in vascular plants. The findings open new possibilities for understanding the language and evolution of communication in land plants.

Key words: plant–plant interaction, volatile organic compounds, plant communication, plant competition, *Hamatocaulis vernicosus*, bryophytes

Introduction

Interactions are crucial for the survival of individuals in ecological communities[1]. Consequently, animals and plants perceive a variety of cues by which they can ascertain what is in the proximity. Until the end of the twentieth century, however, the active sharing of information seemed solely the domain of animals. Plants were viewed as passive, stationary organisms, with only basic interactions with other organisms[2], apart from pollinators. With the discovery of plant communication[3,4], it became evident that plants use light[5], touch[6,7,8,9], vibrations[10] and chemicals[11,12,13] to communicate in an intricate web of multitrophic interactions that affect functioning of ecosystems.

Volatile organic compounds (VOCs) are involved in communication in eukaryotic and prokaryotic organisms including animals and vascular plants[14], bacteria[15], brown algae[16], and fungi [17]. These secondary metabolites with low molecular weight and high vapour pressure at ambient temperature can move freely through the air. They are produced in cytosol (organelles or cytoplasm) and are possibly transported outside the cell through lipophilic carriers (in aqueous environments of cytosol and cell wall) and ABC transporters (through lipophilic plasma membrane; [18, 19]). The production of VOCs by plants depends on genetic identity of the individual, life history and health, plant organ, photoperiod, light quality (e.g., red to far-red (R/FR) ratio), symbiotic organisms and other factors[1,20,21,22,23]. Hence, each organism has a specific VOC blend including compounds unique for the given taxon[24] as well as chemicals with specific ecological meaning (e.g. [25]). Species that can detect and decipher the encoded information can use VOCs in interactions, as a source of information.

Plant–plant VOC interaction often takes the form of eavesdropping. Plants can estimate the strength of their neighbouring competitors and, accordingly, adjust their growth[26]. Parasitic plants can use VOCs to locate their hosts[24]. VOCs could be even used as indicators of unfavourable environmental conditions[15,27] that eavesdroppers survive better by inducing tolerance or resistance to the stress. Yet VOC production in plant–plant interactions may be beneficial for the emitter itself, e.g., when it serves as a quick information transfer between different plant parts, particularly in plants that are unable to transmit that information through vascular tissue (e.g. desert and semi-desert plants[28]). Similarly, VOCs can be used as cues of impending danger, where

the danger is averted more easily when plants employ inter- or intraspecific interactions (e.g. reducing plant attractiveness for herbivores and limiting their population development[29], and by attracting predators of herbivores[30]).

Our knowledge about plant communication has been gathered almost solely from angiosperms, particularly crop species[14], and information about other plant groups is limited or lacking. We know that gymnosperms can communicate through volatiles[31], however, we know nothing about phylogenetically more basal groups of vascular plants (such as ferns) and nonvascular plants (green algae, bryophytes).

To our knowledge, plant–plant volatile interactions has never been studied in bryophytes. There are indications that mosses might use VOCs in interactions in similar ways as vascular plants do; in animal-mediated pollination and seed dispersal, mosses can use odours to facilitate spore and spermatozoid dispersal. Some of the coprophilous mosses (family *Splachnaceae*) are entomophilous, i.e. they use brightly coloured, scented sporophytes to attract flies that disperse their spores to suitable substrate[32]. Similarly, fertile female shoots of at least some moss genera produce odours more attractive to microarthropods than the rest of the population, facilitating spermatozoid dispersal[33].

The basic interaction with insects and microarthropods suggests bryophytes might be able to communicate through VOCs on a sophisticated level. Hence, we hypothesize that, similarly to angiosperms, bryophytes can use VOCs to evaluate the competitive strength of their neighbours and adjust accordingly their shoot growth to avoid competitive exclusion. Competition among bryophytes for light and other resources is tightly linked with their poikilohydry. To maintain hydration, bryophytes often grow in a dense layer (cushions, mats) where light penetrates only one or two centimetres below the surface and the competition is manifested more like a *competition for space*[34]. If an individual grows more slowly than its neighbours, it becomes shaded into darkness; when it overgrows its neighbours, it becomes limited by desiccation. Similar to vascular plants, bryophytes detect spectral changes of light after passing through vegetation[62] that absorbs photosynthetically active light but transmits FR light. However, this mechanism alone cannot distinguish between shading by vascular plants or by overgrowing shoots of a competitor in the bryophyte layer. Thus, individuals with the ability to

recognize the identity of the overshadowing neighbour could have an evolutionary advantage.

If our hypothesis is valid, we may conclude that the capacity to use volatile cues as information in neighbour detection, as we know it from angiosperms, may be, in at least some form, shared by all land plants. We used a pair of competitor moss species from fens, bryophyte-dominated minerotrophic peatlands, to test the following hypotheses:

- *Hamatocaulis vernicosus* (Mitt.) Hedenäs (a rare moss species protected by European law, Natura 2000) will increase its growth in length when exposed to VOCs from its natural competitor *Sphagnum flexuosum* Dozy & Molk. to avoid being out-competed.
- Volatiles released by S. *flexuosum* will change the VOC production of *H. vernicosus*, possibly as a cue for surrounding *H. vernicosus* individuals. Such a response has been observed in vascular plants[23].
- Light quality (increased proportion of far-red light imitating shade by vegetation) will affect VOC production in both species and increase their growth in length, as seen in vascular plants[22].

Materials and Methods

Moss material

Bryophyte plant-plant VOCs interactions were studied in a laboratory experiment using artificial poor fen solutions and an air-flow system. We selected two fen moss species – *Hamatocaulis vernicosus* (Mitt.) Hedenäs (rare, Natura 2000 protected species with an optimum in rich fens) and *Sphagnum flexuosum* Dozy & Molk. (strong competitor dominating poor fens). The species naturally coexist in (moderately) rich fens (terminology follows)[73]; *H. vernicosus* grows in hollows and low hummocks, *S. flexuosum* occupies low and high hummocks. If the pH and [Ca^{2+}] are lowered in the moss carpet, *S. flexuosum* can outcompete *H. vernicosus* and slowly switch the moderately rich fens to poor fens[74,75]. *H. vernicosus* was used as responder, *S. flexuosum* as inducer. Each species was collected from two fens in South or West Bohemia, Czech Republic (detailed description in Table S1; *H. vernicosus* is locally common in sampled localities and the *H. vernicosus* collection did not endanger local populations).

Cultivation experiment

H. vernicosus and *S. flexuosum* were cultivated in an air-flow system of connected transparent containers placed in a growth chamber. Containers for inducers (*S. flexuosum*/empty plate = control) were made from 22-L polyethylene boxes (36.5×25.5×26.5 cm, Ikea), and containers for responders (*H. vernicosus*) from 600 mL polypropylene bottles with cut upper parts (11.5×3.5× 15.0 cm, Tissue Culture Flask, Sarstedt). The containers were sealed by transparent polyethylene film secured by paraffin film. Adhesive properties of the film together with slight negative pressure in the container (created by air flow) prevented unwanted air escape to the growth chamber. Each inducer container was connected by transparent polyethylene tubes with four responder containers, creating an individual *container unit* (Fig. 1).

Containers were filled with artificial poor-fen solution (K – 0.8 mg L^{-1}, Ca – 0,8 mg L^{-1}, Mg – 0,5 mg L^{-1}, N – 1.4 mg L^{-1}, P – 0,5 mg L^{-1}, Cl – 1,4 mg L^{-1}, Mn – 5.4 µg L^{-1}, B – 5.3 µg L^{-1}, S – 1.4 mg L^{-1}, Na – 1 mg L^{-1}, I – 1 µg L^{-1}, Zn – 1 µg L^{-1}, Br – 0.9 µg L^{-1}, Co – 0.8 µg L^{-1}, Cu – 0.7 µg L^{-1}), replaced every 9 days. Each responder/ inducer container contained 400 mL/17 L of the solution. Lower walls of the containers were darkened to suppress algal

growth. Shoots of *H. vernicosus* and *S. flexuosum* were arranged in their natural density into holes made in thin plates of expanded polystyrene floating above the solution. The arrangement ensured sufficient water supply to shoot's apical parts so moisture would not be growth-limiting. *H. vernicosus* carpet had an oval shape and was composed of 45 apical shoot fragments (16 mm long) growing in 15 holes 0.5 cm apart (three fragments per each hole, Fig. 1). The bed of *S. flexuosum* was rectangular (20×15 cm) and composed of approximately 20 mm long apical fragments (about 10 mg on dry mass basis; one or two shoots per hole, holes 0.8 cm apart).

Air flow was created by a pump producing unidirectional flow of approximately 0.1 L min^{-1}. The air inlet of inducer containers was at the level of moss shoots. Air was drawn through the bed of *S. flexuosum* (or a control chamber with solution and empty plate) and via connecting tubes to the responder chamber through the *H. vernicosus* stand. The air from the responder chambers was then vented from the room. Consequently, *H. vernicosus* individuals were exposed to VOCs emitted by shoots of either surrounding *H. vernicosus* (inducer chamber without *S. flexuosum*) or to both, inducer and surrounding *H. vernicosus*.

The growth chamber was illuminated by fluorescent lamps with 14:10 h light:dark. Temperature in the room was 23 ± 1 °C and 25 ± 1 °C in the containers around the mosses . The intensity of photosynthetically active radiation at the moss cover was approximately 120 μmol m^{-2} s^{-1}. In addition to artificial day light, some of the container units (FR+ treatment) were supplemented by far-red (FR) light of 730 nm (one 10-W SMD LED module per container unit) that resulted in R/FR ratio of 0.23.

The growth chamber was equipped with two models of fluorescent tubes of slightly different light spectra: Osram L 36W/865 Lumilux Cool Daylight (colour temperature 6500 K) and Osram FQ 80W/840 HO Constant Lumilux Cool White (4000 K), Germany; the light colour 865 having about two times higher blue light emission than 840 (Fig S1, S2). Using tubes of different colour temperatures was originally not intended but the experimental design required entire capacity of the growth chamber where the two types of illumination were constructed independently. However, this arrangement allowed us to test the side effect of blue light on plant elongation and volatiles emission. Tubes of both colours provide light of high R/FR ratio. Although fluorescent tubes emit light of partly discrete spectral lines, tubes of both

colours have been successfully used in small-scale cultivation for decades (now being replaced by LED-based light sources with continuous light spectra). The placement of the lamps and container units in the chamber was designed to minimise spatial differences in light quality.

Four container units (two with inducer, two controls) were placed in artificial daylight (FR–; *S. flexuosum* unit and control unit under each lamp type), two *container units* (one with *S. flexuosum*, one control) were placed under Osram FQ 80W/840 with added FR light (L1FR+ treatment) and three container units (two with *S. flexuosum*, one control) under Osram L 36W/865 with added FR light (L2FR+ treatment; Fig. S1). Each container unit encompassed 16 *H. vernicosus* triplets (i.e. replicates) used for statistical analysis of fragments growth in length, biomass production and branching, and a *S. flexuosum* carpet (divided to two parts, i.e. 2 replicates) used for statistical analysis of fragments growth in length and biomass production. Bryophytes were cultivated under the described conditions for 30 days, except for period of *H. vernicosus* VOCs collection (21–23 and 28–30 day of cultivation).

VOCs collection

VOCs emitted from *H. vernicosus* and *S. flexuosum* were sampled by dynamic headspace collection (air entrainment). Prior to the entrainment, sampling containers were cleaned with detergent (TEEPOL, 1% w/w) and rinsed with acetone and distilled water. Glass tubes (5 mm diameter) containing the adsorbent Porapak Q (50 mg, mesh 50/80, Supelco, Bellefonte, PA, USA) were cleaned with redistilled dichloromethane and baked overnight at 140 °C under nitrogen flow. Charcoal filters (SGE Analytical Science, Victoria, Australia) were baked overnight at 180 °C under nitrogen flow. PET (polyethylene terephthalate) oven bags (Toppits, Klippan, Sweden) were baked for 2 hours at 140 °C, sampling containers and Teflon connecting tubes were baked overnight at 180 °C.

VOCs sampling was conducted under controlled environment conditions (21 °C, 14/10 h of artificial light/dark). Sampling containers for *S. flexuosum* were made from modified 450 mL glass beakers, sealed by a Petri-dish. Each container contained half a *S. flexuosum* carpet from a container unit (described above). Sampling containers for *H. vernicosus* were made from Duran laboratory glass bottles sealed with material cut from the PET oven bags, each containing all four *H. vernicosus* plates from a container unit

(described above). To avoid desiccation, a small volume of nutrient solution was added to the mosses each day during the VOCs collection.

Charcoal-filtered air was pumped into each container at 400 mL min^{-1} and VOCs-enriched air was drawn out through the Porapak tubes at 300 mL min^{-1} (Fig. 1). The difference in flow rates created a slight positive pressure, minimizing entry of unfiltered air. Volatiles were collected over a period of 72 h. Volatiles from *H. vernicosus* were collected on days 21–23 and 28–30 of cultivation, and volatiles from *S. flexuosum* were collected 1–3 and 5–7 days after the end of the cultivation. *S. flexuosum* carpets remained in the collecting chambers between samplings. *S. flexuosum* shoots from the FR light treatment were exposed to FR light during and 24 h prior to the second VOC collection, while the first collection was conducted without FR light supplement.

VOCs analysis

VOCs were eluted from Porapak tubes with 750 µL redistilled dichloromethane. An internal standard (1-nonene at 20 ng μL^{-1} in the sample) was added and the sample was concentrated to 50 µL under nitrogen flow.

Compounds were identified using coupled gas chromatography/mass spectrometry (GC/MS) as previously described[76]. A 1µL aliquot of each sample was injected onto a HP-1 column (30 m, 0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific, Santa Clara, CA, USA) housed in a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 5975C mass spectrometer. Ionization was by electron impact at 70 eV. The oven temperature was held at 30 °C for 1 min, then programmed at 5 °C min^{-1} to 150 °C, then at 10 °C min^{-1} to 250 °C. The carrier gas was helium with a flow rate of 1 mL min^{-1}. Identifications were made by comparison of spectra with a commercial database (NIST 2008) and by comparing mass spectra and retention times with those of authentic standards where available. Most compounds emitted by both species did not generate a satisfactory match in the commercial database making identification unfeasible; these are designated as ‘unknown compound’. Some compounds generated strong matches in the database but authentic standards were not available; these are designated as speculative identifications. Full mass

spectral data along with retention indices (Kovats) for all compounds quantified are provided in Supplementary Table S3, S4.

Compounds were quantified using gas chromatography (GC). A 1-µL aliquot of each sample was injected onto a HP-1 column (dimensions as for GC/MS) housed in a 6890 GC (Agilent Technologies). The temperature program was as for GC/MS and the carrier gas was hydrogen. Compounds were quantified using the internal standard. The entrained moss material was oven dried (60 °C, 24 h) after the final VOCs collection and VOCs amounts were expressed in relation to moss dry mass (ng g^{-1}).

Moss growth measurement

The effect of FR light and *S. flexuosum* volatiles on the growth of *H. vernicosus* was evaluated as weight and length increments and number of new branches in four triplets of *H. vernicosus* fragments that grew in the middle of *H. vernicosus* floating mat (Fig. 1). The fresh mass (FM) was weighed after careful blotting the fragments between sheets of cellulose filter paper and was transformed to dry mass (DM) by the formula: FM = 3.38 × DM following[75]. The growth response of *S. flexuosum* to FR light was evaluated as shoot length increment.

Statistical analysis

The effect of VOCs and FR light on growth and branching of *H. vernicosus* and the effect of FR light on *S. flexuosum* growth in length and biomass production was evaluated by linear mixed-effect models (LMM, package nlme,[77] in the R statistical language (version 3.4.0; 2017-04-21). Experimental design of *container units* was reflected in the model specification (responder's container nested in inducer's container, both factors were used as random factors). Since the growth of *H. vernicosus* was not affected by light treatments, the effect of *S. flexuosum* VOCs on growth of *H. vernicosus* (length, weight) was evaluated across the two light treatments (FR+, FR−), reducing the problem with a low number of replicates induced by the design complexity.
The effect of *S. flexuosum/H. vernicosus* VOCs production and the effect of *S. flexuosum* VOCs on *H. vernicosus* VOCs production was evaluated by one-

way analysis of variance (ANOVA) in a program Statistica (ver. 8). The evaluation of VOCs production was done individually for each VOCs compound. The two *H. vernicosus* VOCs samplings were pooled together, as well as the two FR treatments of different artificial daylight quality (L1FR+, L2FR+). The data generally met the assumptions of residuals normality and of homoscedasticity for running parametric tests.

Results

Sphagnum flexuosum volatiles affect growth of *Hamatocaulis vernicosus*

H. vernicosus changed its growth pattern when exposed to VOCs produced by *S. flexuosum*. While the overall biomass production remained unchanged (Fig. S3), the shoots increased growth in length but only when the light treatments were pooled together ($F_{1,5}$=8.8, p=0.031), about 0.3 cm and 0.5–0.7 cm in 30 days under normal and supplemented far-red light (FR– and FR+; Fig. 2). The increased growth in length was not significantly compensated by lower shoot branching under FR– ($F_{1,2}$=1.51, p=0.34, Fig. S4) or FR+ ($F_{1,3}$=0.06, p=0.82, Fig. S4). In contrast, FR light induced creation of short branches ($F_{1,7}$=7.2, p=0.031, Fig. S5). Surprisingly, the FR+ did not induce greater growth in length of *H. vernicosus* shoots ($F_{2,5}$=2.6, p=0.17, Fig. S6); however, it induced higher growth in length of *S. flexuosum* shoots ($F_{1,4}$=21.0, p=0.01; Fig. 3) without changing overall biomass production ($F_{1,4}$=4.0, p=0.12, Fig. S7).

S. *flexuosum* volatiles affect *H. vernicosus* VOCs emission

In addition to growth changes, VOCs emitted by *S. flexuosum* induced changes in VOCs composition of *H. vernicosus*. Specifically, *S. flexuosum* VOCs induced six times higher emission of methyl 2,6,6-trimethyl-1-cyclohexene-1-carboxylate (MTCC) under FR– ($F_{1,4}$=10.3, p=0.032, Fig. 4), the production of the other 23 detected compounds remained unchanged (Table S2). The changes were not observed under FR+, probably because the FR light itself increased this compound 12 times (Fig. 4, control). The total amount of VOCs released by *H. vernicosus* was not affected by VOCs from *S. flexuosum*.

FR light changes *H. vernicosus* and *S. flexuosum* VOCs emission

FR light did not induce production of new volatile compounds nor change the total amount of VOCs produced. However, it significantly increased production of specific VOCs in both species. *S. flexuosum* emitted higher amounts of nine VOCs (β-cyclocitral, $F_{1,10}$=95.6, p<0.0001; MTCC, $F_{1,8}$=67.8, p<0.0001; unknown compounds 29, 30, 23, 31, 33 p=0.02–0.004) when grown continuously under FR light (Table 1, S3). Emission of most of

these compounds remained high even after the FR light had been switched off (Table 1). In addition, switching off the FR light increased emission of two other compounds, unknown compounds 27 and 35. *H. vernicosus* reacted to FR+ by an increased production of β-cyclocitral and MTCC ($F_{1,16}$=5.7, p=0.03 and $F_{1,16}$=12.1, p=0.003 respectively, Fig. 4), compounds that had increased emission in *S. flexuosum* under the same conditions.

VOCs blend of *S. flexuosum* and *H. vernicosus*

In total, we detected 29 VOCs produced by *S. flexuosum* and 24 VOCs produced by *H. vernicosus* (Table S2, S3, S4). Four compounds (β-cyclocitral, MTCC, α-copaene and unknown compound 4 (likely a sesquiterpene) were emitted by both species. Except for MTCC, which was produced in similar quantities by both species, the production of individual volatiles was 6–10 times higher in *H. vernicosus* than in *S. flexuosum*. Similarly, the total VOCs production of *H. vernicosus* was approximately four times higher than that of *S. flexuosum*.

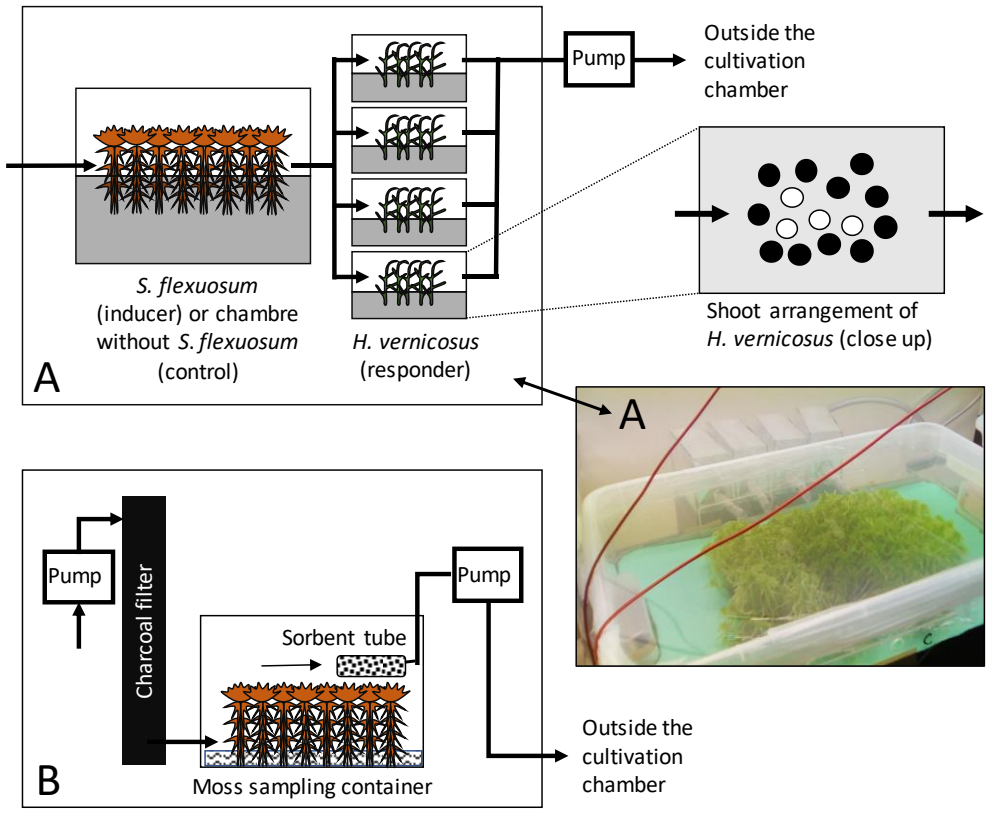

Fig. 1. Experimental setup of A) One of the *Cultivation units* inside the cultivation chamber (Fig. S1) and B) VOCs sampling design.
A) Cultivation unit: The air was drawn through the inducer (*Sphagnum flexuosum*) or control (without *S. flexuosum* chamber to the four responder (*Hamatocaulis vernicosus*) chambers and pumped out of the cultivation chamber. *H. vernicosus* grew on floating mat bearing 15 holes, each accommodating three shoots of *H. vernicosus*. The four white circles in *H. vernicosus* plate indicate shoot triplets used for growth measurements.
B) VOCs sampling: The air was pumped through a charcoal filter over the moss carpet. Air enriched by VOCs was drawn through an adsorbent (Porapack tube) and then vented outside the cultivation chamber.

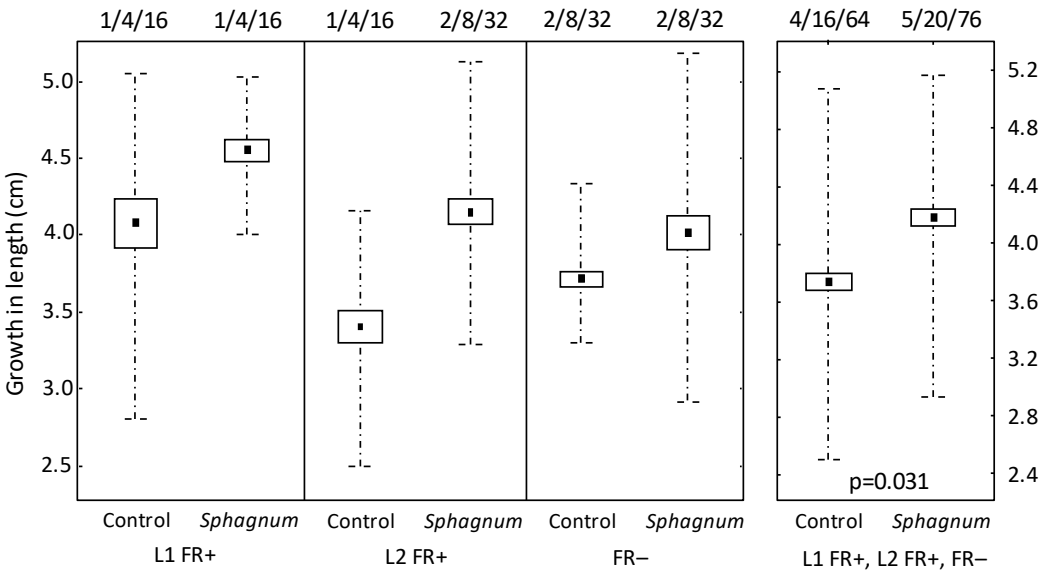

Fig. 2. The length increment of *H. vernicosus* shoots grown under artificial light without FR light addition (FR−) and added FR light (L1 FR+, L2 FR+) in cultivation units (Fig. 1) for 30 days (L2 FR+ had more blue light than L1 FR+, see methods for details). The shoots were exposed to VOCs produced by surrounding *H. vernicosus* individuals and to VOCs from *S. flexuosum* chamber (*Sphagnum*) or chamber without *S. flexuosum* (*Control*). The box and whiskers depict ± s.e. and minimum/maximum values, the numbers above depict number of inducer chamber/responder chamber/ *H. vernicosus* replicates. The *H. vernicosu*s growth increment increased significantly when the shoots were exposed to *S. flexuosum* VOCs ($F_{1,5}$=8.8, p=0.031, tested across all light treatments; the experimental design and number of replicates did not allow to test the light treatments individually).

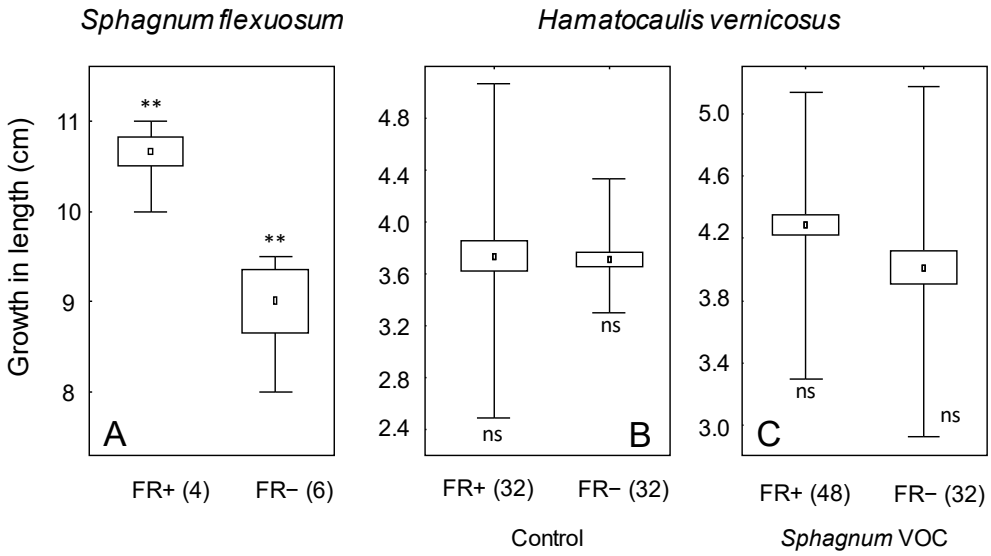

Fig. 3. Growth in length of *S. flexuosum* (A) and *Hamatocaulis vernicosus* (B – control shoots, C – shoots exposed to *Sphagnum* VOCs) cultivated in growing chamber in cultivation units (Fig. 1) under artificial light without FR light addition (FR−) and added FR light (FR+) for 30 days. The box and whiskers depict ± s.e. and minimum/maximum values, the numbers beside light treatments depict number of replicates. Significant differences between treatments (** P=0.01; ANOVA test).

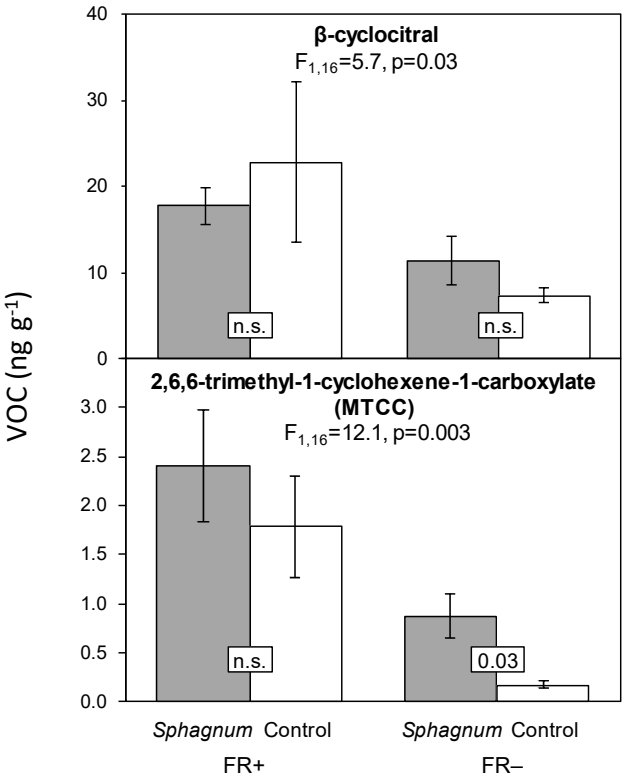

Fig. 4: Quantity of volatile organic compounds (ng g^{-1}) whose production by *Hamatocaulis vernicosus* carpets was influenced by FR light addition (MTCC, B-cyclocitral) or exposure to *Sphagnum* VOC (MTCC under FR–). *H. vernicosus* shoots in the carpets were exposed only to VOCs released from neighbouring *H. vernicosus* individuals (control) or to VOCs released by surrounding *H. vernicosus* individuals and to VOC blend from *S. flexuosum* carpet (*Sphagnum* exposure), for more details see methods, Fig. S1 and Table S2.. Both species were cultivated under artificial light conditions without FR light addition (FR−) or added FR light (FR+). VOCs were collected for 72 h. The error bars depict ± s.e. of means.

Table 1: Quantity of significantly FR light-dependent volatile organic compounds (ng g^{-1}) produced by *Sphagnum flexuosum* cultivated under artificial light conditions without FR light addition (FR−) and supplemented FR light (FR+). The volatiles were sampled under artificial light conditions without added FR light (Standard sampling) or FR light was added to shoots exposed to FR light during cultivation experiment (FR light sampling). (One-way ANOVA performed separately for each compound and sampling treatment, * $p < 0.01$, ** $p < 0.05$.) VOCs were collected for 72 h. For more detail see methods, Fig. S1 and Table S3

	Standard sampling		**FR light sampling**	
Sphagnum flexuosum	**FR+**	**FR−**	**FR+**	**FR−**
methyl 2,6,6-trimethyl-1-cyclohexene-1-carboxylate	2.85 (±0.25)	0.27 (±0.70) **	2.69 (±0.65)	0.31 (±0.08) **
β-cyclocitral	5.55 (±0.40)	0.98 (±0.38) **	4.52 (±0.90)	0.80 (±0.18) **
unknown 23	2.65 (±0.48)	0.90 (±0.52) *	15.40 (±4.96)	2.96 (±0.99) *
unknown 27 (possible sesquiterpene)			0.91 (±0.12)	0.11 (±0.03) **
unknown 28 (possible sesquiterpene)	0.47 (±0.06)	0.08 (±0.02) **	1.58 (±0.38)	0.18 (±0.08) **
unknown 29 (possible sesquiterpene)	1.07 (±0.15)	0.19 (±0.06) **	2.73 (±0.70)	0.17 (±0.04) **
unknown 30	1.68 (±0.21)	0.33 (±0.12) **	5.45 (±1.34)	0.52 (±0.14) **
unknown 31	8.17 (±1.48)	2.97 (±1.07) **	4.81 (±0.83)	1.72 (±0.46) **
unknown 33			5.45 (±1.40)	0.51 (±0.14) **
unknown 35	3.89 (±0.44)	2.58 (±0.83) *		

Discussion

The results show that a non-vascular plant, the moss species *Hamatocaulis vernicosus* can detect VOCs from their neighbour. These volatile cues could potentially be used to evaluate the competitive strength of the neighbour. The air-borne volatiles may serve as growth rate cues for nearby bryophyte eavesdroppers that use the information in regulating their own growth. This type of plant–plant interaction observed in bryophytes resembles responses discovered in vascular plants[22,26] and suggests that plant–plant volatile interaction is developed in the whole Embryophyta division.

H. vernicosus changes growth and VOCs emission in response to volatiles produced by *S. flexuosum*

The accelerated growth in length of *H. vernicosus* in response to *S. flexuosum* VOCs closely resembled a shade-avoidance syndrome that plants, including bryophytes, use as a survival strategy against overshadowing neighbours[35]. The physiological mechanism of shade avoidance has been traditionally connected with a plant's ability to perceive changes in spectra and intensity of the radiation reflected by foliage of adjacent plants through photoreceptors (i.e. low R/FR ratio, lower amount of blue light). We have demonstrated that shade avoidance is also connected with VOCs detection, at least in bryophytes, where the survival of individual shoots is strictly dependent on keeping the growing apex in the upper illuminated part of the bryophyte canopy. While vascular plants react to VOCs from neighbouring competitors with changes in growth strategy[22,26], increased growth in length has not been reported; thus, the role of VOCs perception in the shade avoidance syndrome of vascular plants is unclear.

Apart from growth changes, *H. vernicosus* reacted to *S. flexuosum* VOCs by altering its own VOCs emission, specifically increasing production of a compound tentatively identified as methyl 2,6,6-trimethyl-1-cyclohexene-1-carboxylate (MTCC). We tentatively identified MTCC based on matching in a commercial mass spectral library (NIST 2008), since no authentic standard was available. The tentative compound does however appear to share structural similarity with β-cyclocitral (2,6,6-trimethyl-1-cyclohexene-1-carbaldehyde), which was also released by *H. vernicosus* (and confirmed with an authentic standard). To our knowledge, MTCC has not been previously reported as a plant-produced volatile compound, however emission of β-

cyclocitral by a moss, and compounds with structural similarity to MTCC have been reported[36,37]. Roles for β-cyclocitral in plant stress signalling[38] and allelopathy[39] have been described, and it is conceivable that the structurally related MTCC has similar activity.

The alteration of VOCs blend in response to volatiles from herbivore or pathogen-damaged[40,41,42] and undamaged neighbours[29,43,44] has been documented in vascular plants. The change can be beneficial for both the emitters and receivers upon engaging in tritrophic interactions. For example, volatiles received from emitters due eavesdropping evoked changes in terpenoid[29,43,44] or alkane[29] production by receivers, making their VOCs blend less attractive for herbivores (or pathogens) and more attractive for herbivore predators, thus protecting the whole plant community. Since bryophytes are known to have a large variety of terpenoid secondary metabolites with repellent (or even toxic) effects on herbivores and pathogens[37], their involvement in VOCs interaction is plausible.

Similar principles of cooperation and warning might be expected in bryophyte communities when dealing with competition. Peatland bryophytes (including *H. vernicosus*) have a clonal growth strategy creating genetically identical clusters in the moss layer[45]. Since bryophytes compete predominantly for space[46,47], species forming mats can withstand competition from a stronger competitor for longer than individual shoots. Consequently, the use of VOCs as stress warning cues between conspecific or even genetically identical neighbours would enhance survival of the micropopulation. As the cue is passed among closely related individuals, transfer of the information is much more efficient than if it would be carried to distant relatives or to different species[21].

Volatile organic compounds responsible for bryophyte interaction

Although plant communication has been studied for more than 30 years, the principles behind a 'language' of plant signalling remain unclear, particularly regarding competitive interactions. Our study, as well as previous studies[22,26] clearly shows that plants adjust their growth in response to VOCs from neighbouring plants. However, it is still unknown in what situations VOCs carry information about an emitter's genetic identity and to what extent a receiver (other than parasitic plants[24]) can evaluate the information.

Alternatively, the VOC blend might represent some general cue about a neighbour's presence or other traits characterizing an emitter's competitive strength.

The identity of chemical compounds (or blends) responsible for information transfer in volatile interaction is also speculative. Runyon (2006)[24] showed that, at least in some situations, the bearers of taxon-specific information in plant–plant signalling are terpenoids. In our study we isolated 29 volatiles produced by *S. flexuosum* that mostly differed from those emitted by *H. vernicosus*. The compounds we were able to tentatively identify were terpene-related. Apart from (+)-cyclosativene they are known to be produced by other mosses or liverworts[48,49,50,51,52]. Consequently, if the VOCs blend carried information about the genetic identity of *S. flexuosum* and the information was encoded by VOCs detected in our study, the key part of the cue could be (+)-cyclosativene, one of the unidentified compounds or a specific combination and/or concentration of the detected chemicals. A large number of terpenoid compounds have been identified from bryophytes, but relatively few from the mosses[37], and little mass spectra data are reported. Further, volatile emission by *Sphagnum* species has not been studied in detail, limiting our ability to identify VOCs specific for the genus *Sphagnum* or even *S. flexuosum*.

A chemical compound considered as a potential cue to indicate future plant competition but not analysed in our study is the plant hormone ethylene. Ethylene, in concentrations physiologically active in vascular plants: (i) had no effect on growth of a moss *Fontinalis squamosa*[53], (ii) reduced growth of a moss *Physcomitrella patens*[54] and (iii) inhibited auxin-evoked seta elongation in a liverwort *Pellia epiphylla*[55]. Therefore, ethylene seems to have negative effect on shoot elongation in bryophytes and is unlikely to be responsible for the observed elongation of *H. vernicosus*. The airflow and the low amount of emitter biomass (less than 35 g of DM) in our study may have prevented the build-up of ethylene to physiologically active concentrations 1 ppb[56] or the even higher concentrations reported to affect experimental plants in previous studies[22,56,57,58].

Bryophytes change VOCs emission in response to light quality

Our mosses did not reduce their total VOCs production when grown under light with a low R/FR ratio, i.e. illumination simulating shading by vegetation. This contrasts with the response of vascular plants to low R/FR ratios[22,59]. However, similar to vascular plants, both mosses changed the composition of their VOCs blends, increasing production of β-cyclocitral and MTCC as well as several unidentified compounds. MTCC concentration was also increased on receiving volatiles of a stronger competitor and may potentially function as a common volatile cue of competition in bryophytes.

Low R/FR light generally evokes shade avoidance syndrome in bryophytes[60,61] and, accordingly, it led to a strong shoot elongation in *S. flexuosum* in our study. In contrast, the growth of *H. vernicosus* was not significantly affected by supplemental FR light. A lack of response to FR light has been previously recorded in bryophytes[62]. Moreover, it is known that different populations of the same taxa can react differently to low R/FR[63]. Thus, it is possible to conclude that the strength of the response to shading differs between species or even populations and might be influenced by light conditions in the current microhabitat[63].

The response of bryophytes to R/FR ratio in our experimental system was affected by blue light, emitted in different quantity from the two types of fluorescent tubes. Besides R/FR-sensitive phytochromes, plants detect canopy shade as blue light attenuation via blue light-sensitive cryptochromes. Although each type of photoreceptor has its own signalling pathway, the final response is a result of their integration[64,65]. Consequently, elevated blue light inhibits elongation evoked by low R/FR ratio in vascular plants[48, 58,66]. This explains why both control and *Sphagnum*-exposed *H. vernicosus* shoots had slightly lower (though not significantly) elongation rates under light sources richer in blue light (L2FR+).

Prospective model of plant–plant interactions by VOCs in bryophyte communities

The *Sphagnum–Hamatocaulis* interaction reported here demonstrates that bryophytes can use VOCs as warning cues in detection of future competition. This may be one of the cues enabling centuries-long species coexistence in

stable bryophyte communities such as in peatlands[68,69]. There, competitive exclusion is thought to be avoided by short-term, often seasonal fluctuations in ecological factors (e.g., water availability and chemistry) that alternately favour individual species[47,70]. Consequently, the ability to detect and interpret VOCs emitted by a stronger competitor may provide the weaker 'eavesdropper' with an ecological advantage, enabling it to match its growth with the stronger neighbour and thus bridge the short time span of unfavourable conditions.

Although there are similarities between plant–plant VOCs interactions in bryophytes and vascular plants, these two fundamental groups of land plants differ principally in their ecological strategies. Lacking well-developed anatomical structures allowing efficient water management (vascular tissues, stomata, cuticle), bryophytes must rely on biochemical adaptations to cope with desiccation and related environmental stresses. Therefore, we postulate that VOCs emitted by desiccated or repeatedly rehydrated bryophyte shoots might be decoded as warning cues providing the receivers with time for biochemical acclimation (hardening), since bryophyte desiccation tolerance is largely an inducible trait[71]. Analogous responses to VOCs emitted upon environmental stress are known in vascular plants[23,27]. Consequently, the ability to eavesdrop on desiccation-mediated VOCs cues would present clear ecological advantages, preventing diebacks during drought[72].

Conclusions

Our results provide the first evidence of VOCs-mediated interspecific plant-plant interaction in bryophytes, a phylogenetically basal group of land plants. Since the interaction closely resembles that in vascular plants (morphological response to VOCs stress cues, changed VOC blend of the responder, similar chemistry of VOCs cues), one might speculate it evolved in a common ancestor of land plants. Future research on VOC-mediated interactions among bryophytes dealing with biotic (competition, pathogenesis) and abiotic (water) stress may shed new light on the functioning of bryophyte communities and bryophyte-dominated ecosystems.

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Author Contributions

EV, TH, RG and VN designed the experiment, EV conducted the experiment, EV and RG made VOCs collection, RG made VOCs analysis, EV and PS analysed the data. All authors contributed to writing of the manuscript and gave final approval for publication.

Additional information

Additional supporting information may be found in the online version of this article.

Competing interests

The authors declare no competing interests.

Data availability statement

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Supplementary materials

Table S1: Detailed description of source localities for the mosses *Sphagnum flexuosum* and *Hamatocaulis vernicosus* used in the experiments.

Locality name	Mire type	Localization	GPS coordinates	Altitude (m a.s.l.)	pH	$[Ca^{2+}]$ (mg L^{-1}) / κ (µS cm^{-1})	Sampled species
Dlouhá louka	moderately rich fen	Plzeň region, Czech Republic	49°54'44"N 13°10'43"E	570	6.3	6.5/—	*Sphagnum flexuosum*
Hrádecká bahna	moderately rich fen	Plzeň region, Czech Republic	49°42'47"N 13°39'31"E	400	7.0	—/225	*Sphagnum flexuosum*
Řeka	rich fen	Vysočina region, Czech Republic	49°39'59"N 15°51'11"E	550	7.2	50/—	*Hamatocaulis vernicosus*
Bouskův mlýn	moderately rich fen meadow	České Budějovice region, Czech Republic	48°52'59"N 14°40'58"E	450	6.5	—/159	*Hamatocaulis vernicosus*

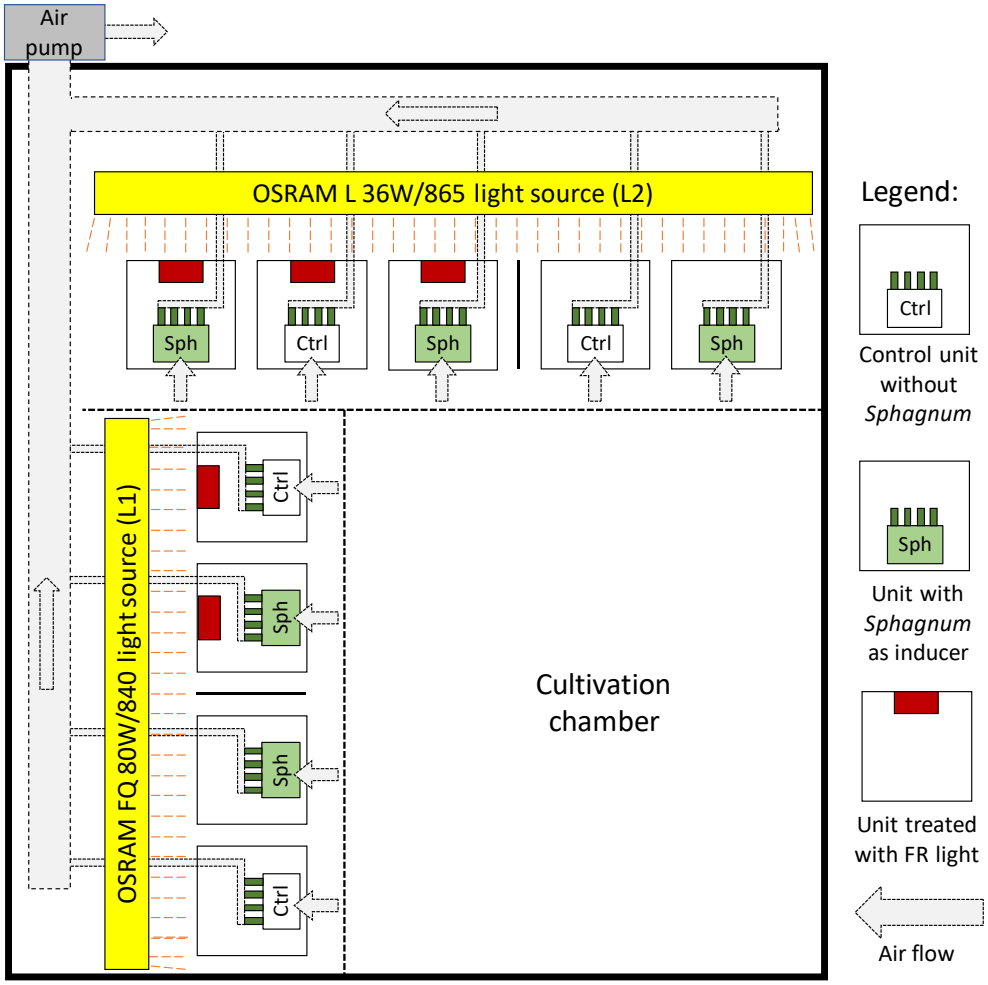

Fig. S1. The setup of *cultivation units* in the cultivation chamber during the experiment (the cultivation unit drawn in Fig. 1). The units were illuminated by two sources of artificial daylight: Osram FQ 80W/840 (four units) and Osram L 36W/865 (five units). The FR light was added to five units; two were under Osram FQ 80W/840 (L1FR+ treatment), three under Osram L 36W/865 (L2FR+ treatment). Units with different light regimes were separated by non-transparent partitions. See Fig. S2 for spectral properties of the two light sources.

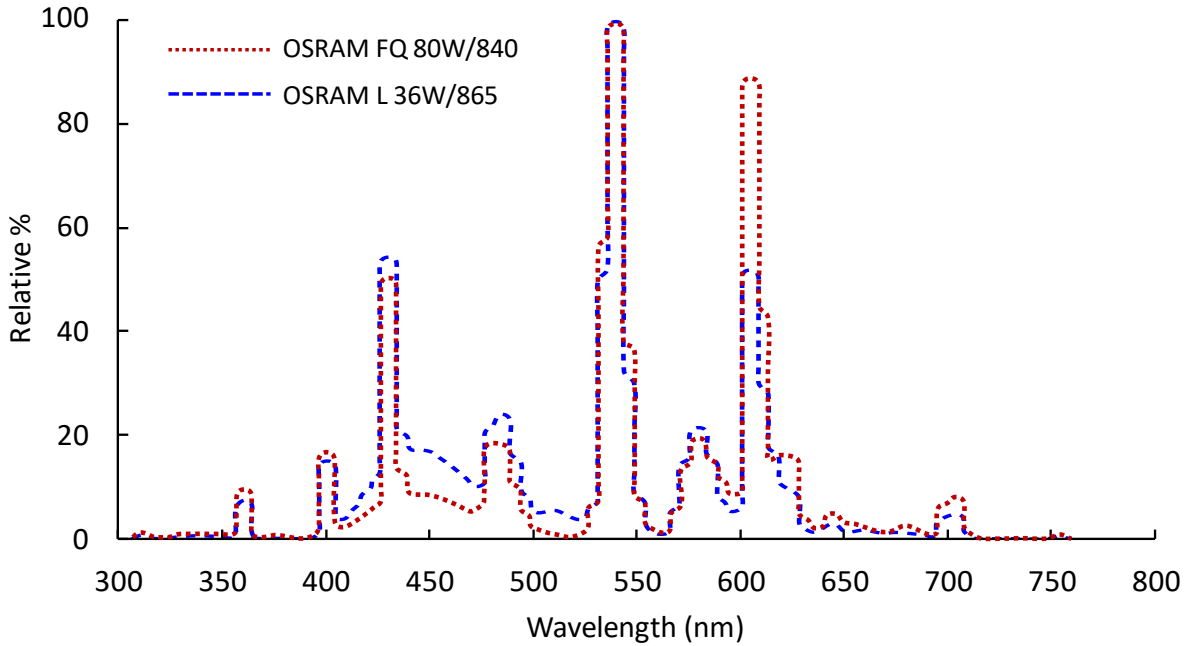

Fig. S2. Spectral power distribution diagram of two fluorescent Osram light sources used in the growth chamber. Redrawn from product datasheets (Osram GmbH, Germany).

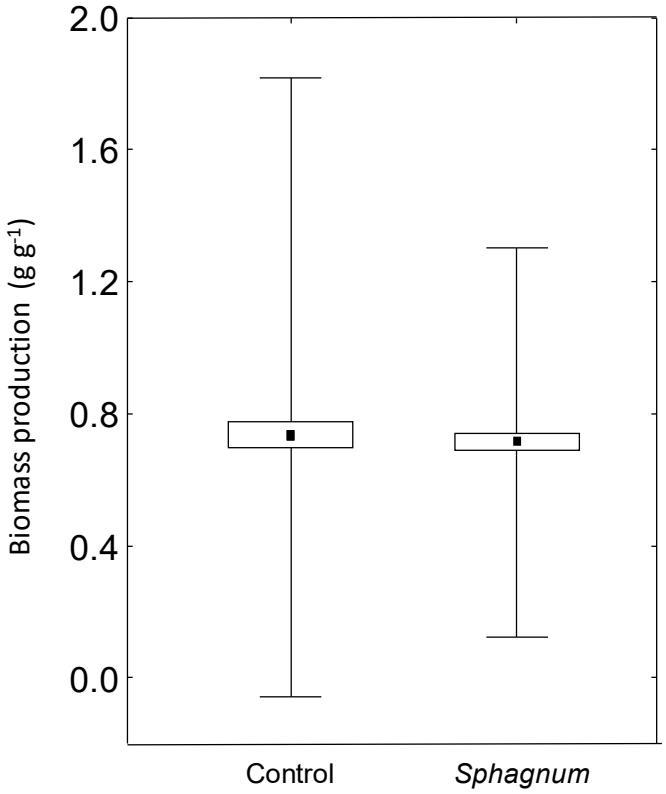

Fig. S3. The biomass production of *H. vernicosus* shoots grown in cultivation units (Fig. 1) for 30 days under different light treatments (light treatments were pooled together for the statistical analysis, see methods for details). The shoots were exposed to VOCs produced by surrounding *H. vernicosus* individuals and to VOCs from *S. flexuosum* chamber (*Sphagnum*) or chamber without *S. flexuosum* (*Control*). The box and whiskers depict ± s.e. and minimum/maximum values. The VOCs treatment has no effect on biomass production of *H. vernicosus* ($F_{1,7} = 6.8$, $p = 0.81$).

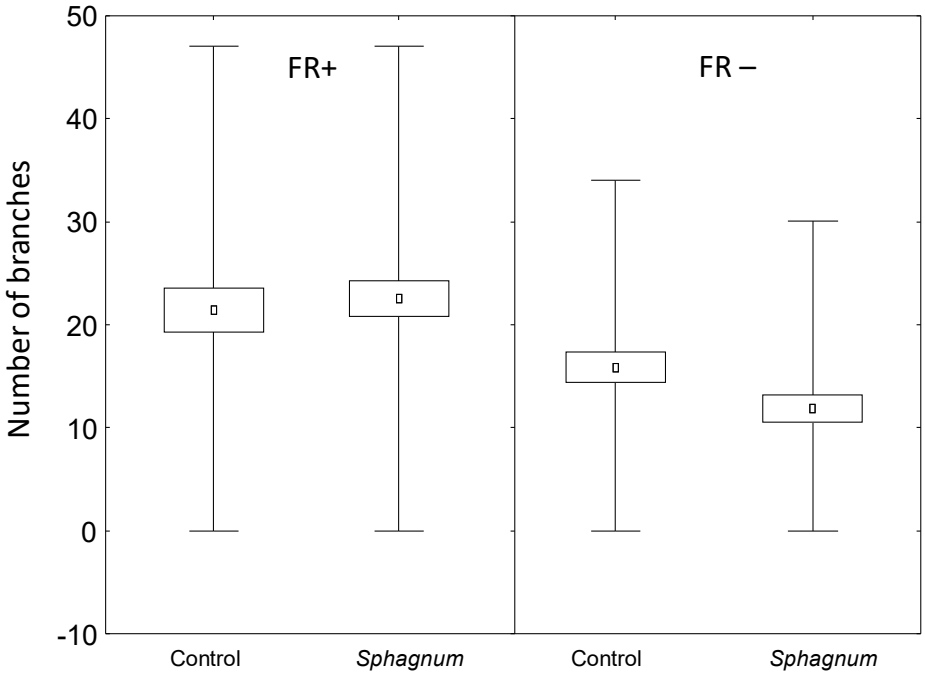

Fig. S4. The number of branches created by *H. vernicosus* shoots grown in cultivation units (Fig. 1) for 30 days under artificial light with and without FR light supplementation (FR+ and FR−). The shoots were exposed to VOCs produced by surrounding *H. vernicosus* individuals and to VOCs from *S. flexuosum* chamber (*Sphagnum*) or chamber without *S. flexuosum* (Control). The *S. flexuosum* VOCs had no effect on number of branches created by *H. vernicosus* under FR− ($F_{1,2}$ = 1.5, p = 0.34) or FR+ ($F_{1,3}$=0.06, p=0.82). The box and whiskers depict ± s.e. and minimum/maximum values.

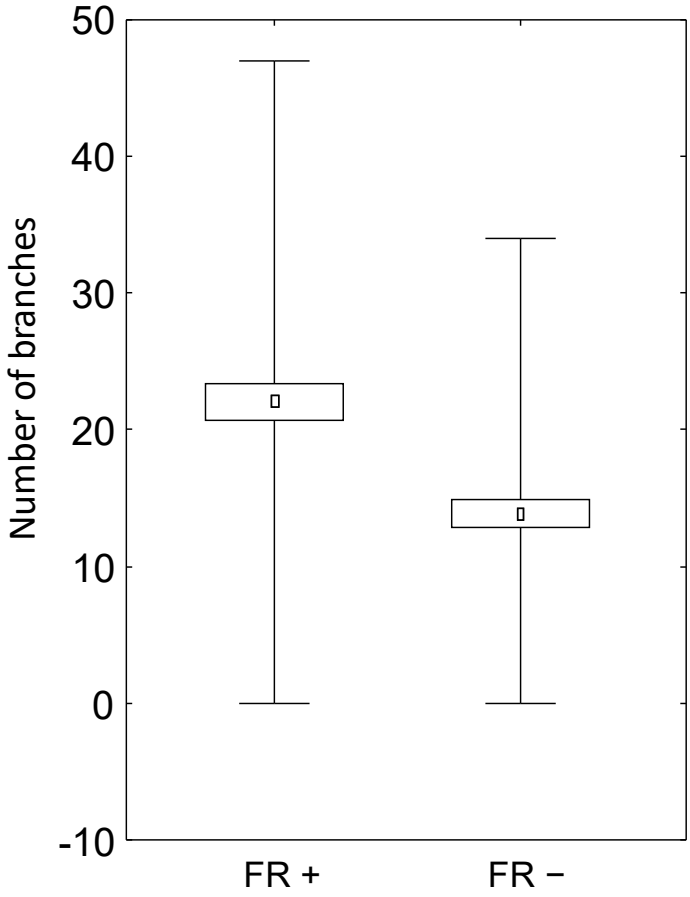

Fig. S5. The number of branches created by *H. vernicosus* shoots grown under artificial light with and without FR light supplementation (FR+ and FR−) in cultivation units (Fig. 1) for 30 days (FR+ includes L1 and L2 FR+, see methods for details). The shoots exposed and unexposed to *S. flexuosum* VOCs were pooled together for the statistical analysis. FR+ induced creation of more short branches ($F_{1,7}$=7.2, p=0.03). The box and whiskers depict ± s.e. and minimum/maximum values.

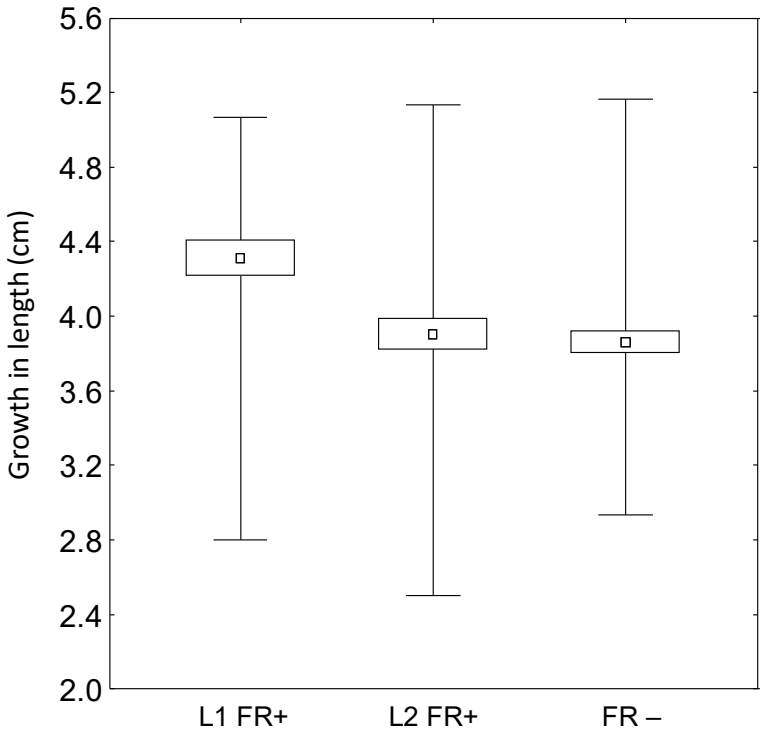

Fig. S6. The length increment of *H. vernicosus* shoots grown under artificial light without FR light addition (FR−) and added FR light (L1 FR+, L2 FR+) in cultivation units (Fig. 1) for 30 days (L2 FR+ had more blue light than L1 FR+, see methods and Fig. S2 for details). The shoots exposed and unexposed to *S. flexuosum* VOCs were pooled together for the statistical analysis. The *H. vernicosu*s growth increment was not significantly affected by different light treatments ($F_{2,5}$=2.6, p=0.17, see also Fig. 3 for different data presentation). The box and whiskers depict ± s.e. and minimum/maximum values.

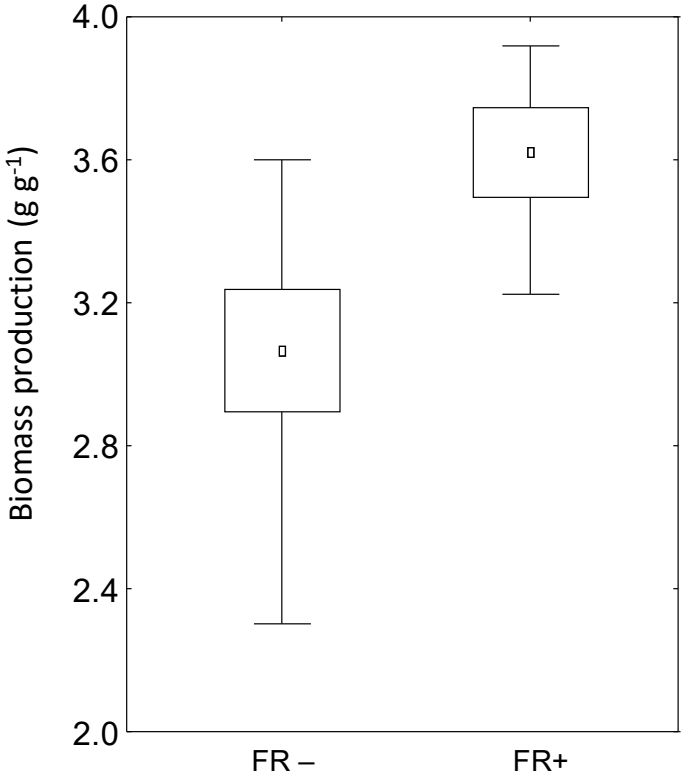

Fig. S7. The biomass production of *S.flexuosum* shoots grown in cultivation units (Fig. 1) for 30 days under artificial light without FR light addition (FR−) and added FR light (FR+; L1 and L2 FR+ treatments pooled together). The light treatment has no effect on biomass production of *S. flexuosum* ($F_{1,4}$=4.0, p=0.12; experimental design included in the test). The box and whiskers depict ± s.e. and minimum/maximum values.

Table S2. Volatile organic compounds (ng g^{-1}) produced by *Hamatocaulis vernicosus* carpets, exposed to *S. flexuosum* VOC (*Sphagnum*) or empty chamber (*control*), that were cultivated under standart light condition (FR–) of added FR light (FR+).
The *H. vernicosus* shoots were exposed either to VOCs released from neighbouring *H. vernicosus* individuals or to *H. vernicosus* VOCs and VOCs coming from *S. flexuosum* carpets. For more detail see methods.

Light	Control / *Sphagnum* exposure	Total	β-cyclocitral	Methyl 2,6,6-trimethyl-1-cyclohexene-1-carboxylate	Unknown 1	Unknown 2	Unknown 3	α-copaene	Unknown 4	Unknown 5	(Z)-β-farnesene	(E)-β-farnesene	Unknown 6	Unknown 7
FR+	control	**121**	11.55	0.58	0.19	0.36	0.24	1.12	1.19	0.46	0.15	0.18	5.83	0.60
FR+	control	**130**	6.58	1.29	0.13	0.31	0.28	1.27	0.76	0.84	0.26	0.33	6.88	0.76
FR+	control	**392**	48.11	2.95	0.80	1.07	0.96	1.35	1.12	1.31	0.77	1.46	28.81	2.54
FR+	control	**257**	25.03	2.28	0.25	0.48	0.33	1.13	1.13	0.45	0.27	1.35	8.70	1.03
mean		**225**	**22.82**	**1.78**	**0.34**	**0.55**	**0.45**	**1.22**	**1.05**	**0.77**	**0.36**	**0.83**	**12.56**	**1.23**
FR–	control	**135**	6.35	0.09	0.06	0.11	0.34	1.21	0.34	0.72	0.28	0.12	0.22	0.48
FR–	control	**185**	9.18	0.08	0.25	0.38	0.28	1.41	1.27	0.74	0.35	0.49	8.16	0.81
FR–	control	**99**	5.56	0.22	0.10	0.20	0.10	0.61	0.28	0.57	0.17	0.19	3.57	0.42
FR–	control	**198**	7.85	0.30	0.18	0.54	0.43	1.96	0.85	0.49	0.54	0.80	9.58	1.03
mean		**168**	**7.23**	**0.17**	**0.15**	**0.31**	**0.29**	**1.30**	**0.68**	**0.63**	**0.33**	**0.40**	**5.38**	**0.69**
FR+	*Sphagnum*	**216**	18.56	2.00	0.20	0.43	0.41	1.65	1.10	0.59	0.32	0.72	9.51	0.72
FR+	*Sphagnum*	**497**	20.49	1.36	0.55	1.04	0.85	5.07	2.01	0.82	0.73	1.80	32.08	2.39
FR+	*Sphagnum*	**146**	21.69	3.15	0.10	0.26	0.60	0.48	0.29	0.40	0.16	0.72	2.91	0.66
FR+	*Sphagnum*	**170**	13.36	4.90	0.51	1.30	0.40	1.32	7.14	0.55	0.47	0.86	5.71	1.23
FR+	*Sphagnum*	**242**	22.66	1.77	0.28	0.54	0.51	2.17	1.24	0.36	0.35	1.50	11.19	0.94
FR+	*Sphagnum*	**380**	9.77	1.23	0.37	0.86	0.82	3.91	2.02	1.07	0.63	1.44	22.84	2.02
mean		**275**	**17.76**	**2.40**	**0.34**	**0.74**	**0.60**	**2.43**	**2.30**	**0.63**	**0.44**	**1.17**	**14.04**	**1.33**
FR–	*Sphagnum*	**150**	6.97	0.71	0.21	0.27	0.24	1.01	0.68	0.85	0.28	0.32	5.75	0.75
FR–	*Sphagnum*	**548**	18.82	1.55	0.63	1.17	1.00	4.87	3.12	1.54	0.92	2.07	29.64	2.12
FR–	*Sphagnum*	**150**	6.97	0.71	0.21	0.27	0.24	1.01	0.68	0.85	0.28	0.32	5.75	0.75
FR–	*Sphagnum*	**256**	12.95	0.50	0.32	0.60	0.44	2.46	1.14	0.59	0.39	1.34	14.13	1.03
mean		**276**	**11.43**	**0.87**	**0.34**	**0.58**	**0.48**	**2.34**	**1.40**	**0.96**	**0.47**	**1.01**	**13.82**	**1.16**

Table S2 (continued).

Light	Control / *Sphagnum* exposure	Unknown 8	Unknown 9	Unknown 10	Unknown 11	Unknown 12	Unknown 13	Unknown 14	Unknown 15	Unknown 16	Rimuene	Unknown 17	Unknown 18
FR+	control	0.95	5.04	16.61	5.50	1.48	2.87	1.19	11.11	0.43	11.60	41.48	0.70
FR+	control	1.39	9.39	11.62	13.06	1.83	3.84	0.74	8.22	0.81	49.13	9.33	0.87
FR+	control	6.31	37.57	67.55	27.26	6.93	14.53	5.66	27.98	6.84	97.72	1.82	0.93
FR+	control	2.82	18.57	31.78	12.36	5.03	9.85	3.99	25.11	0.85	69.11	33.56	1.80
mean		**2.87**	**17.64**	**31.89**	**14.55**	**3.82**	**7.77**	**2.89**	**18.10**	**2.23**	**56.89**	**21.55**	**1.08**
FR–	control	0.22	1.32	1.15	0.44	0.50	0.23	0.26	2.24	0.89	16.47	99.61	1.40
FR–	control	1.88	8.42	28.36	14.12	3.40	6.82	3.46	18.96	1.01	46.31	27.79	0.74
FR–	control	0.61	4.73	7.92	6.41	1.62	2.71	1.18	4.69	0.49	48.10	8.02	0.88
FR–	control	2.40	7.60	24.38	15.74	4.71	7.98	2.84	14.43	2.53	56.32	33.36	0.77
mean		**1.28**	**5.52**	**15.45**	**9.18**	**2.56**	**4.43**	**1.94**	**10.08**	**1.23**	**41.80**	**42.19**	**0.95**
FR+	*Sphagnum*	2.03	11.43	23.73	12.33	2.67	6.00	2.83	19.64	0.91	68.26	28.53	1.21
FR+	*Sphagnum*	7.29	47.06	79.95	73.30	7.04	14.70	6.39	55.79	3.43	51.16	81.22	0.86
FR+	*Sphagnum*	0.59	8.27	9.08	1.46	0.56	0.50	1.14	9.46	0.49	76.32	5.14	1.72
FR+	*Sphagnum*	1.42	11.99	4.59	14.26	1.68	4.37	0.81	5.14	4.35	79.92	1.04	2.58
FR+	*Sphagnum*	3.17	23.47	31.23	21.16	3.90	6.62	2.62	25.46	1.49	50.09	28.79	0.93
FR+	*Sphagnum*	5.22	31.47	57.51	39.46	7.88	15.08	4.16	47.97	3.15	18.92	101.04	1.30
mean		**3.29**	**22.28**	**34.35**	**26.99**	**3.95**	**7.88**	**2.99**	**27.25**	**2.30**	**57.45**	**40.96**	**1.43**
FR–	*Sphagnum*	1.59	7.76	14.42	8.79	2.24	4.27	1.40	12.39	0.84	56.20	20.99	1.15
FR–	*Sphagnum*	7.15	30.70	60.84	47.71	7.57	15.59	5.96	38.32	4.40	188.85	69.96	3.99
FR–	*Sphagnum*	1.59	7.76	14.42	8.79	2.24	4.27	1.40	12.39	0.84	56.20	20.99	1.15
FR–	*Sphagnum*	3.97	20.18	39.79	18.81	4.90	10.35	3.72	33.32	1.68	45.91	36.39	1.38
mean		**3.58**	**16.60**	**32.37**	**21.02**	**4.24**	**8.62**	**3.12**	**24.11**	**1.94**	**86.79**	**37.08**	**1.92**

Table S3. Volatile organic compounds (ng g^{-1}) produced by *Sphagnum flexuosum* carpets, cultivated under standart light condition (FR–) of added FR light (FR+).
The VOCs sampling was done either under standart light condition (SS) or the FR light was added to shoots exposed to FR light during cultivation (FRS).

Sampling	Light	Total	α-pinene	β-myrcene	(E)-ocimene	β-cyclocitral	Methyl 2,6,6-trimethyl-1-cyclohexene-1-carboxylate	(+)-cyclosativene	α-copaene	Unknown 4	(±)-geosmin	(+)-sativene	Unknown 19	Unknown 20	Unknown 21	Unknown 22	Unknown 23
SS	FR–	**121**	0.33	0.41	3.28	2.02	0.67	0.90	0.98	0.29	0.89	1.94	0.44	13.22	3.19	10.14	2.43
SS	FR–	**40**	0.63	0.32	0.60	1.06	0.20	0.23	0.48	0.32	0.20	0.61	0.13	3.68	0.63	2.30	0.57
SS	FR–	**13**	0.02	0.11	0.45	0.31	0.08	0.01	0.03	0.03	0.04	0.06	0.02	1.39	0.18	0.11	0.07
SS	FR–	**25**	0.04	0.20	0.69	0.55	0.14	0.07	0.09	0.08	0.08	0.17	0.04	2.31	0.70	0.15	0.51
mean		**50**	**0.25**	**0.26**	**1.26**	**0.99**	**0.27**	**0.30**	**0.39**	**0.18**	**0.30**	**0.69**	**0.16**	**5.15**	**1.18**	**3.17**	**0.90**
SS	FR+	**59**	0.03	0.29	1.27	5.94	2.03	0.02	0.18	0.16	0.10	0.04	0.13	4.49	1.29	0.19	4.19
SS	FR+	**70**	0.07	0.33	2.18	7.28	3.93	0.08	0.28	0.07	0.11	0.16	0.19	5.14	1.31	0.44	3.60
SS	FR+	**76**	0.06	0.21	0.93	4.79	2.77	0.10	0.30	0.08	0.23	0.28	0.20	7.05	1.66	0.41	2.89
SS	FR+	**85**	0.11	0.44	2.46	4.53	2.65	0.40	0.43	0.06	0.31	0.75	0.23	9.10	1.93	1.92	1.01
SS	FR+	**103**	0.05	0.28	1.30	5.64	3.00	0.23	0.32	0.12	0.27	0.59	0.36	8.34	1.72	2.31	2.42
SS	FR+	**83**	0.08	0.37	2.70	5.14	2.72	0.19	0.19	0.12	0.27	0.38	0.29	8.08	2.04	1.76	1.78
mean		**79**	**0.07**	**0.32**	**1.81**	**5.55**	**2.85**	**0.17**	**0.28**	**0.10**	**0.21**	**0.37**	**0.23**	**7.03**	**1.66**	**1.17**	**2.65**
FRS	FR–	**85**	0.58	1.42	14.94	1.14	0.51	1.46	1.02	0.38	0.81	3.00	0.71	10.26	3.46	6.30	5.53
FRS	FR–	**51**	0.03	0.10	0.50	0.52	0.14	0.78	1.70	0.46	0.33	1.73	0.30	3.01	1.96	9.29	3.18
FRS	FR–	**30**	0.08	0.57	2.41	0.47	0.22	0.34	0.06	0.08	0.21	0.78	0.15	2.71	0.57	1.26	0.72
FRS	FR–	**27**	0.03	0.21	1.06	1.11	0.37	0.11	0.10	0.12	0.08	0.28	0.10	2.10	0.56	0.69	2.39
mean		**48**	**0.18**	**0.57**	**4.72**	**0.81**	**0.31**	**0.67**	**0.72**	**0.26**	**0.35**	**1.45**	**0.32**	**4.52**	**1.64**	**4.38**	**2.96**
FRS	FR+	**60**	0.09	0.58	3.19	3.35	2.21	0.09	0.30	0.08	0.10	0.27	0.31	4.18	1.14	0.68	8.20
FRS	FR+	**95**	0.16	0.87	2.75	8.87	5.76	0.23	0.60	0.13	0.14	0.17	0.46	5.73	1.29	0.47	2.64
FRS	FR+	**68**	0.10	0.55	2.68	3.49	2.70	0.18	0.26	0.08	0.15	0.41	0.25	5.32	1.00	1.12	6.02
FRS	FR+	**100**	0.08	0.66	3.55	4.41	1.97	0.10	0.46	0.16	0.10	0.19	0.15	3.89	1.48	0.50	32.80
FRS	FR+	**86**	0.09	0.65	3.50	4.32	2.46	0.12	0.38	0.08	0.13	0.15	0.27	3.88	1.62	0.44	26.86
FRS	FR+	**47**	0.02	0.17	1.01	2.71	1.05	0.04	0.22	0.14	0.16	0.09	0.15	1.63	0.63	0.21	15.88
mean		**76**	**0.09**	**0.58**	**2.78**	**4.52**	**2.69**	**0.13**	**0.37**	**0.11**	**0.13**	**0.21**	**0.26**	**4.11**	**1.19**	**0.57**	**15.40**

Table S3 (continued).

Sampling	Light	Unknown 24	(-)-calamenene	Unknown 25	Unknown 26	Unkown 27	Unknown 28	Unknown 29	Unkown 30	Unknown 31	Unknown 32	Unknown 33	Unknown 34	Manoyl oxide	Unknown 35
SS	FR–	2.89	0.92	0.96	11.33	0.76	0.12	0.35	0.66	5.25	5.18	2.10	39.64	5.17	4.93
SS	FR–	1.24	0.30	0.58	3.55	0.24	0.07	0.19	0.24	4.37	2.01	0.15	11.08	1.95	2.29
SS	FR–	0.36	0.04	0.06	1.53	0.09	0.02	0.07	0.11	0.99	0.68	0.08	3.82	0.71	1.05
SS	FR–	0.29	0.14	0.07	2.82	0.15	0.10	0.16	0.33	1.29	1.24	0.43	9.09	1.49	2.03
mean		**1.19**	**0.35**	**0.42**	**4.81**	**0.31**	**0.08**	**0.19**	**0.33**	**2.97**	**2.28**	**0.69**	**15.91**	**2.33**	**2.58**
SS	FR+	1.03	0.09	0.22	4.28	0.40	0.43	1.04	1.80	5.46	1.73	0.74	16.01	2.28	3.50
SS	FR+	1.32	0.10	0.21	4.29	0.30	0.73	1.68	2.47	6.18	2.18	1.57	18.02	2.50	2.98
SS	FR+	1.68	0.09	0.22	5.22	0.39	0.54	1.32	1.94	6.20	2.64	2.38	25.11	2.78	3.51
SS	FR+	2.00	0.44	0.31	6.36	0.29	0.44	0.90	1.34	6.65	2.94	0.81	29.62	3.47	3.42
SS	FR+	1.63	0.26	0.29	6.70	0.17	0.42	0.87	1.53	15.00	3.19	2.25	33.87	3.44	5.98
SS	FR+	1.56	0.27	0.37	6.06	0.02	0.26	0.61	0.99	9.51	2.64	0.27	26.76	3.18	3.96
mean		**1.54**	**0.21**	**0.27**	**5.48**	**0.26**	**0.47**	**1.07**	**1.68**	**8.17**	**2.55**	**1.34**	**24.90**	**2.94**	**3.89**
FRS	FR–	3.04	0.70	0.90	6.57	0.15	0.27	0.21	0.91	2.35	4.68	0.74	4.15	5.04	3.27
FRS	FR–	0.95	1.93	0.46	2.33	0.10	0.37	0.09	0.48	2.66	1.42	0.58	12.53	1.47	1.98
FRS	FR–	0.75	0.14	0.10	2.23	0.16	0.03	0.12	0.44	1.04	1.58	0.10	10.12	1.67	1.09
FRS	FR–	0.61	0.11	0.14	1.70	0.04	0.04	0.27	0.26	0.85	1.15	0.65	9.57	1.23	1.40
mean		**1.34**	**0.72**	**0.40**	**3.21**	**0.11**	**0.18**	**0.17**	**0.52**	**1.72**	**2.21**	**0.51**	**9.09**	**2.35**	**1.94**
FRS	FR+	0.94	0.13	0.25	3.10	0.41	0.75	1.41	3.07	4.94	2.21	4.04	10.16	1.91	2.14
FRS	FR+	1.54	0.16	0.36	6.15	0.79	3.20	5.90	11.64	7.70	3.31	1.59	15.18	3.60	3.37
FRS	FR+	1.19	0.23	0.27	3.40	0.83	0.89	1.57	2.87	6.43	2.21	6.38	12.82	1.98	2.82
FRS	FR+	1.01	0.11	0.39	2.25	1.21	2.11	2.69	6.00	4.08	2.12	11.63	11.89	1.89	2.05
FRS	FR+	1.02	0.08	0.49	2.12	1.14	1.47	3.19	5.46	3.72	2.30	5.26	11.38	1.57	2.20
FRS	FR+	0.50	0.23	0.06	0.95	1.05	1.08	1.63	3.67	2.01	0.88	3.81	5.03	0.78	1.10
mean		**1.03**	**0.16**	**0.30**	**2.99**	**0.91**	**1.58**	**2.73**	**5.45**	**4.81**	**2.17**	**5.45**	**11.08**	**1.95**	**2.28**

Table S4. Volatile organic compounds collected from *Sphagnum flexuosum* (SF) and *Hamatocaulis vernicosus (HV)* . Tentative compound identification, retention time and retention index (Kovats index) on HP-1 column, mass spectral data (m/z and relative abundance).

Tentative compound id	Retention time (min)	KI (HP-1)	m/z fragments	Suggested chemical class of unknown	Occurs in species
β-cyclocitral	11.84	1201	137 (100), 152 (86), 109 (74), 123 (74), 81 (58), 67 (56), 41 (39), 91 (34), 79 (31), 77 (27)		HV, SF
Methyl 2,6,6-trimethyl-1-cyclohexene-1-carboxylate	12.10	1220	135 (100), 123 (52), 107 (51), 167 (36), 91 (23), 79 (22), 41 (18), 81 (16), 151 (16), 77 (15)		HV, SF
Unknown 1	13.88	1354	105 (100), 119 (82), 161 (75), 91 (53), 93 (39), 121 (32), 120 (28), 81 (25), 41 (25), 133 (18), 204	sesquiterpene	HV
Unknown 2	13.94	1359	119 (100), 41 (59), 55 (48), 105 (44), 133 (39), 91 (35), 56 (30), 93 (25), 120 (23), 107 (23), 204	sesquiterpene	HV
Unknown 3	14.18	1377	105 (100), 119 (92), 93 (74), 120 (67), 161 (54), 91 (53), 49 (45), 41 (39), 92 (35), 121 (27), 204	sesquiterpene	HV
α-copaene	14.24	1382	119 (100, 105 (98), 161 (79), 93 (48), 91 (45), 41 (30), 92 (27), 81 (24), 120 (22), 77 (21), 204		HV, SF
Unknown 4	14.37	1392	161 (100), 41 (54), 105 (46), 55 (41), 57 (37), 91 (34), 81 (27), 79 (24), 67 (23), 120 (23), 204	sesquiterpene	HV, SF
Unknown 5	14.76	1424	105 (100), 161 (76), 91 (63), 119 (59), 107 (51), 189 (45), 93 (42), 204 (41), 79 (39), 41 (38)	sesquiterpene	HV
(Z)-β-farnesene	14.96	1440	69 (100), 41 (67), 93 (54), 91 (38), 79 (34), 55 (31), 77 (30), 92 (29), 161 (28), 120 (26), 204		HV
(E)-β-farnesene	15.07	1449	69 (100), 41 (91), 93 (58), 91 (35), 105 (33), 55 (29), 79 (29), 133 (28), 67 (28), 161 (26), 204		HV
Unknown 6	15.21	1461	119 (100), 121 (87), 93 (70), 79 (56), 91 (45), 189 (42), 105 (41), 41 (40), 81 (37), 77 (33), 204	sesquiterpene	HV
Unknown 7	15.28	1467	43 (100), 119 (94), 177 (88), 149 (77), 93 (69), 91 (68), 121 (64), 105 (57), 77 (42), 79 (40), 204	sesquiterpene	HV
Unknown 8	15.37	1475	119 (100), 121 (68), 105 (62), 93 (59), 91 (58), 79 (49), 41 (48), 81 (42), 107 (38), 189 (37), 204	sesquiterpene	HV
Unknown 9	15.60	1493	161 (100), 105 (56), 119 (44), 91 (43), 41 (31), 43 (24), 81 (21), 55 (19), 93 (18), 79 (16), 204	sesquiterpene	HV
Unknown 10	15.83	1512	161 (100), 43 (64), 105 (62), 119 (50), 91 (46), 41 (40), 81 (37), 93 (37), 55 (31), 79 (31), 204	sesquiterpene	HV
Unknown 11	15.95	1523	105(100), 43 (99), 220 (76), 91 (76), 106 (70), 81 (69), 41 (54), 93 (50), 147 (47), 137 (46)		HV
Unknown 12	16.07	1534	108 (100), 126 (99), 43 (95), 81 (60), 82 (47), 41 (47), 55 (46), 109 (39), 67 (37), 83 (35)		HV
Unknown 13	16.35	1558	55 (100), 41 (96), 137 (82), 109 (80), 81 (77), 43 (72), 207 (67), 95 (61), 149 (51), 107 (49)		HV
Unknown 14	16.64	1583	161 (100), 105 (59), 207 (59), 43 (54), 91 (38), 119 (37), 41 (30), 81 (28), 93 (19), 55 (18)		HV
Unknown 15	17.02	1617	161 (100), 59 (72), 81 (59), 93 (49), 204 (42), 79 (42), 119 (41), 91 (39), 105 (39), 41 (34)		HV
Unknown 16	19.31	1835	161 (100), 91 (22), 105 (20), 41 (20), 119 (15), 133 (14), 107 (14), 93 (13), 162 (13), 147 (12)		HV
Rimuene	20.30	1937	257 (100), 80 (58), 91 (42), 93 (42), 81 (42), 121 (40), 55 (39), 41 (38), 79 (39), 105 (37), 272	diterpene	HV
Unknown 17	20.64	1972	161 (100), 41 (45), 82 (40), 55 (34), 105 (32), 69 (28), 93 (28), 91 (27), 121 (24), 43 (23)		HV
Unknown 18	21.33	2047	91 (100), 41 (78), 105 (77), 55 (61), 69 (60), 79 (59), 81 (59), 133 (58), 95 (54), 123 (51)		HV
α-pinene	7.89	941	93 (100), 91 (43), 92 (37), 77 (32), 79 (25), 41 (18), 105 (15), 121 (13), 94 (11), 80 (11), 136		SF
β-myrcene	8.66	988	93 (100), 41 (93), 69 (71), 91 (23), 79 (17), 77 (15), 53 (14), 67 (13), 92 (11), 94 (10), 136		SF
(E)-ocimene	9.33	1032	93 (100), 91 (45), 92 (40), 79 (36), 77 (31), 41 (24), 106 (16), 80 (16), 55 (15), 121 (11), 136		SF

Table S4 (continued).

Tentative compound id	Retention time (min)	KI (HP-1)	m/z fragments	Suggested chemical class of unknown	Occurs in species
(+)-cyclosativene	14.15	1375	105 (100), 91 (72), 119 (71), 161 (71), 94 (66), 120 (50), 107 (49), 93 (47), 41 (43), 133 (33), 204		SF
(±)-geosmin	14.42	1395	112 (100), 55 (23), 41 (23), 111 (21), 43 (18), 108 (18), 125 (14), 93 (13), 126 (12), 97 (12), 182		SF
(+)-sativene	14.50	1401	108 (100), 91 (87), 161 (78), 105 (76), 93 (58), 119 (57), 147 (48), 79 (44), 133 (44), 41 (43), 204		SF
Unknown 19	14.75	1423	161 (100), 119 (53), 189 (50), 105 (49), 204 (35), 91 (35), 162 (33), 147 (28), 133 (27), 41 (25)	sesquiterpene	SF
Unknown 20	14.89	1434	147 (100), 105 (94), 91 (53), 93 (43), 119 (41), 107 (33), 41 (30), 190 (30), 79 (30), 175 (29)		SF
Unknown 21	15.19	1460	175 (100), 105 (33), 91 (31), 119 (31), 93 (23), 121 (23), 41 (23), 190 (21), 133 (20), 95 (19)		SF
Unknown 22	15.41	1477	43 (100), 137 (92), 109 (50), 93 (49), 161 (47), 41 (43), 81 (43), 121 (41), 105 (39), 95 (38), 222		SF
Unknown 23	15.65	1496	121 (100), 93 (84), 105 (81), 107 (55), 91 (54), 41 (46), 79 (43), 119 (38), 161 (36), 94 (34), 204	sesquiterpene	SF
Unknown 24	15.74	1504	173 (100), 188 (16), 174 (14), 128 (12), 143 (9), 129 (9), 158 (9), 115 (9), 145 (8), 141 (8)		SF
(-)-calamenene	15.85	1514	159 (100), 160 (13), 128 (13), 129 (13), 131 (10), 144 (9), 202 (8), 115 (8), 143 (6), 105 (6)		SF
Unknown 25	15.90	1519	161 (100), 119 (76), 105 (70), 134 (68), 91 (50), 204 (40), 41 (33), 81 (30), 133 (23), 162 (23)	sesquiterpene	SF
Unknown 26	16.44	1566	43 (100), 121 (86), 109 (81), 175 (56), 105 (55), 93 (54), 190 (49), 107 (44), 91 (41), 108 (39)		SF
Unkown 27	16.51	1572	43 (100), 91 (94), 119 (82), 159 (80), 205 (78), 131 (64), 41 (54), 145 (50), 105 (46), 117 (46)	sesquiterpenoid	SF
unknown 28	16.57	1577	43 (100), 91 (63), 41 (57), 79 (48), 105 (45), 81 (40), 159 (40), 107 (39), 96 (38), 69 (36), 220	sesquiterpenoid	SF
Unknown 29	16.61	1581	43 (100), 41 (65) 105 (65), 107 (61), 93 (55), 91 (55), 69 (48), 109 (47), 55 (45), 81 (44), 220, 222	sesquiterpenoid	SF
Unkown 30	16.70	1588	43 (100), 107 (92), 41 (75), 109 (73), 93 (70), 105 (64), 81 (63), 69 (62), 91 (59), 161 (54), 222	sesquiterpenoid	SF
Unknown 31	16.76	1593	109 (100), 43 (86), 136 (76), 121 (64), 93 (51), 147 (47), 105 (45), 175 (42), 91 (41), 41 (35), 208		SF
Unknown 32	17.67	1676	95 (100), 107 (40), 41 (25), 123 (21), 55 (21), 121 (21), 91 (20), 93 (18), 81 (16), 79 (15), 220		SF
Unknown 33	18.26	1732	41 (100), 91 (85), 105 (71), 67 (71), 55 (67), 79 (64), 93 (58), 81 (57), 95 (55), 43 (52)		SF
Unknown 34	20.83	1992	95 (100), 107 (89), 191 (43), 121 (38), 81 (36), 55 (31), 79 (30), 41 (29), 93 (29), 91 (27)		SF
Manoyl oxide	21.06	2017	43 (100), 55 (86), 81 (86), 95 (69), 67 (65), 257 (65), 41 (64), 69 (60), 275 (54), 137 (51), 290		SF
Unknown 35	21.99	2121	95 (100), 107 (34), 55 (17), 121 (15), 191 (15), 41 (13), 93 (12), 91 (12), 81 (10), 79 (10)		SF

Tentative identification, mass spectrum and KI match with autentic standard

Speculative identification, strong match in commercial library, no standard available

Unknown compound, no satisfactory match in commercial library

Chapter 6 – General conclusions

Composition and survival of bryophyte communities in peatlands of a given local history and geography is determined mainly by chemistry and availability of groundwater, which, besides others, impact interactions and competitive abilities of individual species. The environment and interactions also determine the probability of establishment and invasion of species outside the community that can greatly influence life and survival of local species. Rare alkaline fen communities are particularly affected by the expansion of acidifying *Sphagnum* mosses that triggers succession of these rare habitats to poor fens. The competitive hierarchies between "brown mosses" of alkaline fens and *Sphagnum* species of poor fens are rather complex. As demonstrated in **Paper I**, peatland bryophytes have wide fundamental niches. Alkaline-fen brown mosses can grow and germinate in the environment of poor fens; however, their growth is slower than that of *Sphagnum* species. Calcitolerant sphagna can survive and germinate along the whole poor–rich gradient of calcium bicarbonate concentration, but their growth is inhibited by calcium bicarbonate in alkaline conditions. Calcifuge sphagna (the species known to trigger alkaline–poor fen switch) are eliminated by flowing alkaline water, although their protonemata can grow in stagnant alkaline water. If the groundwater is rich in potassium, which alleviates the calcium stress, the growth and survival of *Sphagnum* species is even better. In contrast, elevated nitrogen concentration could facilitate growth of some rare brown mosses (e.g., *Hamatocaulis vernicosus*) more than other species if there is no light limitation, although unshaded conditions rarely happen in the field.

The wide fundamental niches and the complex competitive hierarchies point out the risk that dry weather conditions pose to bryophyte communities of alkaline fens (dry weather leads to the groundwater table lowering). As shown in **Paper II**, the water chemistry of alkaline fen hummocks is suitable for *Sphagnum* survival and expansion. Sphagna can even improve own immediate environment by lowering pH in capillary water of their shoots. Paradoxically, short severe dry weather spells cause fatal desiccation to the *Sphagnum* species preventing their expansion.

However, if these spells lower the groundwater table, the residual non-toxic concentration of calcium bicarbonate could reduce the post-desiccation damage of *Sphagnum* and facilitate its expansion.

Timely warning and subsequent preparation for a stress by acclimation allows bryophytes to survive in situations that would be otherwise lethal (Hájek and Vicherová 2014). As shown for the first time in **Paper IV**, bryophytes can use volatile organic compounds to ascertain what happens in their proximity. By sensing the presence of a stronger competitor, they can adjust growth to avoid being outcompeted. This ability can be used during short unfavourable periods, e.g. when the environmental conditions favour expansion of *Sphagnum* at the expense of brown mosses. It is very probable that VOCs would be also perceived as warning cues against environmental stress (as known in vascular plants, Caparrotta et al. 2018), particularly in situations where timely biochemical acclimation (hardening) is crucial for species survival (e.g. during desiccation stress).

The reasons behind the diverse reaction of peatland bryophytes to calcium bicarbonate (the calcifuge–calcicole behaviour) is also very complex and despite our contribution to the problematics in **Paper I and III**, still not fully understood. It is clear that bryophytes suffer from the combination of high [Ca^{2+}] and high pH (Clymo 1973, Paper I) and the reasons behind calcium toxicity are intracellular (we have excluded the processes on cell wall or nutrient limitations as they have no significant impact, Paper I). Since the calcicole and calcifuge species differ in the rate of intracellular Ca^{2+} accumulation and have different composition and/or regulation of Ca^{2+} influx, the Ca^{2+} influx/efflux mechanism seems to be important component of adaptation to the alkaline environment. Calcicole species also have a constant high cytosolic concentration of reduced glutathione (GSH), a molecule that protects cells against oxidative stress. We speculate that GSH could directly regulate concentration of free Ca^{2+} in cytosol and thus help to maintain calcium homeostasis, even in calcareous environment.

Prospects of future studies

Discovery of *plant communication* in bryophytes points to a new aspect of peatland and bryophyte ecology that should be considered when studying competition in bryophyte communities or effects of the environment on species survival in peatlands, including alkaline–acidic-fen succession induced by *Sphagnum* mosses. The future studies of *plant communication* in bryophytes should verify the existence of VOCs as cues of environmental stress. It could also concentrate upon finding volatiles responsible for the identification of competitively stronger neighbour, as they could be either species specific (Runyon et al. 2006) or connected with quick biomass production.

Despite the detailed study of calcicole species adaptations to calcareous environment, the mechanisms behind calcium toxicity is still not fully understood. The future research should concentrate upon visualisation of Ca^{2+} fluxes between cell wall and cytosol and interaction of free Ca^{2+} in cytosol with metal chelators, and specifically verify our hypothesis that reduced glutathione can chelate calcium ions and thus helps to regulate intracellular calcium homeostasis in alkaline environment.

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Chapter 7 – Shrnutí (General conclusions in Czech)

Složení společenstev slatiništních mechorostů dané oblasti závisí především na chemismu a dostupnosti podzemní vody. Tento faktor prostředí následně ovlivňuje interakce a konkurenční schopnosti jednotlivých druhů. Faktory prostředí a interakce rovněž určují, zda na slatiniště mohou expandovat druhy z jiných rašeliništních společenstev, které následně často výrazně ovlivní složení místního slatiništního společenstva. Zvláště v současné době klimatické změny expandují do biotopu vzácných bazických slatinišť kyselomilné rašeliníky, které následně spustí sukcesi k relativně běžnému biotopu přechodového rašeliniště. Konkurenční hierarchie *hnědých mechů* bazických slatinišť a rašeliníků je značně spletitá. Jak jsme ukázali v našem článku I, rašeliništní mechorosty mají velice široké fundamentální niky. *Hnědé mechy* mohou růst a klíčit v prostředí přechodových rašelinišť, jejich růst je však pomalejší než růst rašeliníků. Vápnomilné druhy rašeliníků mohou přežít i vyklíčit ve všech typech slatinišť, jejich růst je však v bazickém prostředí limitován vyšší koncentrací hydrogenuhličitanu vápenatého. Kyselomilné rašeliníky (druhy spouštějící sukcesi od bazických ke kyselým slatiništím) nepřežijí v tekoucí vodě bazických slatinišť, ale jsou schopny vyklíčit ve stojaté vodě bazických slatinišť. Pokud je podzemní voda obohacena draslíkem, přežívání rašeliníků je úspěšnější, neboť draslík sníží toxicitu vysoké koncentrace vápenatých iontů při vysokém pH. Naproti tomu zvýšená koncentrace dusíku překvapivě zvýhodňuje růst některých vzácných *hnědých mechů* (konkrétně druhu *Hamatocaulis vernicosus*), pokud světlo není limitujícím faktorem. V terénních podmínkách však tento případ v podstatě nenastává.

Široké fundamentální niky a složitá konkurenční hierarchie poukazuje na to, jak zranitelná jsou společenstva bazických slatinišť v suchém počasí posledních let. Jak je ukázáno v článku II, chemismus kapilární vody na bultech v bazických slatiništích je vyhovující pro přežití a expanzi rašeliníků. Rašeliníky si po uchycení v bultu mohou dále okyselit kapilární vodu v prostoru lodyžek, což usnadňuje jejich přežívání a urychluje jejich

rozrůstání. Paradoxně, krátké intenzivní sucho ve vegetační sezóně způsobí úmrtí expandujících rašeliníků, ovšem pouze v případě, že sucho nevede k oslabení podzemních pramenů a tedy k celkovému snížení hladiny podzemní vody na lokalitě. V takovém případě naopak zbytkové koncentrace hydrogenuhličitanu vápenatého zmírní poškození buněk rašeliníků zapříčiněné extrémním suchem a expanze se naopak může urychlit.

Včasné varování a následná příprava na přicházející stres umožňuje mechorostům přežít v situacích, které by pro ně jinak byly smrtelné (Hájek a Vicherová 2014). V článku IV jsme jako první ukázali, že mechorosty umí pomocí těkavých organických látek (volatile organic compounds, VOC) zjistit, co se děje v jejich okolí. V případě, že se v jejich blízkosti nachází silnější konkurent, jsou schopni přizpůsobit svůj růst. Tato schopnost je pro mechorosty důležitá v době krátkodobé změny podmínek prostředí, např. v době, kdy faktory prostředí krátkodobě umožnují expanzi rašeliníků do společenstva *hnědých mechů*. VOCs velice pravděpodobně slouží i jako varovné signály v situacích, kdy společenstva rašeliništních mechorostů začínají trpět stresem způsobeným faktory prostředí, jak je známo v případě cévnatých rostlin (Caparrotta et al. 2018). Zvláště užitečná by tato schopnost byla v situacích, kdy včasné přizpůsobení biochemických procesů v buňkách (aklimatizace) vede k přežití nadcházejícího stresu, jako je tomu např. při stresu suchem.

Příčiny stojící za rozdílnou tolerancí hydrogenuhličitanu vápenatého rašeliništními mechorosty jsou rovněž velice složité. Navzdory k našemu přispění k problematice v článcích I a III nejsou příčiny toxicity vysoké koncentrace hydrogenuhličitanu vápenatého stále objasněny. Je zcela zřejmé, že kombinace vysoké koncentrace Ca^{2+} s vysokým pH je toxická pro vápnostřezné mechorosty (Clymo 1972, článek I) a příčiny toxicity jsou vnitrobuněčné (v článku I jsme vyloučili možnost limitace živinami či příčiny spojené s procesy na buněčné stěně). Vzhledem k tomu, že se vápnomilné a vápnostřezné druhy liší v rychlosti vnitrobuněčné akumulace Ca^{2+} a mají rozdílné složení či regulaci membránových komplexů zodpovědných za toky Ca^{2+} z /do cytosolu, speciální regulace toků Ca^{2+} z /do buněk se zdá být zásadním přizpůsobením vápnomilných mechorostů

bazickému prostředí. Vápnomilné druhy rovněž mají stálou vysokou koncentraci redukovaného glutathionu (GSH) v cytosolu. Tato molekula chrání buňky před oxidativním stresem. Je rovněž možné, že GSH by mohl být schopen chelatovat volné vápenaté ionty v cytoplazmě a tím pomáhat kalcitolerantním mechorostů v udržení Ca^{2+} homeostázy v bazickém prostředí vápnitých slatinišť.

Možný směr dalšího výzkumu

Objev rostlinné komunikace u mechorostů poukazuje na nový aspekt ekologie rašelinišť a mechorostů, který by měl být zohledněn při budoucím studiu konkurence mezi mechorosty a při studiu tolerance jednotlivých druhů k faktorům prostředí. Rostlinná komunikace mezi mechorosty bude mít pravděpodobně vliv i na expanzi rašeliníků do bazických slatinišť a následnou sukcesi tohoto biotopu k biotopu přechodových rašelinišť. Budoucí studie komunikace u mechorostů by měly ověřit možnost funkce VOCs jako identifikátorů stresu vyvolaného faktory prostředí. Bylo by rovněž vhodné se zaměřit na nalezení konkrétních látek, které identifikují konkurenta. Tyto látky by mohly být buď druhově specifické (Runyon et al. 2006) nebo spojené s rychlou produkcí biomasy.
Navzdory detailnímu studiu adaptací vápnomilných druhů k prostředí bazických slatinišť zůstává mechanismus toxicity vápenatých iontů stále ne zcela objasněn. Budoucí studium by se mělo zaměřit na sledování toků Ca^{2+} mezi buněčnou stěnou a cytosolem a na vazbu volných vápenatých iontů v cytoplazmě s molekulami schopnými chelatovat kovy. Konkrétně navrhujeme ověřit hypotézu, že redukovaný glutathion je schopen chelatovat vápenaté ionty a tím pomáhat v udržování Ca^{2+} homeostázy v buňkách v prostředí bazických slatinišť.

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Chapter 8 – Curriculum Vitae

Surname and first name: Vicherová Eliška

Birth: 27.8.1987, Plzeň

Contact: vicherova.e@seznam.cz

Education:

2013–2020

Doctoral study program, University of South Bohemia, Faculty of Science, Department of Botany, thesis: **Ecology of peatland bryophytes** (supervisor T. Hájek)

2010–2013

Master's study program, University of South Bohemia, Faculty of Science, Department of Botany, thesis: **Desiccation tolerance of *Sphagnum*: A puzzle solved** (supervisor: T. Hájek, defended in January 2013)

2006–2010

Bachelor's study program, University of South Bohemia, Faculty of Science, Department of Botany, thesis: **The effect of trees and water table on a vegetation of two mountain raised bogs in Šumava** (supervisor: J. Kučera, defended in January 2010)

Interships:

2016: V. Ninkovic laboratory, SLU Uppsala, Sweden, (2 months)

2016: K. Trebacz laboratory, Maria Curie-Sklodowska University, Lublin, Poland (3 months)

Study programs:

2015: Ph.D. course „Plant communication and trophic interactions", Ekenäs, Sweden.

2013: Bryophyte field course, Research School in Biosystematics, Norway.

2012: Erasmus study program, University of Helsinki, Finland (4 months)

2011: Field course of tropical ecology, Papua New Guinea (1 month)

2009: Summer course of peatland ecology, Uppsala University, Sweden (1 month)

Conferences attended:

2016: „VI International Meeting on the Biology of *Sphagnum*“, Khanty-Mansyisk, Russia, (presentation: Plant communication in bryophytes)

2015: „International Symposium on Plant Signaling and Behavior“ 2015, Paris, France, (poster: Establishment of calcium-intolerant *Sphagnum* mosses in alkaline peatlands).

2012: „The 5th International Meeting on the Biology of *Sphagnum*“, Turku, Estonia (poster: The establishment of Sphagnum in fens: dealing with calcium toxicity and drought).

2011: Joint Meeting of Society of Wetland Scientists and Weatland Biogeochemistry Symposium (poster: Desiccation tolerance of *Sphagnum*: A solved puzzle?).

2011: Mokřady a klimatická změna, Blansko (poster: The effect of trees and water table on a vegetation of two mountain raised bogs in Šumava).

Work experience, grants:

2017–2020: Nature conservation agency of the Czech Republic (AOPK ČR), Správa CHKO Broumovsko, position: botanist

2016: University of South Bohemia in České Budějovice, GAJU: Mechanisms of calcifuge–calcicole behaviour in bryophytes (grant number: 009/2016/P)

2014–2016: Institute of Botany, Czech Academy of Sciences, Department of Functional Ecology, position: PhD. student

2016: Faculty of Science, University of South Bohemia, grant: Metodika druhové ochrany bezcévných rostlin – TAČR Beta TB050MZP005, position: příprava metodik mapování vybraných druhů mechorostů, ověření jejich recentního a historického rozšíření.

2015–2016: cooperation on grant: TAČR: "Příprava a zavedení sledování stavu předmětů ochrany EVL", position: příprava metodiky monitoringu druhu *Orthotrichum rogeri*

2012–2020: bryological inventarizations and monitoring of rare bryophytes

Teaching:

KBO/137 Základní kurz botaniky, fykologie a mykologie/ Basic course in botany, phycology and mycology – practical part, 1 lecture (bryology)

KBO/132 Botanika vyšších rostlin – malá/ Botany of higher plants – basic – practical part, 1 lecture (bryology)

KBO/138 Botanika vyšších rostlin – velká / Botany of higher plants – advanced – practical part, 1 lecture (bryology)

KBO/004 Biologická laboratorní technika / Laboratory techniques in biology – practical part

KEBR/220 Fyziologie rostlin / Plant Physiology – practical part, 1 lecture (measurement of water potential)

Publications in journals with IF:

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