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RESEARCH ARTICLE

REVISED Interspecific competition affects spore germination and gametophore development of mosses

[version 2; peer review: 3 approved, 1 approved with reservations]

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Abstract

Background

Interactions between moss species in their earliest growth stages have received little attention. To what extent interspecific competition or priority effects influence spore germination, protonemal development and gametophore emergence is unknown. We evaluated such effects in pairwise interaction between six common bryophyte species: *Atrichum undulatum*, *Bryum argenteum*, *Ceratodon purpureus, Funaria hygrometrica*, *Hypnum cupressiforme*, *Leptobryum pyriforme*

Methods

Interspecific interactions were assessed *in vitro*. Spores were sterilized and sown on agar plates in three treatments: 1) as single species cultures (controls), 2) as pairwise species cultures inoculated simultaneously, and 3) with a time lag of 20 days between species. Data on time needed for spore germination, germination rate, the time needed for gametophore differentiation, number of gametophores per germinated spore and average diameter of colonies were collected. We also performed spore germination tests in single-species cultures at the start and end of the study, as well as tests for density-dependency at spore germination and gametophore formation.

Results

We observed strong pairwise interactive effects when sowing spores of different species simultaneously or with a delay of 20 days. The results indicate that spore germination is often inhibited by

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interspecific competition. The first species has an advantage as compared to the later colonizing species, i.e., an apparent priority effect. Interspecific interactions were also evident during gametophore development and included both inhibition and facilitation.

Conclusion

We found pronounced differences in the relative performance of species in interaction with other species during spore germination and gametophore formation. Allelopathic effects are the most probable explanation for these observations. Our results under sterile lab conditions are likely to reflect processes that occur in the wild, governing biotic filtering and bryophyte community assembly during primary and secondary colonization.

Keywords
mosses, spore germination, gametophores, competition, allelopathy, Atrichum undulatum, Bryum argenteum, Ceratodon purpureus, Funaria hygrometrica, Hypnum cupressiforme, Leptobryum pyriforme



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REVISED **Amendments from Version 1**

We have remedied a couple of typing errors, notably in Table 2 (missing %-sign for spore germination rate at the end of experiment) and Table 3 (second heading should be "Correlation coefficient" rather than "Mean no. of spores"). We have also made some clarifications in response to suggestions from the reviewers, mainly in the methods and results sections. For example, the text describing the nested ANOVAs has been more detailed, and Figure 4 has been updated to make it easier to understand. Several referees have asked about the selection of species included in the study, so we have added information about this in the methods section. We also added a few references suggested by the reviewers for improved context in the introduction and the discussion. References to Bopp (1959) and Watson (1981) represent earlier studies on spore germination. The reference to Monteiro *et al.* (2023) adds some recent findings concerning bryophyte assembly rules. The study of Barbé *et al.* (2016) used a sampling of bryophyte diaspores in the field on agar plates for comparison with the extant community composition, which can be related to our cultures on axenic agar plates. The reference to During (1979) includes a description of different life strategy categories relevant to our species selection. The section "Concluding remarks" has been updated to incorporate suggestions from the reviewers regarding comparison to rhizosphere exudates, selection of species and life strategy categories, and some study limitations. We have not made any changes concerning data included, statistical treatment or interpretation of results. There are no changes concerning the author list or author contributions. We thank the referees for their helpful and constructive suggestions for improvement.

Any further responses from the reviewers can be found at the end of the article

Introduction

Plant community assembly – mechanisms by which the local communities are formed from the species pool – have been intensively studied during past decades (Kraft & Ackerly, 2014). Much emphasis is given to the relative contribution of deterministic and stochastic processes in the search for principles to explain the assembly of local plant communities. Götzenberger *et al.* (2012) conclude from a meta-analysis of 59 articles that "non-random occurrence of plant species is not a widespread phenomenon", i.e., plant communities often show an individualistic nature.

Interspecific interaction or competition is an important factor determining the structure and dynamics of plant communities (Aerts, 1999). Due to considerable differences in biology, it has been proposed that competition plays different roles in the community assembly of vascular plants and bryophytes. There are few studies of competition among mosses (During & van Tooren, 1990), and most are focused on *Sphagnum* and other peat-forming mosses (e.g., Robroek *et al.*, 2007; Rydin, 1986; Sonesson *et al.*, 2002). Some results based on co-occurrence suggest less efficient competition and predominant mutualism in bryophytes (Steel *et al.*, 2004), whereas other evidence maintain that species exclude each other to the same degree as among vascular plants (Wilson *et al.*, 1995). A recent study suggests that at the fine scale, environmental filtering prevails in stressful habitats, while competitive interactions appear more important in more favourable conditions (Monteiro *et al.*, 2023). However, co-occurrence is not evidence of ecological interactions (Blanchet *et al.*, 2020), and there is a need for more studies with an experimental approach.

Besides biotic and abiotic filtering processes, priority effects could contribute to the diversity of communities dependent on the order and timing in which diaspores of different species arrive at a colonization site (Fukami *et al.*, 2016). However, priority effects are rarely considered in plant community assembly because such effects may be considered of short duration if the community is subject to pronounced successional changes. The study of priority effects also requires repeated screening during the colonization process.

Ancestors to modern bryophytes were amongst the first plants to colonise terrestrial habitats, and many species still act as primary or secondary colonisers. Most bryophytes are dispersed by spores that are small enough (10–20µm) to be efficiently wind-borne (Sundberg, 2013). This means that many viable spores of different species continuously disperse, although the composition of the downfall varies due to different spore dispersal phenologies among species. Attempts to experimentally sow spores in natural habitats have largely failed for unclear reasons (Miles & Longton, 1990; Newton & Mishler, 1994). It is also methodologically challenging to monitor germination of microscopic spores in the field. In contrast, it is often easy to germinate spores *in vitro*. Laboratory culturing has shown that spore germination and protonemal development can be affected by many abiotic factors, e.g., water, light intensity, photoperiod, pH, calcium ions, and plant hormones such as IAA (Indole Acetic Acid) (Cove *et al.*, 2006; Glime, 2013; Glime, 2015). A comparison of the extant bryophyte community with the propagule rain community (trapped on agar plates) in Canadian boreal forest showed that long-distance dispersal prevails over short-distance dispersal and that species' ability to produce large amounts of spores, as well as their environmental tolerance during establishment, may have considerable filtering effects (Barbé *et al.*, 2016).

Interactions between moss species in their earliest growth stages have received little attention (Watson, 1981). For example, it remains unclear if negative interactions are expressed as exploitive (resource) competition or interference (chemical) competition. Plant allelopathy is defined as interference concerning growth due to chemical interactions between plants and other organisms, mediated by the release of plant-produced bioactive or toxic specialized metabolites referred to as allelochemicals (Latif *et al.*, 2017). Such metabolites could have beneficial (stimulatory) or detrimental (inhibitory) effects on target organisms (Cheng & Cheng, 2015; Zhang *et al.*, 2021). Based on studies by (Bopp, 1959) and her own observations, Watson (1981) suggested that differences among species in arrival time and sensitivity to leaked chemicals could affect the outcome of competitive interactions and, thus, community assembly.

The early development of mosses involves crucial steps, which could be targeted by exuded chemicals with allelopathic

effect on neighbouring species. Spore germination, formation of filamentous protonema with two distinct stages (chloronema and caulonema), and initiation of gametophores involve complex physiological processes controlled by internal and external signals involving several phytohormones. As a rule, conspecific moss spores germinate simultaneously with no primary dormancy (Vesty *et al.*, 2016). It is known from studies of *Physcomitrium patens* that abscisic acid (ABA) has a negative effect, whereas certain diterpenoid hormones have a positive effect on germination (Vesty *et al.*, 2016). IAA stimulates the transition from chloronema to caulonema cells (but in high concentrations inhibits the normal development of buds); ABA could inhibit this transition, whereas cytokinins are known to enhance bud formation (from chloronemata or caulonemata) (Decker *et al.*, 2006; Glime, 2013; Reski, 1998).

This project investigated pairwise interactions between six common bryophyte species (*Atrichum undulatum*, *Bryum argenteum*, *Ceratodon purpureus*, *Funaria hygrometrica*, *Hypnum cupressiforme* and *Leptobryum pyriforme*). We sowed spores on agar plates in sterile conditions and compared their germination and growth, aiming to answer the following three questions: 1) Is it possible to observe interspecific interactions between mosses during spore germination and gametophore development? 2) How are spore germination and protonemal growth affected by interspecific interaction? 3) Is the competitive ability of mosses related to the time when they are sown, i.e., is there a priority effect dependent on the first coloniser? To address these questions, we also performed a spore germination test in single-species cultures at the start and end of the study and checked for density-dependency at spore germination and gametophore formation.

Methods

Spore germination, protonemal development and gametophore initiation

Moss spores that deposit on a suitable substrate germinate if enough water is available. Spore germination includes a swelling stage involving water uptake and a distension stage when the cell wall rupture and a germ tube forms (Mogensen, 1978) that divides to initiate the protonema, which is a 2-dimensional algal-like system of branched filaments. The first protonemal stage is called chloronema and typically has hyaline cell walls, with transverse cross-walls and numerous round chloroplasts. The caulonema develops later, with pigmented cell walls, oblique cross-walls, and fewer and smaller plastids. The caulonema is the primary source of gametophore buds. The cauolonema from a single spore can give rise to multiple gametophores. The gametophore is the 3-dimensional and more persistent life stage of mosses which could form either acrocarpous or pleurocarpous shoot systems, which appear as cushions or tufts respectively as spreading carpets or wefts.

Study species

We included six common species, *Atrichum undulatum, Bryum argenteum, Ceratodon purpureus, Funaria hygrometrica, Hypnum cupressiforme* and *Leptobryum pyriforme*, which frequently produce sporophytes with overlapping spore dispersal period in the early autumn. It is, therefore, likely that the spores encounter when deposited on bare ground, although the gametophores only occasionally grow together in nature and do not represent any specific moss community. At this stage, it was more important for us that they represent different phylogenetic lineages (different moss families) and growth forms (Table 1).

Species collection and preparation of spore suspensions

Fully developed specimens with sporophytes and gametophores were collected from Scania, the southernmost province of Sweden, in October (See Table 1 for full information about sampling sites). First, for each species, a ripe unopened capsule was surface sterilized by dipping in 0.5% NaDCC solution for 2 minutes and then thoroughly washed several times in sterile distilled water. After sterilization, each sporophyte was ruptured with a sterile rod in 0.5 ml sterile distilled water

Table 1. Description of the six study species, sampling coordinates, growth form, morphology and ecology.

Species (Family)	Sampling coordinates	Growth Form	Gametophore size	Ecology
Atrichum undulatum (Polytrichaceae)	56°5′32.9″N 13°6′19.9″E	Acrocarp	Stems are up to 7 cm high.	Perennial stayer in shaded, well-drained places.
Bryum argenteum (Bryaceae)	55°42′46.6″N 13°12′19″E	Acrocarp	Stems are up to 1–2 cm high.	Colonist on disturbed ground.
Ceratodon purpureus (Ditrichaceae)	55°42′46.6″N 13°12′19″E	Acrocarp	Stems are up to 3 cm high.	Colonist in exposed habitats, on disturbed ground.
Funaria hygrometrica (Funariaceae)	55°42′44.2″N 13°12′22.3″E	Acrocarp	Stems are about 1 cm high.	Fugitive on exposed and disturbed ground, characteristic of old bonfire sites.
Hypnum cupressiforme (Hypnaceae)	56°5′32.9″N 13°6′19.9″E	Pleurocarp	Stems are prostrate and form dense mats.	Perennial stayer on hard surfaces and on ground.
Leptobryum pyriforme (Meesiaceae)	55°42′44.2″N 13°12′22.3″E	Acrocarp	Stems are up to 1-2 cm high	Colonist in arable fields and disturbed ground.

in a separate sterile microtube. The spore suspensions were homogenized by a vortex mixer (VWR; "lab dancer") and the spore concentration in each suspension was then estimated under a microscope by a haemocytometer. All spore suspensions were diluted to a concentration of approximately 300 spores/100µL. Subsequently, the spore suspensions (one for each species, used throughout the experiment) were stored in darkness in a refrigerator until inoculations.

Growth medium

We used a mineral nutrient medium by Rudolph *et al.* (1988) originally formulated for nutrient-poor *Sphagnum* cultures, with a pH value of 5.6 after sterilization. We applied this medium in a fourfold concentration solidified with 1 % agar. After sterilization in an autoclave, we distributed the nutrient solution into small Petri dishes (diameter=5 cm). Before starting the experiment, we checked that the spores were viable and able to germinate within a short period (5 days) on agar plates.

Experimental design and data analysis

We sowed spores on agar plates in three treatments: (1) as single species cultures (controls), as pairwise species cultures inoculated (2) simultaneously (spore suspension droplets inoculated on top of each other), and (3) with a time lag of 20 days between the first and second species. The single-species cultures were initiated twice, at the start and end of the experiment, to test for loss of germination capacity during storage. We used a nested design for all three treatments with five separate inoculates on each of the three replicated Petri dishes (Figure 1A). All inoculates (=sowing spots) consisted of a single 20 µL droplet of spore suspension distributed with a micropipette. Inoculates contained c. 60 spores (since we calibrated the concentration of spore suspension for each species to about 300 spores per 100µL). Inoculates were distributed in a circle inside Petri dishes to maximize the distance between individual inoculates. All Petri dishes with spores were sealed with medical adhesive tape ("Micropore") since this tape allows for CO^2 and O^2 diffusion between the Petri dishes and the ambient air. The Petri dishes were cultured in a climate chamber at 14°C, at a diurnal cycle of 16 h light/8 h dark. The Petri dishes were repeatedly randomly redistributed during cultivation to reduce position effects. A light fixture from Topanga provided light with a light emitting plasma (LEP) bulb, which gives a spectrum similar to sunlight at a fairly low light intensity, 50 µmol/m²/s. Spore germination and gametophore budding (Figure 1 B–D) was scored with a dissecting microscope. For the fast-growing species *B. argenteum*, *C. purpureus*, *F. hygrometrica* and *L. pyriforme*, the frequency of germinated and ungerminated spores was scored on the 4th day after sowing, while for the slower germinating species *A. undulatum* and *H. cupressiforme*, this data was recorded on the 9th day after sowing (Table 2). The number of gametophore buds for each species was scored on the 40th day after sowing. The exact number of spores in inoculates was somewhat variable. To check if this could

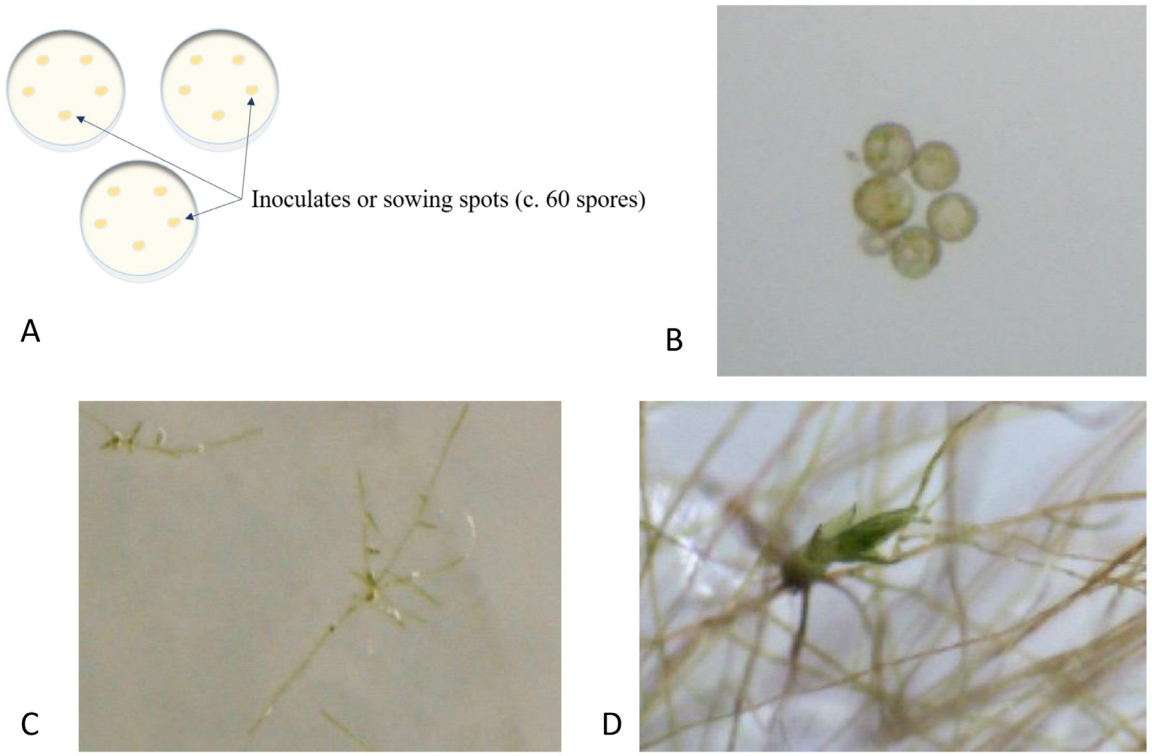

Figure 1. A. Illustration of the nested design (for all three treatments) with five separate inoculates on each of the three replicated Petri dishes. **B**–**D**. Moss development exemplified by *Leptobryum pyriforme*. **B**. Mature spores at sowing on agar plate. **C**. Germinated spores forming protonema, seven days after sowing. **D**. Gametophore budding from the protonema three weeks after sowing.

Table 2. General description of spore size, spore germination and gametophore development for six studied species in single species cultures, mean±standard deviation of 15 replicates in a nested design (five replicates within each of three agar plates).

Species	Spore size (µm)	Germination time (days)	Spore germination rate (%)	Gametophore differentiation time (days)	No. of gametophores per germin-ated spore	Diameter of colony on 15th day (cm)	Diameter of colony on 50th day (cm)	Spore germination rate at the end of experiment (%)
A. undulatum	16-21	3-8	16.6 ±5.9	20-28	0.46 ±0.14	N/A	1.2 ±0.03	1.38 ±0.02
B. argenteum	10-15	2-4	27.3 ±8.7	N/A	0	0.35 ±0.02	0.87 ±0.02	30.1 ±6.5
C. purpureus	10-17	2-4	59.1 ±6.6	20-28	0.05 ±0.05	0.44 ±0.01	0.83 ±0.01	70.8 ±2.4
F. hygrometrica	16-24	2-4	30.6 ±6.7	20-28	0.05 ±0.07	0.45 ±0.01	0.67 ±0.00	26.0 ±7.6
H. cupressiforme	15-25	3-8	31.4 ±6.2	N/A	0	N/A	0.42 ±0.00	32.0 ±7.9
L. pyriforme	9-13	2-4	90.9 ±4.2	20-28	0.19 ±0.13	0.63 ±0.01	1.38 ±0.02	94.4 ±6.29

be a source of error, we tested for density-dependent spore germination in the control treatment by calculating the correlation between spores sown out for each species in relation to the number of spores germinated. We also tested for density-dependent gametophore budding in the control treatment by correlating the number of gametophore buds to the number of spores germinated. The spore germination rates of individual species were compared between the single-species cultivation condition (controls) and each of the two pairwise cultivation conditions (i.e., simultaneous and 20-day time-lag treatments) separately (Figure 2, Table S1–Table S6), as well as gametophore budding rates (Figure 3, Table S1–Table S6), by nested ANOVAs (McDonald, 2014) using a spreadsheet template, fetched from http://www.biostathandbook.com/nestedanova.html. All statistical tests were pairwise, so the only options were either no significance or significance (positive or negative, dependent if facilitation or suppression occurred) in comparison between control and treatment for each species. The data was normally distributed, and no transformations were needed. In many cases, the difference between the treatment and the control was quite strong without or almost with no germination in the treatments.

Results

For the single-species cultures (controls), data on time needed for spore germination, germination rate, the time needed for gametophore differentiation, gametophore per germinated spore, and average colony diameter on the 15th and 50th day after sowing are listed in Table 2. Among the six species, *B. argenteum, C. purpureus, F. hygrometrica,* and *L. pyriforme* germinated quickly after sowing, whereas *A. undulatum* and *H. cupressiforme* germinated much slower. The germination rate ranged from c. 90% in *L. pyriforme* to c. 17% in *A. undulatum*.

Some small, brownish spores (lacking chlorophyll) occurring in the samples of *A. undulatum*, *F. hygrometrica* and *H. cupressiforme* were seemingly unable to germinate. Therefore, they were considered unviable and left out when counting the total number of spores.

The rate of colony expansion differed between species. On the 15th day after sowing, the protonemata of *A. undulatum* and *H. cupressiforme* did not merge into visible colonies because of slow growth rate after germination, but the other four species formed visible round colonies that could be measured by a ruler (Table 2), *L. pyriforme* forming the largest. However, on the 50th day after sowing, the colonies of *A. undulatum* were distinct, being the second largest among the six species.

Most species formed gametophores 20–28 days after sowing; the exceptions were *B. argenteum* and *H. cupressiforme*, which did not develop gametophores at all in single species cultures. *A. undulatum* had the highest amounts of gametophores per germinated spore. The germination rates were unaffected by storage of spore suspensions in a refrigerator for all species except *A. undulatum*, which completely lost germination capacity between the first and the second germination test in single species condition (Table 2). Conspecific spore germination was generally density-independent at the spore concentrations used in the controls, except for *A. undulatum*, which displayed negative density-dependence, suggesting that the spores first to germinate could suppress the germination of other spores (Table 3). Similarly, *A. undulatum* was the only species with density-dependent gametophore budding, suggesting that a higher density of germinated spores induced more buds.

Atrichum undulatum

When cultured with *B. argenteum* and *F. hygrometrica*, respectively, *A. undulatum* did not germinate, whether the spores were sown simultaneously or with a delay. When sown at the same time as *C. purpureus*, *H. cupressiforme* and *L. pyriforme*, the germination rate of *A. undulatum* decreased significantly.

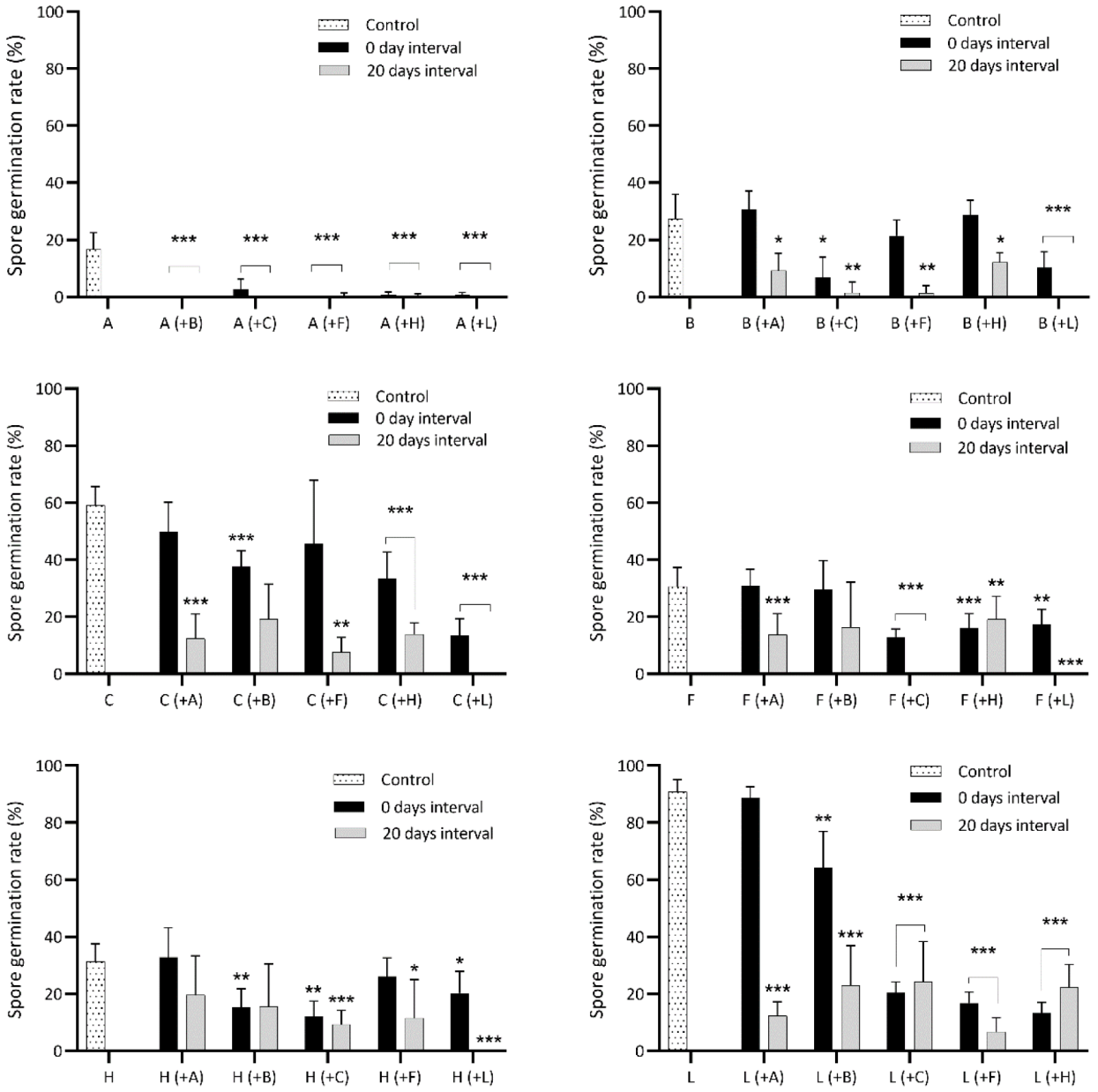

Figure 2. Spore germination rate of six bryophytes when sown in pairwise combinations in axenic culture. Controls are single-species cultures. Each species pair was sown simultaneously and with a delay of 20 days between the first and second species. A, B, C, F, H and L are abbreviations for *A. undulatum, B. argenteum, C. purpureus, F. hygrometrica, H. cupressiforme* and *L. pyriforme*, respectively. Each graph displays the spore germination of controls for a certain species, its spore germination when sown simultaneously with each of the five other species (in parentheses) and its spore germination when sown with a 20-day delay relative to the other species. Bars display mean germination and standard deviation for 15 replicates in a nested design (five replicates within each of three agar plates). The performance of individual species when growing alone (control) versus in combination with another species (sown simultaneously respectively with a 20-day delay) is statistically tested using separate nested ANOVAs. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. See Table S1–Table S6 for detailed comparison between each species' control and treatments.

When sown 20 days later than the three species above, *A. undulatum* showed no germination at all. Considering that *A. undulatum* was the only species that lost spore germinability during storage, the data from the 20 days delay treatment cannot be trusted (Figure 2; Table S1). Only when sown simultaneously with *H. cupressiforme* did *A. undulatum* form gametophores in similar frequency as the control group, and in almost all other cases, no gametophores formed at all (Figure 3; Table S1).

Bryum argenteum

When cultured at the same time as all other five species, *B. argenteum* germinated comparatively well, but its germination rate decreased significantly when grown with

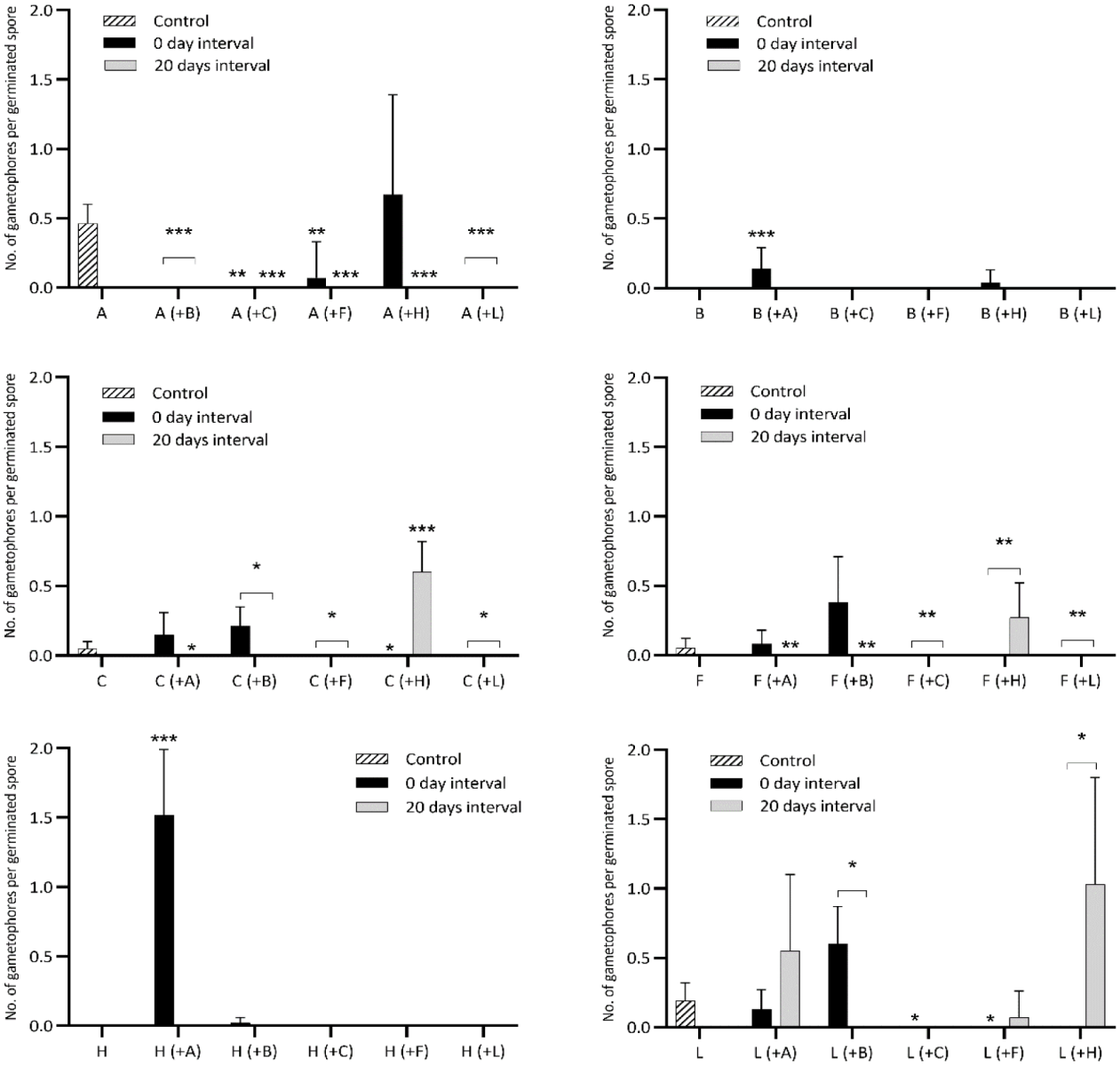

Figure 3. Gametophore budding rate of six bryophytes when sown in pairwise combinations in axenic culture. Controls are single-species cultures. Each species pair was sown simultaneously and with a delay of 20 days between the first and second species. A, B, C, F, H and L are abbreviations for *A. undulatum, B. argenteum, C. purpureus, F. hygrometrica, H. cupressiforme* and *L. pyriforme*, respectively. Each graph displays the gametophore budding in relation to the number of germinated spores of controls for a certain species, its performance when sown simultaneously with each of the five other species (in parentheses) and when sown with a 20-day delay relative to the other species. Bars display mean budding rates and standard deviation for 15 replicates in a nested design (five replicates within each of three agar plates) 40 days after sowing. The performance of individual species when growing alone (control) versus in combination with another species (sown simultaneously respectively with a 20-day delay) is statistically tested using separate nested ANOVAs. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. See Table S1–S6 for a detailed comparison between each species' control and treatments.

C. purpureus and *L. pyriforme*. When sown 20 days later than other species, the germination rate of *B. argenteum* decreased strongly in all species treatments; combined with *F. hygrometrica* and *L. pyriforme* it failed completely to germinate (Figure 2, Table S2). In controls, *B. argenteum* did not form gametophores within 40 days, but when sown at the same time as *A. undulatum,* it did develop gametophores within this period. No gametophores were formed in any cultures with 20 days delay (Figure 3, Table S2).

Ceratodon purpureus

When sown at the same time as the other five species, the germination rate of *C. purpureus* decreased significantly. When sown 20 days later than the others, its germination rate became

Table 3. Correlations between total number of spores and germination frequency respectively between number of germinated spores and number of gametophores for single-species cultures. The correlations are based on 15 replicates in a nested design (five replicates within each of three agar plates).

Species	Mean no. of spores	Correlation coefficient	p	Mean no of gametophores	Correlation coefficient	p
A. undulatum	77	-0.549	**0.034**	5.7	0,518	**0,048**
B. argenteum	51	-0.346	0.201	0	N/A	N/A
C. purpureus	49	0.231	0.408	1.4	0.104	0.712
F. hygrometrica	57	-0.059	0.835	0.86	-0.204	0.465
H. cupressiforme	62	0.132	0.639	0	N/A	N/A
L. pyriforme	62	0.021	0.939	9.9	-0.415	0.123

even lower, especially with *L. pyriforme*, *C. purpureus* did not germinate at all (Figure 2, Table S3). Only when cultured at the same time as *B. argenteum* and 20 days later than *H. cupressiforme* did *C. purpureus* form gametophores, but then with a number of gametophores per germinated spore that was significantly higher than in the controls, and strongly so with *H. cupressiforme*. In all other combinations *C. purpureus* did not form gametophores at all (Figure 3, Table S3). Thus, the development of gametophores of *C. purpureus* could either be promoted or inhibited in co-occurrence with other species (Figure 4).

Funaria hygrometrica

When sown at the same time as the other species the germination rate of *F. hygrometrica* decreased significantly. When sown 20 days later than the other species, germination of *F. hygrometrica* in all treatments was strongly inhibited, with no germination at all when cultured with *C. purpureus* and *L. pyriforme* (Figure 2, Table S4). Only when sown 20 days later than *H. cupressiforme* did *F. hygrometrica* form gametophores and at a significantly higher rate than in the control (Figure 3 and Figure 4, Table S4).

Hypnum cupressiforme

When sown at the same time as other species, the germination rate of *H. cupressiforme* decreased significantly. When sown 20 days later than other species, *H. cupressiforme* was strongly inhibited in the spore germination stage, especially with *L. pyriforme,* it did not germinate at all (Figure 2, Table S5). *H. cupressiforme* formed gametophores only in treatment where it was sown simultaneously with *A. undulatum,* suggesting a promoting effect of *A. undulatum* on its gametophore development, (Figure 3 and Figure 4, Table S5).

Leptobryum pyriforme

In controls, *L. pyriforme* showed a germination rate of 95%, and this germinability was retained nearly so with the fast germinator *B. argenteum*, whereas germinability dropped significantly to around 10-20% with the remaining species. When sown 20 days later than the other five species, the germination rate of *L. pyriforme* was generally around (5-)10-20% (Figure 2, Table S6). When cultured with *C. purpureus*, no matter if sown at the same time or 20 days later, *L. pyriforme* did not form gametophores at all, and almost so with *F. hygrometrica*. When cultured simultaneously with *H. cupressiforme*, no gametophores were formed, but when cultured with 20-day delay, a significantly higher frequency of gametophores budded than the controls (Figure 3). Conversely, gametophores emerged more frequently than the controls when spores were sown at the same time as *B. argenteum* but were absent when sown with a delay (Figure 3, Table S6).

We have summarized the pairwise interactions between species in Figure 4 (a–d). The interactions display a complex pattern involving suppression (blue arrows) and facilitation (yellow arrows).

Discussion

Recent studies of bryophyte community assembly based on occurrence data stress the importance of habitat filtering as well as dispersal capacity (Barbé *et al*., 2016; Lönnell & Hylander, 2018; Tiselius *et al.*, 2019). Whereas the abiotic requirements are relatively well characterized for many species (e.g., for pH values; Tyler & Olsson, 2016), the biotic filtering processes following spore deposition are poorly known. When sown simultaneously, we show pronounced biotic interactions at spore germination involving inhibition of one or the other species. Such effects would impose filtering effects if occurring under natural conditions (Figures 4a, c). The effects were even more evident when we sowed spores with 20 days delay, indicating apparent priority effects, e.g., the dominance of the species first to colonise (Figures 4b, d). This is in line with observations of Watson (1981) suggesting that six *Polytrichum* species differ in their abilities to exclude each other during the juvenile phase. We also observe that some mosses can accelerate the speed of the formation of gametophores in interaction with another species (facilitation) (Figures 4c and 4d).

Density-dependence does not influence the results

Negative density-dependent effects occur commonly in vascular plants when crowded seedlings compete for resources and it seems that the stronger this mechanism is, the higher the

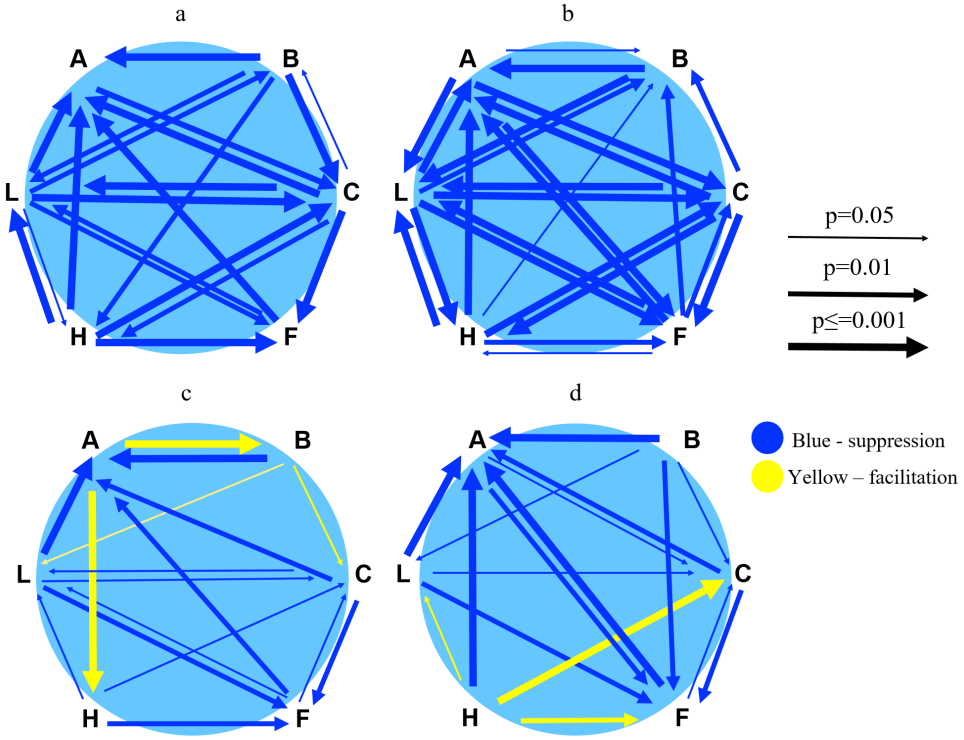

Figure 4. Pairwise interactions between species. a. Matrix displaying interaction in terms of spore germination rate between six species when sown pairwise and simultaneously. **b**. Matrix displaying interaction in terms of spore germination rate between six species when sown pairwise and one species in the pair 20 days later than the first. **c**. Matrix displaying interaction in terms of gametophore budding relative to the number of germinated spores between six species when sown pairwise and simultaneously. **d**. Matrix displaying interaction in terms of gametophore budding relative to number of germinated spores between six species when sown pairwise and one species in the pair with 20 days delay. A, B, C, F, H and L are abbreviations for *A. undulatum, B. argenteum, C. purpureus, F. hygrometrica, H. cupressiforme* and *L. pyriforme*, respectively. Thickness of arrows is proportional to the degree of significance (p=0.05, thin; p=0.01, intermediate; p=0.001, thick). The arrowhead is pointing to the species that is affected by the species at the tail of the arrow. Blue colour indicates suppression and yellow colour indicates facilitation. Arrows are not displayed for non-significant interactions, i.e., when there was no difference between the test and the control.

plant diversity becomes at the global scale (LaManna *et al.*, 2017). Negative density-dependent effects are likely to occur in the early colonization of bryophytes. For example, it is known that high spore densities may cause the failure of caulonema differentiation (self-inhibition) (Zamfir & Goldberg, 2000), which might prevent intraspecific competition among protonemata. Also, Bopp (1959) and later Watson (1981) found that single spores of *Funaria hygrometrica* sown at some distance formed separate protonema that kept apart when encountering, whereas spores sown together freely merged to form a common protonema. We did not see density-dependency in our study at the applied densities, except for *A. undulatum*, which displayed negative density-dependence for spore germination and positive density-dependence at gametophore budding (Table 3), so this factor is unlikely to affect the results.

Spore germination time and lateral expansion differ among species

It is known from the literature (summarized by Glime, 2015) that germination time varies with size, age, available light, water, and nutrients. Acrocarpous species benefit from growing in dense cushions and a single protonema can produce multiple gametophytic buds. In the single species controls, we found that *B. argenteum*, *C. purpureus*, *F. hygrometrica* and *L. pyriforme* germinated as fast as three days after sowing, whereas *A. undulatum* and *H. cupressiforme* needed about seven days (Table 2). Rapid germination would mean that a species could quickly occupy the space (exploitive competition) and earlier turn on the production of allelochemicals (inducing interference competition). In our study, the slowly germinating *H. cupressiforme* performed well when sown simultaneously with the other species (Figure 2) but largely failed to produce gametophore buds (Figure 3). In contrast, the other slow germinator, *A. undulatum* displayed reduced germination, or none at all, when sown simultaneously with the other species (Figure 2). We could see that species showed a different capacity for lateral expansion in single-species cultures, *L. pyriforme* and *A. undulatum* having the most vigorous expansion after 50 days (Table 2).

Except for *A. undulatum*, the germination rate of spores from other species did not decrease after two months storage in 4°C in darkness. The spore germination rate was relatively low for the studied species (except *L. pyriforme*) in

single-species cultures, maybe due to dormancy induced by storage, but this is unlikely to affect the results since the germination rate remained stable over time (except for *A. undulatum*). Data on the longevity of moss spores seems scarce (Pence, 2004) and most studies have tested survival in dry conditions (Proctor *et al.*, 2007), so it is an open question how long they remain viable under cool and dark conditions and to what extent it differs within species.

Exploitive competition in bryophytes

Grime (1973) defined competition as "the tendency of neighbouring plants to utilize the same quanta of light, ion of a mineral nutrient, molecule of water, or volume of space". He also classified plants into three groups with distinct strategies in interspecific interaction: competitive, stress tolerant and ruderal (Grime, 1977). Bryophytes were previously considered ruderal or stress-tolerant due to their small size and low growth rate; however, competition has been shown to be important in structuring bryophyte communities similar to vascular plant communities (Ma *et al.*, 2020; Mulligan & Gignac, 2002; Rydin, 1997). In our study, *A. undulatum* had the lowest spore germination rate (Table 2, Figure 2) and the shortest spore longevity. It also germinated comparatively slowly and responded negatively to crowding. Taken together, it appears that *A. undulatum* is a poor competitor during establishment, as Miles and Longton (1987) concluded from germination studies under field conditions.

Inhibition of spore germination could result from competition for resources such as water, nutrients, and light (exploitive competition). In our experiments, we expect low competition for water and light, the agar medium consisting of water to 98% and being essentially translucent. Competition for nutrients is more realistic since our culture medium is rather low in minerals. We found that *L. pyriforme*, *C. purpureus*, and *F. hygrometrica* occupied the space quicker than *A. undulatum*, *B. argenteum*, and *H. cupressiforme*, suggesting a stronger competitive ability during early colonization.

The case for interference competition

Although most bryophyte studies concern exploitive competition, there is growing evidence that interference competition, in the form of chemical interference (allelopathy), occurs as well (Liu *et al.*, 2020a). For example, growing protonema secretes morphogenetic substances, which regulate the development of other filaments and coordinate the growth and differentiation of neighbouring plants (Reski, 1998). Recent studies of peat mosses have revealed plant-plant interspecific interaction mediated through volatile organic compounds (VOCs) (Vicherová *et al.*, 2020) and also that leachates can affect the microbiome, possibly influencing plant fitness and interspecific competition (Hamard *et al.*, 2019).

It is generally experimentally challenging to distinguish between exploitive and interference competition in natural plant communities (He *et al.*, 2012). Both can operate simultaneously and interact as shown in studies of the grass *Lolium rigidum* Gaud. and the soy bean *Glycine max* L. (San Emeterio *et al.*, 2007). In peatland ecosystems, two closely related co-occurring *Sphagnum* species (from hollow and hummock habitats) showed niche differentiation along a water table gradient due to both resource competition and allelopathic effects (Liu *et al.*, 2020b).

Some observations strongly suggest that chemical (allelopathic) interactions play a vital role in our study. First, the responses were triggered at early spore germination before any resource limitations could arise. Second, facilitation occurred in some pairwise combinations, an unlikely outcome of resource scarcity. As displayed in our matrix figures (Figure 4a and 4b), most interactions during spore germination involve inhibitory effects. Several compounds with negative allelopathic activity (inhibiting germination, growth, and establishment of surrounding plants) have been discovered in bryophytes (Basile *et al.*, 2003; Kato-Noguchi & Seki, 2010; Kato-Noguchi *et al.*, 2010; Nozaki *et al.*, 2007).

On the other hand, we also observed clear beneficial effects (facilitation), particularly during gametophore formation. Notably, *A. undulatum* and *H. cupressiforme* were involved in such interactions (Figure 4). Both *B. argenteum* and *H. cupressiforme* failed to form gametophores in controls. However, they succeeded in doing so when cultured simultaneously with *A. undulatum*, indicating that formation of gametophores was enhanced or speeded up (Figure 3). Rapid formation of gametophores may help mosses occupy new habitats because of earlier resistance to drought and enhanced performance in competition for light.

The roles of competition and facilitation have been assessed in communities of both vascular plants and bryophytes (Bu *et al.*, 2013; Mulder *et al.*, 2001; Pedersen *et al.*, 2001; Rixen & Mulder, 2005), ideally together with environmental factors (Callaway & Walker, 1997). Interspecific facilitation in bryophytes is mainly connected to abiotic factors (light, water levels, different seasons), while intraspecific facilitation is influenced by density-dependent effects and environmental variation (Cornelissen *et al.*, 2007; Pedersen *et al.*, 2001). According to the stress-gradient hypothesis (Bertness & Callaway, 1994), the relative importance of competition decreases, and facilitation increases with an increase in abiotic stress. However, the opposite effect was observed in a *Sphagnum* study (Bu *et al.*, 2013). There are some reports of facilitative effects of bryophytes on vascular plants (e.g., Lett *et al.*, 2018; Soudzilovskaia *et al.*, 2011). Reports that support chemical facilitation in bryophyte communities are few; we have only found two such studies, but they reported contradictory results between laboratory and field experiments and, thus, dependency on environmental conditions (Liu *et al.*, 2020a; Liu *et al.*, 2020b) which suggests a need for further investigation.

Concluding remarks

Bryophytes (mosses, liverworts, and hornworts) are likely to have been competing for space ever since the groups originated and diversified during the Silurian and Devonian periods, long before the advent of seed plants. Our study gives a glimpse of interactions during spore germination and gametophore formation, which are likely to impose profound filtering effects when mosses establish in a new habitat, not the least in places far away from the parent colony (Odu, 1979). Our

results also align with the discrepancy Barbé *et al*. (2016) found between extant bryophyte communities relative to the ones they observed to emerge from aerially dispersed propagules trapped on agar plates. Their data suggest that the spore rain from distant sources often has a stronger influence on community formation than nearby propagule dispersal due to largely unknown filtering mechanisms.

The diversity of compounds produced inside bryophyte tissues is extensively investigated (see, e.g., Asakawa *et al.*, 2013). In contrast, substances released by bryophytes to the ambient environment are almost completely neglected. We envision that bryophyte exudation might compare to rhizosphere exudation in vascular plants, a research area that has exploded in recent years (Bais *et al.*, 2006; Wang *et al.*, 2021).

This study is restricted in the sense that we made it under sterile conditions, with a narrow span of microclimatic variation, which means that most natural stressors and biotic interactions are excluded. Although it is conventional to study interactions between species by comparing their performance in two-species communities with that in single-species culture (Jolliffe, 2000), we acknowledge that this gives a simplified picture of community assembly. We observe considerable differentiation in interactions between species, but the data set is too limited to draw any conclusions related to phylogenetic lineages, habitats or life forms. We avoided focusing on any particular moss community because naturally co-occurring species could have evolved specific adaptations. Oour results exemplify more generalized responses unrelated to any specific habitat context. The species included have relatively small spores but species belonging to the life strategy categories of shuttle species (annual, short-lived and perennial shuttles) sensu During (1979) generally have larger spores as an adaption to frequent disturbance and local secondary recolonization. In future studies, it would be interesting to test if their larger spores infer advantages in exploitive and interference competition. As our study had a narrow genetic basis, including spores from a single sporophyte for each species, testing for variation within species is also desirable.

Our study paves the way for novel experimental designs, which could be used to identify potential bryophyte-released allelochemicals in combination with advanced analytical chemistry techniques. For example, it would be possible to test if the composition of exuded substances differs when the species grow alone or in combination with other species. Such studies, including the comparison of effects under field and laboratory conditions, are crucial to getting deeper insights into the role of interactions and allelopathy in the assembly of bryophyte communities.

Data availability

Dryad. "Spore germination and gametophore development of mosses in response to competition", licensed under a CC0 1.0 Universal (CC0 1.0) Public Domain Dedication license, doi:10.5061/dryad.7d7wm380t.

Authors' contributions

Jingmin Cheng (JC) and Nils Cronberg (NC) designed the experiment. JC made the experimental work and data screening. JC, Isidora Loncarevic (IL) and NC analysed the data and wrote the manuscript.

Supplementary material: Tables S1-6

Table S1. Spore germination rate (no. of germinated spores/total no. spores) and gametophore production (no. of gametophores/no. of germinated spores) for *Artrichum undulatum* (A) as cultured in pairwise combinations with five other species (B = *Bryum argenteum*; C = *Ceratodon purpureus*; F = *Funaria hygrometrica*; H = *Hypnum cupressiforme*; L = *Leptobryum pyriforme*) sown simultaneously (0 days) or with a delay of 20 days (with *A. undulatum* as the second species). Mean value ± standard deviation (SD) is indicated for spore germination rate and gametophore production. The cultures were carried out in a nested design (five replicates within each of three agar plates). The scores from pairwise cultures were analysed against single species cultures using a nested ANOVA. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. S or F in the table stands for the suppression or facilitation in case of significant treatment.

Dependent	Species Treatment	Time Interval	Average±SD	df	F-value	p-value
Spore germination rate	A(with B)	0 day S	0	29	147.9	$2.6\ 10^{-4}$***
		20 days S	0	29	147.9	$2.6\ 10^{-4}$***
	A(with C)	0 day S	2.83%±3.43%	29	84.55	$7.8\ 10^{-4}$***
		20 days S	0	29	147.9	$2.6\ 10^{-4}$***
	A(with F)	0 day S	0.14%±0.38	29	145.0	$2.7\ 10^{-4}$***
		20 days S	0	29	147.9	$2.6\ 10^{-4}$***
	A(with H)	0 day S	0.84%±0.83%	29	127.0	$3.5\ 10^{-4}$***
		20 days S	0	29	147.9	$2.6\ 10^{-4}$***
	A(with L)	0 day S	0.49%±0.78%	29	134.2	$3.2\ 10^{-4}$***
		20 days S	0	29	147.9	$2.6\ 10^{-4}$***

Dependent	Species Treatment	Time Interval	Average±SD	df	F-value	p-value
No. of gametophores per germinated spore	A(with B)	0 day S	0	29	231.6	1.1 10^{-4}***
		20 days S	0	29	231.6	1.1 10^{-4}***
	A(with C)	0 day S	0	29	30.8	0.0052**
		20 days S	0	29	231.6	1.1 10^{-4}***
	A(with F)	0 day S	0.07±0.26	29	30.8	0.0052**
		20 days S	0	29	231.6	1.1 10^{-4}***
	A(with H)	0 day	0.67±0.72	29	1.954	0.235 ns
		20 days S	0	29	231.6	1.1 10^{-4}***
	A(with L)	0 day S	0	29	231.6	1.1 10^{-4}***
		20 days S	0	29	231.6	1.1 10^{-4}***

Table S2. Spore germination rate (no. of germinated spores/total no. spores) and gametophore production (no. of gametophores/no. of germinated spores) for *Bryum argenteum* (B) as cultured in pairwise combinations with five other species (A = *Atrichum undulatum*; C = *Ceratodon purpureus*; F = *Funaria hygrometrica*; H = *Hypnum cupressiforme*; L = *Leptobryum pyriforme*) sown simultaneously (0 days) or with a delay of 20 days (with *B. argenteum* as the second species). Mean value ± standard deviation (SD) is indicated for spore germination rate and gametophore production. The cultures were carried out in a nested design (five replicates within each of three agar plates. The scores from pairwise cultures were analysed against single species cultures using a nested ANOVA. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. S or F in the table stands for suppression or facilitation in case of significant treatment.

Dependent	Species Treatment	Time Interval	Average±SD	df	F-value	p-value
Spore germination rate	B(with A)	0 day	30.65%±6.45%	29	0.5664	0.493 ns
		20 days S	9.24%±6.07%	29	15.99	0.016*
	B(with C)	0 day S	6.81%±7.15%	29	14.00	0.020*
		20 days S	1.49%±3.73%	29	33.48	0.0044**
	B(with F)	0 day	21.43%±5.54%	29	1.707	0.261 ns
		20 days S	1.28%±2.65%	29	32.49	0.0047**
	B(with H)	0 day	28.73%±5.15%	29	0.1061	0.761 ns
		20 days S	12.15%±3.34%	29	10.77	0.0304*
	B(with L)	0 day S	10.29%±5.47%	29	12.80	0.00232**
		20 days S	0	29	38.79	0.0035**
No. of gametophores per germinated spore	B(with A)	0 day F	0.14±0.15	29	163.4	2.2 10^{-4}***
		20 days	0	29	N/A	N/A
	B(with C)	0 day	0	29	N/A	N/A
		20 days	0	29	N/A	N/A
	B(with F)	0 day	0	29	N/A	N/A
		20 days	0	29	N/A	N/A
	B(with H)	0 day	0.04±0.09	29	2.9174	0.163 ns
		20 days	0	29	N/A	N/A
	B(with L)	0 day	0	29	N/A	N/A
		20 days	0	29	N/A	N/A

Table S3. Spore germination rate (no. of germinated spores/total no. spores) and gametophore production (no. of gametophores/no. of germinated spores) for *Ceratodon purpureus* (C) as cultured in pairwise combinations with five other species (A = *Atrichum undulatum*; B = *Bryum argenteum*; F = *Funaria hygrometrica*; H = *Hypnum cupressiforme*; L = *Leptobryum pyriforme*) sown simultaneously (0 days) or with a delay of 20 days (with *C. purpureus* as the second species). Mean value ± standard deviation (SD) is indicated for spore germination rate and gametophore production. The cultures were carried out in a nested design (five replicates within each of three agar plates). The scores from pairwise cultures were analysed against single species cultures using a nested ANOVA. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. S or F in the table stands for suppression or facilitation in case of significant treatment.

Dependent	Species Treatment	Time Interval	Average±SD	df	F-value	P-value
Spore germination rate	C(with A)	0 day	49.89%±10.32%	29	4.800	0.094 ns
		20 days S	12.35%±8.66%	29	128.8	$3.4\ 10^{-4}$***
	C(with B)	0 day S	37.71%±5.52%	29	124.4	$3.7\ 10^{-4}$***
		20 days	19.33%±12.11%	29	0.9701	0.380
	C(with F)	0 day	45.59%±22.33%	29	1.74	0.258 ns
		20 days S	7.66%±5.14%	29	35.40	0.0040**
	C(with H)	0 day S	33.47%±9.27%	29	121.6	$3.8\ 10^{-4}$***
		20 days S	13.89%±4.00%	29	1169	$4.3\ 10^{-6}$***
	C(with L)	0 day S	13.58%±5.83%	29	743.2	$1.1\ 10^{-5}$***
		20 days S	0	29	21.51	$9.7\ 10^{-4}$***
No. of gametophores per germinated spore	C(with A)	0 day	0.15±0.16	29	2.004	0.230 ns
		20 days S	0	29	19.71	0.011*
	C(with B)	0 day F	0.21±0.14	29	15.43	0.017*
		20 days S	0	29	19.71	0.011*
	C(with F)	0 day S	0	29	19.71	0.011*
		20 days S	0	29	19.71	0.011*
	C(with H)	0 day S	0	29	19.71	0.011*
		20 days F	0.43±0.18	29	94.94	$6.2\ 10^{-4}$***
	C(with L)	0 day S	0	29	19.71	0.011*
		20 days S	0	29	19.71	0.011*

Table S4. Spore germination rate (no. of germinated spores/total no. spores) and gametophore production (no. of gametophores/no. of germinated spores) for *Funaria hygrometrica* (F) as cultured in pairwise combinations with five other species (A = *Atrichum undulatum*; B = *Bryum argenteum*; C= *Ceratodon purpureus;* H = *Hypnum cupressiforme*; L = *Leptobryum pyriforme*) sown simultaneously (0 days) or with a delay of 20 days (with *F. hygrometrica* as the second species). Mean value ± standard deviation (SD) is indicated for spore germination rate and gametophore production. The cultures were carried out in a nested design (five replicates within each of three agar plates). The scores from pairwise cultures were analysed against single species cultures using a nested ANOVA. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. S or F in the table stands for the suppression or facilitation in case of significant treatment.

Dependent	Species Treatment	Time Interval	Average±SD	df	F-value	P-value
Spore germination rate	F(with A)	0 day	30.91%±5.77%	29	0.0250	0.882 ns
		20 days S	13.78%±7.38%	29	88.95	$7.0\ 10^{-4}$***
	F(with B)	0 day	29.60%±10.09%	29	0.0718	0.802 ns
		20 days	16.35%±15.82%	29	6.482	0.0636 ns
	F(with C)	0 day S	12.93%±2.78%	29	117.9	$4.1\ 10^{-4}$***
		20 days S	0	29	553.6	$1.9\ 10^{-5}$***
	F(with H)	0 day S	16.13%±4.97%	29	99.9	$5.6\ 10^{-4}$***
		20 days S	19.21%±7.98%	29	36.94	0.0037**
	F(with L)	0 day S	17.31%±5.29%	29	70.36	0.0011**
		20 days S	0	29	553.6	$1.9\ 10^{-5}$***

Dependent	Species Treatment	Time Interval	Average±SD	df	F-value	P-value
No. of gametophores per germinated spore	F(with A)	0 day	0.08±0.10	29	0.3168	0.604 ns
		20 days S	0	29	33.06	0.0045**
	F(with B)	0 day	0.38±0.33	29	4.247	0.108 ns
		20 days S	0	29	33.06	0.0045**
	F(with C)	0 day S	0	29	33.06	0.0045**
		20 days S	0	29	26.39	0.0068**
	F(with H)	0 day S	0	29	33.06	0.0045**
		20 days F	0.27±0.25	29	11.146	0.002**
	F(with L)	0 day S	0	29	33.06	0.0045**
		20 days S	0	29	33.06	0.0045**

Table S5. Spore germination rate (no. of germinated spores/total no. spores) and gametophore production (no. of gametophores/no. of germinated spores) for *Hypnum cupressiforme* (H) as cultured in pairwise combinations with five other species (A = *Atrichum undulatum*; B = *Bryum argenteum*; C= *Ceratodon purpureus;* F = *Funaria hygrometrica*; L = *Leptobryum pyriforme*) sown simultaneously (0 days) or with a delay of 20 days (with *H. cupressiforme* as the second species). Mean value ± standard deviation (SD) is indicated for spore germination rate and gametophore production. The cultures were carried out in a nested design (five replicates within each of three agar plates). The scores from pairwise cultures were analysed against single species cultures using a nested ANOVA. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. S or F in the table stands for suppression or facilitation in case of significant treatment.

Dependent	Species Treatment	Time Interval	Average±SD	df	F-value	P-value
Spore germination rate	H(with A)	0 day	32.87%±10.32%	29	0.0559	0.825 ns
		20 days	26.27%±29.95%	29	0.2009	0.672 ns
	H(with B)	0 day S	15.42%±6.41%	29	26.14	0.0069**
		20 days	15.63%±14.88%	29	3.549	0.133 ns
	H(with C)	0 day S	12.12%±5.37%	29	53.24	0.0019**
		20 days S	9.33%±4.89%	29	255.2	$9.0\ 10^{-5}$***
	H(with F)	0 day	26.12%±6.51%	29	5.933	0.072 ns
		20 days S	11.58%±13.40%	29	12.23	0.025*
	H(with L)	0 day S	20.28%±7.65%	29	16.70	0.015*
		20 days S	0	29	3097	$6.2\ 10^{-7}$***
No. of gametophores per germinated spore	H(with A)	0 day F	1.52±0.47	29	222.6	$1.2\ 10^{-4}$***
		20 days	0	29	N/A	N/A
	H(with B)	0 day	0.02±0.04	29	2.496	0.189 ns
		20 days	0	29	N/A	N/A
	H(with C)	0 day	0	29	N/A	N/A
		20 days	0	29	N/A	N/A
	H(with F)	0 day	0	29	N/A	N/A
		20 days	0	29	N/A	N/A
	H(with L)	0 day	0	29	N/A	N/A
		20 days	0	29	N/A	N/A

Table S6. Spore germination rate (no. of germinated spores/total no. spores) and gametophore production (no. of gametophores/no. of germinated spores) for *Leptobryum pyriforme* (L) as cultured in pairwise combinations with five other species (A = *Atrichum undulatum*; B = *Bryum argenteum*; C= *Ceratodon purpureus;* F = *Funaria hygrometrica*; H = *Hypnum cupressiforme*) sown simultaneously (0 days) or with a delay of 20 days (with *L. pyriforme* as the second species). Mean value ± standard deviation (SD) is indicated for spore germination rate and gametophore production. The cultures were carried out in a nested design (five replicates within each of three agar plates). The scores from pairwise cultures were analysed against single species cultures using a nested ANOVA. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. S or F in the table stands for suppression or facilitation in case of significant treatment.

Dependent	Species Treatment	Time Interval	Average ± SD	df	F-value	p-value
Spore germination rate	L(with A)	0 day	88.69%±3.91%	29	3.614	0.130 ns
		20 days S	12.40%±4.80%	29	3818	$4.1\ 10^{-7}$***
	L(with B)	0 day S	64.25%±12.68%	29	28.43	0.0060**
		20 days S	22.98%±13.89%	29	182.2	$1.7\ 10^{-4}$***
	L(with C)	0 day S	20.39%±3.82%	29	2448	$1.0\ 10^{-6}$***
		20 days S	24.22%±14.13%	29	165.8	$2.1\ 10^{-4}$***
	L(with F)	0 day S	16.77%±3.82%	29	10034	$5.6\ 10^{-8}$***
		20 days S	7.45%±4.77%	29	1593	$2.3\ 10^{-6}$***
	L(with H)	0 day S	13.29%±3.77%	29	5100	$2.3\ 10^{-7}$***
		20 days S	22.40%±7.86%	29	697.9	$1.2\ 10^{-5}$***
No. of gametophores per germinated spore	L(with A)	0 day	0.13±0.14	29	0.734	0.439 ns
		20 days	0.55±0.55	29	2.468	0.191 ns
	L(with B)	0 day F	0.60±0.27	29	11.60	0.027*
		20 days	0	29	8.253	0.045*
	L(with C)	0 day S	0	29	8.253	0.045*
		20 days	0	29	1.655	0.268 ns
	L(with F)	0 day S	0	29	8.253	0.045*
		20 days	0.07±0.19	29	4.109	0.052
	L(with H)	0 day S	0	29	8.253	0.045*
		20 days F	1.03±0.77	29	10.97	0.030*

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Version 2

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✔ **Marko S. Sabovljevic**
Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade, Belgrade, Serbia

The MS can be approved.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Bryophyte Biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 26 February 2024

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✔ **Jennifer K. Rowntree**
University of Plymouth, Plymouth, England, UK

I am satisfied with the authors responses to my comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Ex situ bryophyte conservation and in vitro culture of bryophytes, genetics of species interactions.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 03 November 2023

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?

Jennifer K. Rowntree
University of Plymouth, Plymouth, England, UK

I thought this was a really interesting, and as the authors state, understudied, topic. I found the introduction to be well written and that it did a good job of setting the scene for the research. One typo I noted was that in paragraph 4, I think the fourth sentence should read 'Attempts to experimentally sow…'

I have similar questions to the first reviewer about how the study species were chosen and urge the inclusion of information from the second paragraph of responses in a revised version of the manuscript. I think it is valid that you chose species that could be distinguished from each other on a plate as you describe, but the reader needs to know what motivated the final choice. I also think including the life-strategy categories according to Heinjo During, as you suggest in your responses is a good idea.

I found the description of the experimental design rather unclear and spent some time trying to work out the number of replicate plates you had used per treatment (three I believe). I think inclusion of the N per treatment and total number of plates in the methods would help to clarify this. I do think there are some potential issues with this nested design, which can be overcome with the analysis that you have done, but this does need some careful explanation. If I have understood correctly, there are really 3 true replicates per treatment, each with 5 pseudo-replicated colonies. This is much clearer from the figure legends but needs to also be obvious in the main text. Related to this, and for clarity, please include some details of the nested model used to analyse the data, the statistical software used to analyse the data and information on how the significance values were detected. From the figures I assume that these are differences from the control (Dunnett's test), but this is not necessarily the case.

In light of the design, I also wondered if there was any growth among colonies on a plate as the plates themselves are not that big. This would obviously lead to some uncertainty in the estimates if that were the case.

When you set up the control colonies, did you add two drops of spore solution as you do for the

competition experiments or were these a single drop (and hence half the density of the competition treatments? Please clarify this in the methods.

In the results, you mention removing the small brown spores of some species that did not germinate from estimates of total spores. At was stage was this? Was it in the spore solutions or on the plates as you were counting? Were these really non germinating spores?

In Table 2, can you clarify the final column. I wasn't sure if this was percent germination or absolute number of spores germinated per plate. There is also an unnecessary hyphen in germinated.

In figures 2 and 3, I think it would help to either put the species names at the top of each panel or provide each panel with a letter and then describe the species it relates to in the legend. I know you have the species names in the legends, but it is not that easy to interpret quickly. See also my previous comment about significant differences and what these actually represent.

In Figure 3, I was a bit confused whether the y axis was number of gametophores per germinated spores in each treatment (implied from the axes titles) or the number of gametophores per germinated spores in the control, as stated in the legend. Please clarify this.

Please check the column titles for Table 3 as Mean no. of spores is in there twice, and I assume column 3 is a correlation coefficient. Check also for consistency of decimal point and comma usage in the numbers. I think this table might be better placed earlier in the manuscript, after Table 2 as that is where it is first referred to.

For figure 4 could you add a legend that shows the respective arrow widths and significance values for ease of interpretation?

One final question I had was about the number of sporophytes that the spores originated from. This is driven by a question I have about how more widely applicable these findings might be, or whether there is potential for these particular responses to interactions to be driven by the genetic background of the spores.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Ex situ bryophyte conservation and in vitro culture of bryophytes, genetics of species interactions.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 03 Jan 2024

Nils Cronberg

Thanks a lot for being willing to review our manuscript! We are glad that you appreciated our manuscript! Your detailed and constructive suggestions will allow us to make substantial improvements to the text. I thought this was a really interesting, and as the authors state, understudied, topic. I found the introduction to be well written and that it did a good job of setting the scene for the research. One typo I noted was that in paragraph 4, I think the fourth sentence should read 'Attempts to experimentally sow…' To be changed: 'Attempts to experimentally sow…' *This error has apparently gone unnoticed. We will change this in the updated version.*

I have similar questions to the first reviewer about how the study species were chosen and urge the inclusion of information from the second paragraph of responses in a revised version of the manuscript. I think it is valid that you chose species that could be distinguished from each other on a plate as you describe, but the reader needs to know what motivated the final choice. I also think including the life-strategy categories according to Heinjo During, as you suggest in your responses is a good idea. *We will update this, as already suggested in the response to the first referee.*

I found the description of the experimental design rather unclear and spent some time trying to work out the number of replicate plates you had used per treatment (three I believe). I think inclusion of the N per treatment and total number of plates in the methods would help to clarify this. I do think there are some potential issues with this nested design, which can be overcome with the analysis that you have done, but this does need some careful explanation. If I have understood correctly, there are really 3 true replicates per treatment, each with 5 pseudo-replicated colonies. This is much clearer from the figure legends but needs to also be obvious in the main text. Related to this, and for clarity, please include some details of the nested model used to analyse the data, the statistical software used to analyse the data and information on how the significance values were detected. From the figures I assume that these are differences from the control (Dunnett's test), but this is not necessarily the case. *Yes, we used a nested design for the experiments and then a nested (hierarchical) ANOVA for the statistical analyses. The reason for using this approach was that we had no space to place all replicates on separate agar plates. The strength of a nested*

design is that you can evaluate if there is any effect of the levels in the hierarchy (in this case, 3 agar plates vs 5 spots in each agar plate). There were no such effects. We will point out this in the updated version. We used an Excel spreadsheet provided by the referenced book (McDonald 2014), but this should have been expressed more implicitly. All statistical tests were pairwise, so the only options were either no significance or significance (positive or negative, dependent if facilitation or suppression occurred). So, no tests for contrasts were necessary. Changed text (Methods): The spore germination rates of individual species were compared between the single-species cultivation condition (controls) and each of the two pairwise cultivation conditions (i.e., simultaneous and 20-day time-lag treatments) separately (Figure 2, Table S1–Table S6) by nested ANOVAs (McDonald, 2014) using a spreadsheet template fetched from http://www.biostathandbook.com/nestedanova.html. The gametophore budding rates were in the same way compared for each species between the controls and the budding rates in pairwise culture (Figure 3, Table S1–Table S6). All statistical tests were pairwise, so the only options were either no significance or significance (positive or negative, dependent on facilitation or suppression occurred) in comparison between control and treatment for each species. Data was normally distributed, and no transformations were needed. In many cases, the difference between the treatment and the control was quite strong without or almost with no germination in the treatments.

In light of the design, I also wondered if there was any growth among colonies on a plate as the plates themselves are not that big. This would obviously lead to some uncertainty in the estimates if that were the case. *No, the colonies did not mix into each other during the time the experiment lasted.*

When you set up the control colonies, did you add two drops of spore solution as you do for the competition experiments or were these a single drop (and hence half the density of the competition treatments? Please clarify this in the methods. *No, we added just a single drop for the control colonies. We focused on adding the same amounts of spores for each species in the control and competition treatments. So, the amounts of spores were always the same. The additional water when applying two drops was considered insignificant, given that the agar was 98% water, and the additional water was rapidly absorbed by the agar. However, we will make this clearer in an updated version of the manuscript.* In the results, you mention removing the small brown spores of some species that did not germinate from estimates of total spores. At was stage was this? Was it in the spore solutions or on the plates as you were counting? Were these really non germinating spores? *We considered these spores necrotic, so removing them from the calculations was logical. We also checked the germination frequency at the end of the experiment. The germination frequency remained the same (except for Atrichum), so we expect this is not an error source*.

In Table 2, can you clarify the final column. I wasn't sure if this was percent germination or absolute number of spores germinated per plate. There is also an unnecessary hyphen in germin-ated. *I see that there is a missing (%) in the column for "Spore germination rate at the end of the experiment". I will also check if we can get the hyphen removed in the updated version.* In figures 2 and 3, I think it would help to either put the species names at the top of each panel or provide each panel with a letter and then describe the species it relates to in the legend. I know you have the species names in the legends, but it is not that easy to interpret quickly. See also my previous comment about significant differences and what these

actually represent. *The significances relate to the growth in controls. So, the question is if each species grows better or worse in combination with another species compared to when it grows alone. We will make this clearer in the legend. In addition to the legends, bars display mean germination and standard deviation for 15 replicates in a nested design (five replicates within each of three agar plates). "The performance of individual species when growing alone (control) versus in combination with another species (sown simultaneously respectively with a 20-day delay) is statistically tested using separate nested ANOVAs. Statistical significance levels p=0.05, p=0.01, p=0.001 are marked "*", "**", "***", respectively. "*

In Figure 3, I was a bit confused whether the y axis was number of gametophores per germinated spores in each treatment (implied from the axes titles) or the number of gametophores per germinated spores in the control, as stated in the legend. Please clarify this. *The first bar is the control. The other bars reflect the growth under pairwise conditions. Just like for Figure 2, this needs to be better explained.*

Please check the column titles for Table 3 as Mean no. of spores is in there twice, and I assume column 3 is a correlation coefficient. Check also for consistency of decimal point and comma usage in the numbers. I think this table might be better placed earlier in the manuscript, after Table 2 as that is where it is first referred to. *Yes, you are right. This should be Correlation coefficient. We will update the Table numbering.*

For figure 4 could you add a legend that shows the respective arrow widths and significance values for ease of interpretation? *Ok. We will consider this. In such a case, it would also be good to explain the colours of the arrows in the figure.*

One final question I had was about the number of sporophytes that the spores originated from. This is driven by a question I have about how more widely applicable these findings might be, or whether there is potential for these particular responses to interactions to be driven by the genetic background of the spores. *Yes, the spores came from a single sporophyte for each species. We had a passus about this in an earlier version of the ms, but it was removed when we were forced to reduce the number of words to follow a format restriction. This is, of course, a shortcoming of the experiment, but within-species variation would be possible to investigate in the following studies. We included a new sentence at the end of the discussion: " As our study had a narrow genetic basis, including spores from a single sporophyte for each species, there is also a need to test for variation within species."*

Competing Interests: No competing interests were disclosed.

Reviewer Report 06 October 2023

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Maxine Watson
Indiana University Bloomington, Bloomington, Indiana, USA

This is an interesting, well-written paper, that address – as the authors note – a rarely considered aspect of moss biology. The paper "Interspecific competition affects spore germination and gametophore development of mosses" examines the early interactions of six species of moss in axenic culture to infer the role of competition in their abilities to establish and initiate populations.

My main concern is the study's small size and thus limited replication, and that their findings of significant results may be owed in part to the difficulty some of the species had in establishing in culture. None-the-less, their findings are a unique contribution that may well spark others to pursue similar investigations.

I have another issue of concern and that is their discussion of the role of allelopathy in affecting the relative behaviors of their subject species. Allelopathy as a term is defined differently by different people, and thus is a matter of opinion not yet settled. However, I think that some of the phenomena that the authors report may be due to the leaching of growth hormones into the medium, and that these hormones may be produced at different times and at different stages of development in the different species. So I see their effect as an interplay of developmental phenology, time, and proximity. I realize these are my interests and maybe not those of the authors. At any rate, they may want to take a look at:

Watson, M.A. 1981. Chemically mediated interactions among juvenile mosses as possible determinants of their community structure. J. Chem. Ecol. 7:367-376

and the references to Bopp's work therein.

References

1. Watson MA: Chemically mediated interactions among juvenile mosses as possible determinants of their community structure.*J Chem Ecol*. 1981; **7** (2): 367-76 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant developmental ecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 03 Jan 2024

Nils Cronberg

Thanks a lot for being willing to review our manuscript! We are glad that you appreciated our manuscript! This is an interesting, well-written paper, that address – as the authors note – a rarely considered aspect of moss biology. The paper "Interspecific competition affects spore germination and gametophore development of mosses" examines the early interactions of six species of moss in axenic culture to infer the role of competition in their abilities to establish and initiate populations.

My main concern is the study's small size and thus limited replication, and that their findings of significant results may be owed in part to the difficulty some of the species had in establishing in culture. None-the-less, their findings are a unique contribution that may well spark others to pursue similar investigations. *Regarding the size of the study, we must put forward that a large amount of work was put into the experiments. With 6 species in pairwise comparison, it means 30 contrasts x 3 petri dishes x 5 spots = 450 spore counts for simultaneous spore germination and equally many for delayed germination. The same number of counts for gametophore emergence. On top of that, 6 x 15 spore counts for controls were repeated twice.*

I have another issue of concern and that is their discussion of the role of allelopathy in affecting the relative behaviors of their subject species. Allelopathy as a term is defined differently by different people, and thus is a matter of opinion not yet settled. However, I think that some of the phenomena that the authors report may be due to the leaching of growth hormones into the medium, and that these hormones may be produced at different times and at different stages of development in the different species. So, I see their effect as an interplay of developmental phenology, time, and proximity. I realize these are my interests and maybe not those of the authors. At any rate, they may want to take a look at: Watson, M.A. 1981. Chemically mediated interactions among juvenile mosses as possible determinants of their community structure. J. Chem. Ecol. 7:367-376 and the references to Bopp's work therein. *We agree that allelopathy can be defined in different ways. Our results point to the effects of substances excreted by the bryophytes on the ambient environment. It can be discussed whether these substances are just leaked waste products or if they are actively exudated to exclude or promote other species as a means of competition or facilitation. It is clear that developmental phenology could play a role here. In this case, our intention was to study the very first stages of bryophyte development during spore germination and gametophore budding. We will include references to Watson and Bopp in an updated version of the article. In fact, these references were included in an earlier version of the manuscript but were excluded in favour of a broader review by Janice Glime, when we had to reduce the text to*

meet specific word count restrictions.

Competing Interests: No competing interests were disclosed.

Reviewer Report 04 October 2023

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Linda Fuselier
University of Louisville, Louisville, Kentucky, USA

Attention to competition in colonization processes related to community composition and the study of interspecific interactions in bryophytes (outside of *Sphagnum*) is lacking in the literature. As such, this paper presents a needed study that address a gap in our understanding of bryophyte ecology. That said, studies of competition are not easy to design and implement. I recommend several changes to the manuscript and encourage authors to consider a more "methods" focused paper. Ultimately, focusing on the advantages and disadvantages of the methods and species used will be important to subsequent studies.

Stochastic processes are likely important for bryophyte assemblages that depend on long distance dispersal for colonizers. The first paragraph of the introduction applies mainly to vascular plants rather than bryophytes (see Monteiro et al. 2023 Journal of Ecology).

Need to define colonization – includes germination and establishment; also some of the "colonizers" that are first to appear on bare soil are not those with small spores but rather large spores – you might want to use During's life cycle classifications when specifically explaining bryophyte colonization; the life span may be significant to ultimate community composition.

It is unclear why you chose the species you chose. *Bryum, Ceratodon, Funaria* are early colonizers of bare substrate but *Atrichum, Hypnum* not as much. Please add an explanation of your species choice that supports the comparisons and methods. Were there any species that you attempted to germinate but did not germinate on the agar and thus decided not to include in the study? If you wish to connect this to community assemblage composition, it would make sense to use species that are likely to be germinating and establishing together.

In thinking about a broad readership, you might also explain why you checked for density dependence at the germination stage. Table 1 is nice but if it had to be cut down, the specific sampling coordinates could be omitted; the addition of spore size would be helpful. I suggest moving spore size to table 1 since information in Table 2 is data collected during the study but spore size is more a characteristic of the species. I'm assuming that you did not measure the spore size because it is not mentioned in the methods. Table 3 has a duplicate column heading

(no. of spores).

Germination does not mean establishment. Although gametophores are produced, depending on the species, the plants may mature earlier or later and produce spores or not and influence what can colonize the area. This will also be influenced by time to germination which was much slower in two of the species you used, both of which were perennials. There are many variables to disentangle so it is difficult to draw conclusions from these particular tests (especially for Atrichum and Hypnum). Results using gametophores are more interesting and very different from those of simply germination; this should be the focus of the discussion.

The germination rates for some species seem very low even in control situations and for known colonizers. This could indicate a mismatch between agar conditions and naturally occurring germination conditions.

The conclusions accurately point out that this is a study paving the way for additional studies of exudation, facilitation and competition at this stage of assembly. However, any claims about rhizosphere exudation are a stretch – this was not specifically tested here.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Bryophyte ecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 03 Jan 2024

Nils Cronberg

Thanks for insightful and constructive suggestions! Attention to competition in colonization processes related to community composition and the study of interspecific interactions in bryophytes (outside of *Sphagnum*) is lacking in the literature. As such, this paper presents a needed study that address a gap in our understanding of bryophyte ecology. That said, studies of competition are not easy to design and implement. I recommend several changes to the manuscript and encourage authors to consider a more "methods" focused paper. Ultimately, focusing on the advantages and disadvantages of the methods and species used will be important to subsequent studies. *We already have a rather strong focus on methodology, and we stress in the concluding remarks that we pave the way for novel experimental designs based on our study. We also briefly mention the limitations of our approach. We have expanded the part of the discussion where we discuss the limitations in a new version of the article.*

Stochastic processes are likely important for bryophyte assemblages that depend on long distance dispersal for colonizers. The first paragraph of the introduction applies mainly to vascular plants rather than bryophytes (see Monteiro et al. 2023 Journal of Ecology). *Yes, the introduction is intended to broadly introduce the research area for plants and then focus on bryophytes. The Monteiro publication was unavailable when we finished our ms, but we will incorporate it into an updated text version. As a matter of fact, a major conclusion from the study of Monteiro et al is that at fine scale, environmental filtering prevails in stressful habitats, while competitive interactions appear more important in more favourable conditions. We have also found another quite important reference on this topic, which will be incorporated into the updated version: Barbé M, Fenton N J, Bergeron Y: So close and yet so far away: long-distance dispersal events govern bryophyte metacommunity reassembly. J Ecol. 2016;104(6):1707-1719. https://doi.org/10.1111/1365-2745.12637*

Need to define colonization – includes germination and establishment; also some of the "colonizers" that are first to appear on bare soil are not those with small spores but rather large spores – you might want to use During's life cycle classifications when specifically explaining bryophyte colonization; the life span may be significant to ultimate community composition. *All the included species frequently have sporophytes and small spores, so they are likely to be present in a common spore cloud during early autumn. It is possible that species with large spores and shorter dispersal capacity (shuttle species, sensu During) are more competitive. We did not include any of those species, which would be interesting to study in future experiments. We have added a reference to this in the final part of the discussion.*

It is unclear why you chose the species you chose. *Bryum, Ceratodon, Funaria* are early colonizers of bare substrate but *Atrichum, Hypnum* not as much. Please add an explanation of your species choice that supports the comparisons and methods. Were there any species that you attempted to germinate but did not germinate on the agar and thus decided not to include in the study? If you wish to connect this to community assemblage composition, it would make sense to use species that are likely to be germinating and establishing together. *Yes, we are aware that the included species differ in various respects, such as life span duration and life strategies. We chose to include species representing different taxonomic groups and different life strategies because this seemed to be a logic first step for a pioneering study. Also, we had to use species that had spores and gametophore buds, which could be pairwise discriminated (by size, shape and colour) on agar plates in a microscope. We did not test any*

other species. As stated under "Study species", the species included in the study do not occur in any specified community, although they can sometimes be found growing adjacent to each other in southern Sweden. All are growing on the ground (Hypnum also on other substrates) and at similar pH levels. At this stage, there is no data to assume that species forming communities have stronger or weaker pairwise interactions. Likewise, it is not evident how species representing different life strategies would differ in terms of interactions during germination. We have modified the text under the concluding remarks to make this clearer.: "We observe considerable differentiation in interactions between species, but the data set is too limited to draw any conclusions related to phylogenetic lineages, habitats or life forms. We avoided focusing on any particular moss community because naturally co-occurring species could have evolved specific adaptations – our results should be seen as examples of more generalized responses." The most important point is that the included species all produce abundant sporophytes and release their spores during the same period in the late summer. This is pointed out under "Study species" and will be further emphasized in the revised version. The earlier mentioned study by Barbé et al. supports our conception that abundant spore production is important for the regional spore cloud and the chance of encounter at spore deposition.

In thinking about a broad readership, you might also explain why you checked for density dependence at the germination stage. *We have made this clearer by a small addition: "The exact number of spores in inoculates was somewhat variable. To check if this could be a source of error, we tested for density-dependent spore germination in the control treatment..."* Table 1 is nice but if it had to be cut down, the specific sampling coordinates could be omitted; the addition of spore size would be helpful. I suggest moving spore size to table 1 since information in Table 2 is data collected during the study but spore size is more a characteristic of the species. I'm assuming that you did not measure the spore size because it is not mentioned in the methods. Table 3 has a duplicate column heading (no. of spores). *We avoid making unnecessary changes in the tables because of the risk of introducing new errors. However, the duplicate column (no. of spores) is a mistake, the second column should be correlation coefficient.*

Germination does not mean establishment. Although gametophores are produced, depending on the species, the plants may mature earlier or later and produce spores or not and influence what can colonize the area. This will also be influenced by time to germination which was much slower in two of the species you used, both of which were perennials. There are many variables to disentangle so it is difficult to draw conclusions from these particular tests (especially for Atrichum and Hypnum). Results using gametophores are more interesting and very different from those of simply germination; this should be the focus of the discussion. *We disagree to some extent here. Germination is the first step of colonization after dispersal. The spore germination tests were performed both with simultaneous sawing and with a delay. The results are similar but stronger with a delay. We are pointing out the limitations in the interpretation of Atrichum.*

The germination rates for some species seem very low even in control situations and for known colonizers. This could indicate a mismatch between agar conditions and naturally occurring germination conditions. *Yes, maybe also the fact that we stored spores in a fridge in a water solution could be responsible for the germination rates. However, we tested the germination frequency at the start and end of the experiments and it differed only for Atrichum,*

so it should not affect the results for the other species.

The conclusions accurately point out that this is a study paving the way for additional studies of exudation, facilitation and competition at this stage of assembly. However, any claims about rhizosphere exudation are a stretch – this was not specifically tested here. Ok. We reformulate a bit. "We envision that bryophyte exudation might compare to rhizosphere exudation...". We actually have some data on the chemical composition of exudates from bryophytes, which is quite diverse. Also, other recent publications point in this direction, but remember where you read it first!

Competing Interests: No competing interests were disclosed.

Reviewer Report 31 August 2023

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Marko S. Sabovljevic
Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade, Belgrade, Serbia

The study itself is pioneer and thus very interesting. However, the design remains blurred and thus the results seems to be rather unsupported. There are too many assumptions. Some of the species are ecologically unrelated and not in natural competition at all. Some have naturally rapid and short life span and the others are perennial, thus completely different life strategies suggesting quick vs. slow germination rate cannot advance in nature always. Some literature can be found on these phenomena.

Also, it is unclear how did the authors distinguished the spores and protonema of different species once you mix them. Are you sure the one protonema is originated from one spore when calculating bud formation. What about tmema cells, ruptures or even brachycites? Not mentioned at all. When did caulonema occur?

Exogenously substances in different concentrations can affect some process positively and other negatively. These can be the answer for many of your assumptions as shown already by Martin Bopp in the 1980s. Also, the effect of spores, protonema or bud stages is not linked and easy to followed in the manuscript.

Life strategies, life forms and finally biological features of various mosses tested here including life span are no considered.
What about photoblastic, light quality and/or recalcitrance in spore germination? There are some of these phenomena mentioned in the previous literature not mentioned in this study.

Also, spore germinability is not equal to spore viability. Some spores are prepared to remain in natural spore banks and to be transferred by various vectors in nature! Not considered here.

Author mentioned that they are aware of understudied bryophyte spore biology but it is not considered within the manuscript. Thus it makes the good idea rather preliminary.

Thus, I suggest major revision and full rewrite of the manuscript.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Bryophyte Biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 08 Sep 2023

Nils Cronberg

Author response to Reviewer 1 Marko S. Sabovljevic

Thanks a lot for being willing to review our manuscript! We appreciate that you checked our manuscript swiftly with many thoughtful suggestions! The referee texts are written in italic text and our responses in bold text. Changed texts are underlined. We will make a revised version of the manuscript based on the suggestions communicated here. We will do this when we have received reports from additional referee(s).

The study itself is pioneer and thus very interesting. However, the design remains blurred and thus the results seem to be rather unsupported. There are too many assumptions. Some of the species are ecologically unrelated and not in natural competition at all. Some have naturally rapid and short life span and the others are perennial, thus completely different life strategies suggesting quick vs. slow germination rate cannot advance in nature always. Some literature can be found on these phenomena.

Yes, we are aware that the included species differ in various respects such as life span duration and life strategies. We chose to include species representing different taxonomic groups and different life-strategies because this seemed to be a logic first step for a pioneering study. Also, we had to use species that had spores and gametophore buds which could be pairwise discriminated (by size, shape and colour) on agar plates in a microscope. As stated under "Study species" the species included in the study do not occur in any specified community, although they can sometimes be found growing adjacent to each other in southern Sweden. All are growing on the ground (Hypnum also on other substrates) and at similar pH levels. At this stage, there is no data to assume that species forming communities have stronger or weaker pairwise interactions. Likewise, it is not evident how species representing different life strategies would differ in terms of interactions during germination. The most important point is that the included species all produce abundant sporophytes and release their spores during the same period in the late summer. This is pointed out under "Study species" but needs to be further emphasized in a revised version. The spores that become airborne are likely to mix with each other in the air, much like wind-dispersed pollen. During rainfall, they will be washed to the ground and are likely to encounter on potential colonization spots on bare ground.

Also, it is unclear how did the authors distinguished the spores and protonema of different species once you mix them. Are you sure the one protonema is originated from one spore when calculating bud formation. What about tmema cells, ruptures or even brachycites? Not mentioned at all. When did caulonema occur?

As explained above, the spores could be discriminated by differences in size, shape and colour on agar plates in a microscope. We used two variables in the analyses: spore germination frequency and the frequency of gametophore buds relative to the number of spores germinated. We scored the spore germination and gametophore numbers at fixed time points. It was difficult, but doable, to identify the spores in the species pairs. It was somewhat easier to identify the gametophore buds. We included pictures of germinating spores and gametophore buds for all species in an earlier version but decided to exemplify with just one species in the present version. We did not score any details of the stages between germination and gametophore bud formation. This could have been interesting but would have required an enormous amount of work, given the number of replicates we had in the study. For this reason, we did not try to compare the development of protonemata between species, nor did we quantify the amounts of protonemata or set a timepoint when the switch between chloronemata and caulonemata took place. This would be interesting to investigate in a subsequent study. It is clear that one spore can produce more than one

gametophore bud, but it was not our focus to investigate the number of gametophores produced by individual spores. This would have required us to work with single-spore cultures. We scored the number of gametophores in each replicate spot and divided this number by the number of germinated spores for the same species (see Table 2 for single-spore tests and Table S1-S6 for pairwise tests). In most cases, the mean number of gametophores per germinated spore was less than one. The number of emerging gametophores will likely increase over time, but we did not check that. We did however check for density dependence.

Exogenously substances in different concentrations can affect some process positively and other negatively. These can be the answer for many of your assumptions as shown already by Martin Bopp in the 1980s. Also, the effect of spores, protonema or bud stages is not linked and easy to followed in the manuscript.

We are aware of the studies by Martin Bopp testing the effect on protonema by different plant hormones. We have included some more recent references on this topic to Vesty et al. 2016, Decker et al. 2006 and Reski 1998. The topic is also reviewed in the referenced texts by Glime. However, we will include references to the research of Bopp in a new version of the manuscript and consider how the research could aid in the interpretation of our data. However, as far as we know he has not studied interspecies interaction between protonemata.

A reference to Maxine Watsons work is also relevant in the introduction (changes underlined):

Plant allelopathy is defined as interference concerning growth due to chemical interactions between plants and other organisms, mediated by the release of plant-produced bioactive or toxic specialized metabolites referred to as allelochemicals (Latif *et al.*, 2017). Such metabolites could have beneficial (stimulatory) or detrimental (inhibitory) effects on target organisms (Cheng & Cheng, 2015; Zhang *et al.*, 2021). Interactions between moss species in their earliest growth stages have received little attention (Watson, 1981). Based on studies by (Bopp, 1959) and own observations Watson (1981) suggested that "differences among individuals (taxa) in arrival time and susceptibility to leaked chemicals would appear likely to effect the outcome of competitive interactions among them". As for the discussion: Negative density-dependent effects occur commonly in vascular plants when crowded seedlings compete for resources and it seems that the stronger this mechanism is, the higher the plant diversity becomes at the global scale (LaManna *et al.*, 2017). Negative density-dependent effects are likely to occur in the early colonization of bryophytes. For example, it is known that high spore densities may cause the failure of caulonema differentiation (self-inhibition) (Zamfir & Goldberg, 2000), which might prevent intraspecific competition among protonemata. Also, Bopp (1959) and later Watson (1981) found that single spores of *Funaria hygrometrica* sown at some distance formed separate protomema that kept apart at encounter, whereas spores sown together freely merged to form a common protonema. We did not see density-dependency in our study at the applied densities, except for *A. undulatum*, which displayed negative density-dependence for spore germination and positive density-dependence at gametophore budding (Table 3), so this factor is unlikely to affect the results. Bopp, M. (1959). Versuche zur analyse von Wachstum

und Differenzierung des Laubmoosprotonemas. *Planta, 53*, 178-197. Watson, M. A. (1981). Chemically mediated interactions among juvenile mosses as possible determinants of their community structure. *Journal of Chemical Ecology, 7*(2), 367-376.

Life strategies, life forms and finally biological features of various mosses tested here including life span are no considered.
What about photoblastic, light quality and/or recalcitrance in spore germination? There are some of these phenomena mentioned in the previous literature not mentioned in this study.

The growth forms and life strategies are briefly accounted for in Table 1. We will expand on this and more strictly follow the life-strategy categories according to Heinjo During. This will also be followed up more in depth in the discussion of an updated version of the manuscript. However, it is difficult, based on the limited number of species in the data set, to trace patterns based on the life-strategy concept. The study was performed in a growth chamber. With respect to light, we used a light emitting plasma (LEP) fixture which has a light spectrum which is similar to sun light. The light intensity was fairly low, but uniform, at level of the table we used for cultures. We were aware of the risk for position effects, so the Petri dishes were randomly redistributed at regular intervals during the study. This is described under "Experimental design and data analysis". The important point was that all replicates were subject to the same conditions to avoid interfering effects of phenomena mentioned by the reviewer, such as photoblastic differences, differential response to light quality or dormancy which surely occur among the species. In the statistical treatment we did not see any systematic effects relating to the Petri dish level or the individual spot within Petri dish.

Also, spore germinability is not equal to spore viability. Some spores are prepared to remain in natural spore banks and to be transferred by various vectors in nature! Not considered here.

Yes, good point. As the spore germination was far from complete in most of our species, dormancy is one possible explanation. We should stress this. We cite a study by Vesty et al 2016 which states (partially based on other studies) that primary dormancy lacking in several bryophytes. We will look more into this, but as stated by Glime, there are few studies of dormancy in bryophytes and even fewer that test the potential for differential degree of dormancy in individual sporophytes. On the other hand, there are many studies indicating diaspore banks in the soil, sometimes persistent during decennia, but this is somewhat outside the scope of this study. However, primary dormancy is unlikely to affect our results. We tested the germination frequency of all species in single-species agar plates at the start as well as the end of the experiment. We did this to check for changes in spore germinability over time. The spore germination rate did not differ, except for Atrichum which dropped considerably in spore germination frequency, probably due to spore necrosis during storage.
As for the discussion:

Except for *A. undulatum*, the germination rate of spores from other species did not decrease after two months storage in 4°C in darkness. The spore germination rate was relatively low

for the studied species (except *L. pyriforme*) in single-species cultures, maybe due to dormancy induced by storage, but this is unlikely to affect the results since the germination rate remained stable over time (except for *A. undulatum*). Data on the longevity of moss spores seems scarce (Pence, 2004) and most studies have tested survival in dry conditions (Proctor *et al.*, 2007), so it is an open question how long they remain viable under cool and dark conditions and to what extent it differs within species.

Author mentioned that they are aware of understudied bryophyte spore biology but it is not considered within the manuscript. Thus it makes the good idea rather preliminary.

Yes, bryophyte spore biology is a generally neglected research area. In our introduction and discussion, we concentrate on interactions between bryophyte species at germination as this is the focus of our study.

Competing Interests: No competing interests were disclosed.