Interactions in bryophytes using a new in vitro culture method reveal negative and positive interspecific effects in the sporelings of two moss species

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Abstract

In vitro culture experiments are crucial for the studies of chemical-mediated interactions in plants. However, distinguishing spores and sporelings of different species of bryophytes in mixed cultures poses a serious drawback for research on early developmental stages. Here we propose a modification of the sandwich technique, a standard method to explore allelopathic effect of plants, and present a case-study using two common mosses. As in the standard sandwich method, we have created a physical barrier using gelled medium, and inoculated spores of *Tortula muralis* and *Syntrichia ruralis* in two layers. To assess their intra- and interspecific interactions, we measured protonemata green coverage using image analysis, and degree of sporeling development using a categorical index. We successfully obtained physically separated sporelings of target and emitters from spores of these two species. The green-coverage analysis showed no differences in any of the comparisons. However, the developmental index shows a negative effect of *T. muralis* on *S. ruralis*, while *S. ruralis* apparently promotes the development of *T. muralis*. The method here proposed is successful for culturing moss spores, so that the different inocula are physically separated while allowing diffusion of water-soluble and volatile substances. For testing interactions in these early stages of the gametophyte, we recommend measuring the degree of development of moss sporelings rather than their coverage. Our results have revealed the existence of both positive and negative interspecific relationships between *T. muralis* and *S. ruralis* sporelings, supporting that positive interactions in bryophytes might be more common than previously thought.

INTRODUCTION

Plant interactions are often regulated by the release of biochemicals that can act as inhibitors or promoters of other plants (Whittaker et al. 1970). These chemical interactions, both positive and negative, have large ecological significance as they affect species distributions, condition the interactions and have an important role in the maintenance of biodiversity (Hierro and Callaway, 2021). There are many studies on chemical interactions in vascular plants (Rice, 1984; Zhang et al. 2021), but bryophytes have lagged behind (Whitehead et al. 2018). Recent studies have substantially advanced our knowledge showing the importance of chemical interactions for competitive dynamics between bryophytes in peatlands (Fenton and Bergeron, 2006 p. 2010; Turetsky et al. 2010; Bu et al. 2013; Liu et al. 2020b), characterizing the production of secondary metabolites including allelopathic compounds (Tsubota et al. 2006; Nozaki et al. 2007; Horn et al. 2021) and VOCs (Vicherová et al. 2020), and studying the effect of bryophytes over seed germination (Tsubota et al. 2006; Michel et al. 2011; Soudzilovskaia et al. 2011; Bhadauriya et al. 2016).

However, there are still important gaps in our knowledge about chemical effects.

On the one hand, it is unclear how frequent positive effects are among bryophytes. Field studies suggest that they might be more frequent in this plant group than in vascular plants (Bergamini et al. 2001;

Bowker et al. 2017), but experimental studies are scarce and show contradicting results (Bopp 1963; Watson 1981; Liu et al 2020).

Besides, most studies on competition and chemical-mediated effects focus on the gametophore (the adult gametophyte), usually larger and predominant in the bryophyte life cycle, neglecting early stages of gametophyte development, so crucial to understand bryophyte colonization and establishment. Additionally, the low morphological variation in spores and protonemata (Nehira 1983) makes it difficult to differentiate them for most species when growing together in competition experiments.

Still, protonema is probably the most sensitive stage of the bryophyte cycle. In fact, the few studies that have analysed biotic interactions in sporelings have shown interesting results. In the last century, research on the moss *Funaria hygrometrica* showed complex intraspecific interactions, both autoinhibitory and facilitatory, depending on the developmental stage of the sporelings (Bopp 1963; Watson 1981). In turn, protonemata of *F. hygrometrica* (Johri and Desai 1973) and protoplasts of *Physcomitrium patens* (Schween et al. 2003) have shown intraspecific inhibitory effects that follow a negative dose-response relationship.

In order to extend the studies of bryophyte interspecific interactions, we need to explore new methodologies addressing the difficulties posed by the early stages of the gametophyte development, namely the small size and interspecific similarity of spores and sporelings.

Here we present a modification of the sandwich method designed by Fujii et al. (2003), a standardized test for allelopathic effects of vascular plants (Fujii et al. 2003, 2004). The method uses a gel as a permeable barrier, allowing both the physical separation of the samples under study, and the flux of potentially bioactive substances. In the original design, the dried and powdered material from the emitter (putative allelopathic plant) is disposed between two agar layers, while the standard target of these possible effects, lettuce seeds, are arranged over the upper agar layer (Fig. 1). Through measurements on the length of roots and hypocotyls of the lettuce seedlings, this assay allows a quick identification of the presence of allelopathic compounds in vascular plants (Fujii et al. 2003) or bryophytes (Tsubota et al. 2006). Nevertheless, in the original protocol the emitter material is dead and unable to synthesize compounds actively, so this method does not allow assessing interactions between living plants.

Our modification of Fujii's sandwich method involves two different suspensions of moss spores as living inocula. Again, the emitter inoculum (now consisting of living spores) is placed between the two gelled layers. In place of the lettuce seeds used as the target in the standard Fujii's assay, the second inoculum of spores is placed as target on top of the upper layer. This allows both the germination of the emitter and target inocula, and also the air diffusion and substance interchange between them, as we show subsequently.

As a case-study we use two allied species, *Tortula muralis* Hedw. and *Syntrichia ruralis* (Hedw.) D.Mohr, both in fam. Pottiaceae. These species were selected because they are very common in Mediterranean

areas, also in the Iberian Peninsula (Guerra and Cross, 2006), and often coexist, making it more relevant to study their possible interactions.

MATERIALS AND METHODS

Plant material —

For our experiments, we selected two widespread moss species: *Tortula muralis* Hedw. and *Syntrichia ruralis* (Hedw.) Weber & D.Mohr. Both belong to fam. Pottiaceae and are very common in Mediterranean areas. *T. muralis* is a saxicolous, basophilous species, whereas *S. ruralis* may grow on soils and on rocks and is able to colonize both basic and slightly acid substrates. Both species often coexist, so it is relevant to assess their possible interactions. Their spores are quite similar: in *Syntrichia ruralis* they are 10–15 µm in size, while the spores of *Tortula muralis* are 7.5–12.5 µm (Guerra and Cross, 2006), and in both species they often contain large lipid droplets and are protected by a brownish, rugose sporoderm.

We collected fresh material with mature sporophytes of both species, as follows:

Tortula muralis: Spain, Madrid: Stone wall bordering Arroyo Meaques, 40.417020, -3.735322, 600 m. Leg. & det.: B. Estébanez, 20 June 2019

Syntrichia ruralis: Spain, Madrid, Rascafría: Stone wall near Arroyo del Artiñuelo, 40.9043, -3.8819, 1160 m. Leg. & det.: B. Estébanez, 17 Jul. 2020.

Voucher specimens are kept at MA-UAM herbarium (Universidad Autónoma de Madrid).

Culture conditions —

We used a modified Murashige-Skoog (MS) (Murashige and Skoog 1962) culture medium (Rowntree 2006; Sargent 1988), without organic compounds (Sigma, *Murashige-Skoog basal medium 5519)* and at half strength (MS ½: 2.15 g L^{-1}), with pH adjusted to 6.5 with NaOH.

As gelling agent, we employed gellan gum at half the concentration generally used (i.e., 2.15 g/l instead of 4.3 g/L, Gelzan™, Sigma G1910) with heptahydrate sulphate ($MgSO_4$ 7 H_2O, 0.5 g/L), after Rowntree and Ramsay (2005). Gellan gum allows greater hormone effects than agar in *in vitro* cultures (Jansson et al. 1983; Hadeler et al. 1995) and has a crystal-clear transparency, which is important to assure adequate light transmission for the germination and development of the spore inoculum beneath.

Mature, operculate capsules were disinfected *prior to* culture with dichloroisocyanurate (DCI) at 0.1% (Sigma-Aldrich, Troclosennatrium 96%, 892-8). After disinfection, we washed the capsules three times with distilled water and two times with MS ½. Then we squeezed the capsules of each species in an Eppendorf tube with MS ½, suspended the spores, and decanted the capsule debris to obtain an initial spore suspension of 65000–85000 spores.

Modification of the Sandwich Method —

We conducted the modified Fujii's sandwich experiments in Nalgene® transparent jars (Thermo Scientific™ 11-815-10B: 60 mL, ⌀ 55 mm, 45 mm length). As seen in Figs. 1 and 2, our culture sandwich consists of 1) a bottom layer of gelled medium, 8 mL2) a thin film of spore suspension (i.e., the inoculum of the emitter species), 1 mL, 3) an upper layer of gelled medium, 8 mL, and, on top of all, 4) a thin film of spore suspension (inoculum of target species), 1 mL. Each layer of gelled medium was 0.5 cm high in the jar (Fig. 2). The spore suspension of the emitter species, between both gelled layers, is 1 ml of its initial suspension, with a high spore density (65000–85000 spores). In turn, the spore suspension of the target species is 1 ml of a 1:50 dilution of the initial suspension, with lower spore density (to minimize auto-inhibitory effects). This system allows the germination of both inocula and the diffusion of hydrosoluble and volatile substances from the suspension of the emitter species through the gelled upper layer.

We performed a factorial design with all intra- and interspecific paired interactions, using 6 replicates per experiment, and a control per species, using also the low-density suspension as target species, but with just 1 mL of gelled medium instead of the emitter inoculum (also 6 replicates). The cultures were left to grow for 15 days at 24 ºC and a photoperiod of 16:8 light/darkness.

Quantification of the development of protonemata —

As in the standard Fujii's assay, all growth measurements were taken only in the target species. In our method, the emmiter inoculum is alive, but because of its sandwiched placement and of its high density, only suboptimal growth is to be expected. Nevertheless, we also inspected this emmiter layer visually to assure its germination and development (i.e., that it was still living and able to interact with the target species). As indicators of protonematal development of the target species, in all culture jars we 1) measured the green coverage and 2) counted the number of cells of 50 randomly sampled spores or protonemata after the culture period.

We used image analysis to estimate the surface covered by the sporelings (germinated spores and protonemata). First, we cut-off the upper gelled layer of the sandwich (the one with the target inoculum on its top) and took two photographs of the surface at 80x magnification. For each photograph, we selected 20 well-focused squares of 2 mm² per sample (10 squares x 2 photos). We segmented the photos into RGB colour channels and selected the channel with the highest contrast (the blue channel). Then we established a colour threshold to separate protonemata from background. Finally, in each of the squares, we measured the area occupied by protonemata and the percentage they represented with respect to the area of the square. All images were analysed with Fiji 1.52 (Schindelin et al. 2012), the code used to analyse the images with Fiji is available as supplementary material (ESM 1).

To quantify the number of cells per protonema, we added a few drops of water over the culture and gently scratched the surface, then we pipetted the suspended spores and protonemata onto a microscope slide; and we classified 50 random spores or protonemata into 5 categories: one for germinated (undivided) spores, and four for protonemata: with 2-20-cells, with 21-40-cells, with 41-100-cells and with more than 100-cells (Fig. 3).

Statistical analysis —

To analyse whether the surface covered by protonema (green coverage) was affected by the presence of an emitter we fitted a linear mixed model, with the green coverage as the dependent variable, the type of emitting material (control, *Tortula muralis* or *Syntrichia ruralis*) as the independent variable, and a random variable associated with every jar. This model considers that the squares in each jar are not independent samples and corrects differences that may be due to particular factors of each jar. These analyses were done using the package agricolae (Mendiburu, 2021) in R (R Core Team, 2018) and the code is available as Electronic Supplementary Material (ESM 2).

To analyse the effect of the emitter on the protonemal development we fitted an ordinal logistic regression model. We chose this type of model as it is appropriate for categorical variables with a clear order between the different categories (in this case, degree of development see, for example Christensen, 2019). The model includes the effect of ordinal categories, considering the accumulative probability for each of our categories. In other words, it considers that to reach the 21–40 cells a given protonema must reach 2–20 cells first. The output of the model is the log of the odds ratio of the emitter vs. the control. After transforming the variables with an exponential function, the transformed coefficients express the odds ratio for the emitter when compared to the control. That is, if the transformed coefficient is 1.5 the odds of increasing the degree of development are 1.5 times higher in the samples with the emitter than the control. For these analyses, we used the package ordinal (Christensen, 2019) in R 3.6.0 software (R Core Team, 2018) and an $\alpha = 0.05$ was considered as the threshold of significance. This code is available as Electronic Supplementary Material (ESM 3).

RESULTS

Validation of the method to grow spores —

The spores of the emitter grown embedded between two gelled layers of the culture sandwich successfully developed into living protonemata in both species studied, maintaining their vitality throughout the entire experiment (Fig. 1). In turn, also for both species, the sporelings of the target inocula exhibited high survival rates (Fig. 2).

Development of protonemata —

The image analysis of surface covered by the protonemata resulted in an average coverage ranging from 0.218 to 0.302 mm^2 for *T. muralis*, and from 0.055 to 0.092 mm^2 for *S. ruralis.* We did not find statistically significant differences between the diverse experiments (including the controls and the different cross-paired cultures) (Tables 2 and 3). The pictures taken and the ROI squares selected for the analysis are respectively available as Electronic Supplementary Material 4 and 5, additionally the results are also available as EMS 6.

Table 1
Factorial design of our experiment with paired interactions (intra- and interspecific pairs).

Emitter	Target	Type of interaction	Nº of jars
T. muralis	*T. muralis*	intraspecific	6
	S. ruralis	interspecific	6
S. ruralis	*T. muralis*	interspecific	6
	S. ruralis	intraspecific	6

Table 2
Mean of the surface covered by the protonemata in the controls and the treatments. P (p-values) show the results of the ANOVA, SE the standard error and df the degrees of freedom. Tm, *Tortula muralis*; Sy *Syntrichia ruralis*.

	Tm target					Sr target				
	Mean	SE	Df	t value	P	Mean	SE	df	t value	P
Control	0.218	0.042	-	-	-	0.089	0.018	-	-	-
Tm emitter	0.302	0.042	32	1.433	0.173	0.055	0.016	31	1.395	0.162
Sr emitter	0.289	0.044	32	0.102	0.257	0.092	0.016	31	1.153	0.919

Table 3
Mean number of cells per protonema of the target species and standard deviation (sd). Tm, *Tortula muralis*; Sy *Syntrichia ruralis*.

	Tm target		Sy target	
	mean	sd	mean	sd
Control	115	115	44.9	55.5
Tm emitter	172	126	43.0	16.4
Sr emitter	135	123	24.0	38.2

As for the sporeling development, we did not observe unicellular spores in any of the two species. In the experiments with *T. muralis* as the target species (Fig. 4), the control treatment resulted in an average of 115 cells per protonema (Table 4). Around 27% of the protonemata developed more than 100 cells, and the majority fell in one of the other categories. In the intra-specific interaction experiment in which *T. muralis* acted both as the emitter and the target species, the sporeling development in the target inoculum showed no significant differences with the control (Table 4). However, when *T. muralis* was exposed to an inoculum of *S. ruralis* as emitter, the development of *T. muralis* protonemata was 2.48 times higher compared to the control. This difference primarily resulted from an increase in the number

of protonemata with more than 100 cells at the expense of the categories of 2–20 and 21–40 cells (marginally significant result, see Table 4, and Fig. 4). The results of category classification obtained for the cultures with *T. muralis* as target species are available as Electronic Supplementary Material 7.

Table 4
Results of the ordinal logistic regression showing the effect of the intraspecific and interspecific treatments on the number of cells per protonema type;. <0.1; *<0.05; **<0.01; ***<0.001. Tm, *Tortula muralis*; Sy *Syntrichia ruralis*. Exp E, the coefficients transformed exponential values; df, degrees of freedom P, p-values.

	Tm target			
	Exp E	df	P	z value
Tm emitter	1.28	6	0.614	0.505
Sy emitter	2.48	6	0.063.	1.863
	Sy target			
	Exp E	df	P	z value
Sy emitter	1.40	6	0.407	0.830
Tm emitter	-2.66	6	0.010*	-2.363

In the experiments with *S. ruralis* as target species, the control treatment showed an average of 44.9 cells per protonema. On average, 10% of the protonemata had between 2–20 cells. The majority of the protonemata fell in the categories of 21–40 cells or 41–100 cells, with less than 4% of the protonemata developing more than 100 cells. Also, the intra-specific interaction experiment with *S. ruralis* as both emitter and target species, showed no significant differences with the control (Table 4, Fig. 4). A significant difference emerged in the inter-specific interaction with *T. muralis* spores as emitter, where the development of *S. ruralis* was inhibited by 2.66 times (Table 4 and Fig. 4). This inhibition was apparent in the lack of protonemata with more than 100 cells, the reduction in protonemata with 41–100 cells, and the increase in the categories of protonemata with 2–20 and 21–40 cells. The results of category classification obtained for the cultures with *T. muralis* as target species are available as Electronic Supplementary Material 8.

DISCUSSION

This modified sandwich method grants a homogeneous physical barrier between the spores of the emitter and the target species. Although in the original Fujii's method the emitter was dried and powdered plant material (Fujii et al. 2003), we show that this method allows the survival, germination and growth of the spores of the emitter species, albeit embedded between the two gelled layers, so it

seems an appropriate technique to test intra and interspecific effects during the spore germination and initial developmental stages of the moss gametophyte.

Using this method, we did not detect negative intraspecific interactions in any of these two species. Also, we show that interspecific interactions were strongly negative for the protonemal development of *Syntrichia ruralis*, and positive for *Tortula muralis*.

Of the two methods used here to estimate germination and development success, we obtained statistically significant results only when quantifying the degree of development of the protonemata. The analysis of surface covered by protonemata (green coverage) does not indicate differences between the controls and any of the intra- or interspecific cross-paired cultures. Hu et al. (2011) found that estimates in surface cover are more imprecise in plants with complex shapes. Thus, the lack effects may be due to a limitation of the method related to the complex, profusely branched, mycelium-like shape of the protonemata of both species. Still, further developments of the method, such as improving the image quality and including other colour channels in the analyses (Hu et al. 2011) could help improve the technique.

However, the image acquisition was a time-consuming procedure, that involved cutting the upper layer of the sandwich, taking clear photos (without glare, reflections or bubbles), and selecting 10 squares per photo before starting the automatic stage of the analysis. Johnson et al. (2016), in their comparison between automatic analysis and visual-human analysis for interpreting foliage herbivory, concluded that human estimates can be accurate and precise, faster and cheaper, when the individual is properly trained. Therefore, although we recommend direct observation and quantification of the protonemata development rather than green coverage measurements for these experiments, we suggest also trying a human-visual quantification of the surface covered by protonemata in other species.

The results on the development index suggests that the emitter secretes some allelopathic substances that can get across the gelled layer, which is in agreement with previous knowledge of inhibitory substances in vascular plants (Whittaker et al. 1970) and bryo-phytes (Basile et al. 2003).

Our experiment does not show any significant intraspecific interaction in the sporeling development of any of the two species. This result is at odds with the prevalence of negative interspecific relationships among vascular plants highlighted in Adler et al. (2018). Previous results with the moss *F. hygrometrica* (Bopp 1963) found a promoting effect by the protonemata with less than eight days old, and a negative effect by older protonemata. Thus, it seems that the interactions in bryophytes could be neutral or positive more often than in vascular plants.

In contrast, intraspecific effects were clearly observed. First, *T. muralis* spores inhibit the protonemal development of *S. ruralis*. We do not know which substance may be involved in inhibiting the development of *S. ruralis*, although the previously reported antimicrobial capacity of *T. muralis* (Asakawa, 1995; Üçüncü et al. 2010), shows that this species probably possesses a molecular stock to interact with other organisms. In turn, the spores of *S. ruralis* (or probably some substance produced by these spores)

seem to enhance the development of *T. muralis* (marginally significant result, intermediate effect size). Most of the studies about plant-plant interactions have focused in negative allelopathic effects and other negative competition related effects (Adler 2018). Indeed, a recent meta-analysis shows that there is a publication bias towards negative effects in the studies about allelopathy (Zhang et al. 2021). However, there are some reports of positive intra and interspecific effects too. In vascular plants, for example, leaf litter promotes seed germination (Facelli and Facelli, 1993; Bosy and Reader, 1995) or aqueous extracts from *Eucalyptus urophylla* promote the growth of *Cinnamomum camphora* roots and stems, as well as *Helicia cochinchinensis* at low concentrations (Qin et al. 2018). As with intra-specific interactions, it is possible that mosses have more neutral and positive interactions that vascular plants with complex responses that arise from the entanglement of neutral, positive and negative interactions. This idea is in line with the results of field experiment in adult plants of the genus *Sphagnum* in peatlands that have shown entangled facilitation and inhibition interactions in adult populations. Hummock-forming *Sphagnum* species promote the development of hollow species when the environment is more humid (Fenton and Bergeron, 2006). However, under the same conditions, the hollow species inhibit hummock species by producing allelopathic substances (Liu et al. 2020a). The effects are probably mediated by the ability of hummock species to retain moisture and generate and adequate environment for hollow species, but interspecific interactions also seem to have a role as growth modulators (Liu et al. 2020a; b). Also, intraspecific positive effects in very young sporelings that shifted to negative effects were known previously (Bopp, 1963; Watson, 1981). However, interspecific positive effects in sporelings have not been previously reported. Thus, our results are unique in showing positive interactions among early development stages in the gametophyte (the dominant phase) of bryophytes. However, we believe that our method will allow other researchers testing many other species, and, as the volume of research grows, reports of such positive interactions will likely grow.

Effects of interactions in the protonemal development are still largely unexplored (but see Bopp, 1963 and Watson 1981). These neglected interactions may play a critical role in the distribution shifts of species. For instance, in ferns, intraspecific competition between prothalli, the gametophytic and most sensitive phase of the fern's life cycle (Testo and Watkins, 2013; Testo et al. 2014), has been identified as a likely cause of the decline of the threatened fern, *Asplenium scolopendrium* var. *americanum*. In this species, the spore germination was inhibited by the presence of other fern spores, which together with other environmental factors has provoked its decline (Testo and Watkins, 2013). We suggest that, also in mosses, both positive and negative interactions during the early developmental stages might affect decisively the establishment of the plant, and ultimately play a major role in determining the actual distribution of the species.

Although we must consider that *in vitro* results are hard to extrapolate to natural scenarios, our interspecific results with *T. muralis* and *S. ruralis* may provide a more realistic understanding of the interactions of both species in the field. *S. ruralis* is able to growth in diverse substrates, both as terricolous and as saxicolous, with wide tolerance to substrate pH, whereas *T. muralis* seems to specialize in basophilous, saxicolous substrates, where it is one of the most common moss species in the Mediterranean region (Guerra and Cros, 2006). If this moss is not limited by negative intraspecific

interactions with adult shoots and is able to profit from organic substances from their potential moss competitors, and, at the same time, inhibits their growth, a behaviour that is consistent with the interspecific relationships here shown, its success in its habitat is to be expected, as well as its dominance over *S. ruralis* in their overlapping habitat.

As concluding remarks: 1) The modified sandwich method, as implemented in this study, can be used to grow moss spores by separating them with a layer of gelled medium, allowing both the development of protonemata from the two sets of spores, and the gas exchange and diffusion of compounds they may emit. 2) Our results indicate the existence of relationships between sporelings, likely mediated by water-soluble substances that are able to diffuse through the gelled layer of the medium: although no intraspecific interaction has resulted from our experiments, the spore inocula of *T. muralis* inhibit the development of *S. ruralis* protonemata, while the presence of *S. ruralis* spores and sporelings probably promotes the protonematic development of *T. muralis.* 3) We recommend quantifying the sporeling development after direct observation of the protonemata (for instance, as here, with an index based on their number of cells), rather than using a semiautomatic measurement of green coverage.

We understand that *in vitro* results do not necessarily correspond with those in the field, but the modified sandwich method can be used as a potential tool to guide posterior field experiments. We believe this method can be used with a large variety of bryophytes, yielding interesting possibilities in the study of biotic interactions: early-stage constrictions in the establishment of coexisting species, invasive potential, etc. Therefore, we consider that the modified sandwich method could make a significant contribution in bryophyte ecology.

Declarations

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Figures

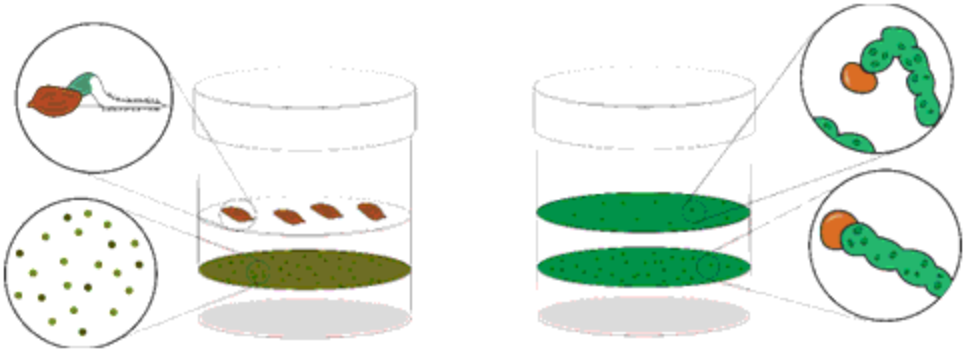

Figure 1

On the left, schematic representation of the original sandwich experiment, in which lettuce seeds are exposed to dried and powdered vegetative material from potentially allelopathic plants. On the right, the modified sandwich method for growing different populations of moss spores together. Note that in the new method spores and protonemata of both layers are alive

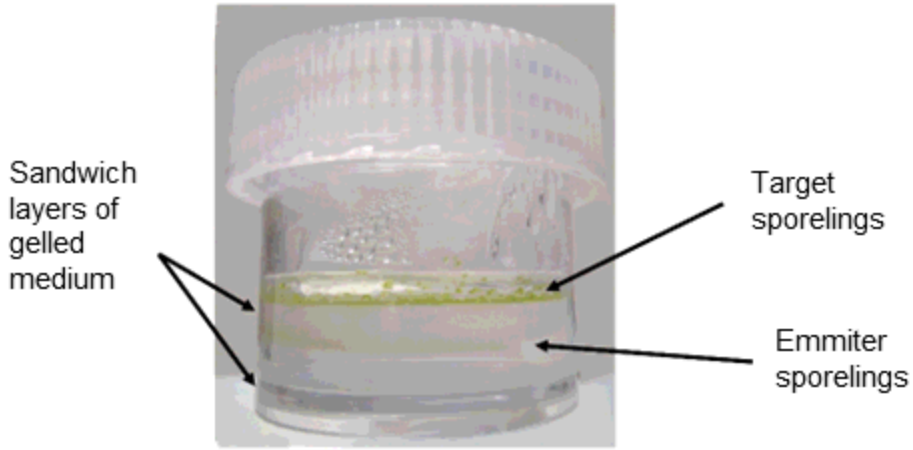

Figure 2

Photo of an experiment of the modified sandwich technique. Note the greening of the emitting population, indicating that it has survived embedded between the two layers of gelled medium

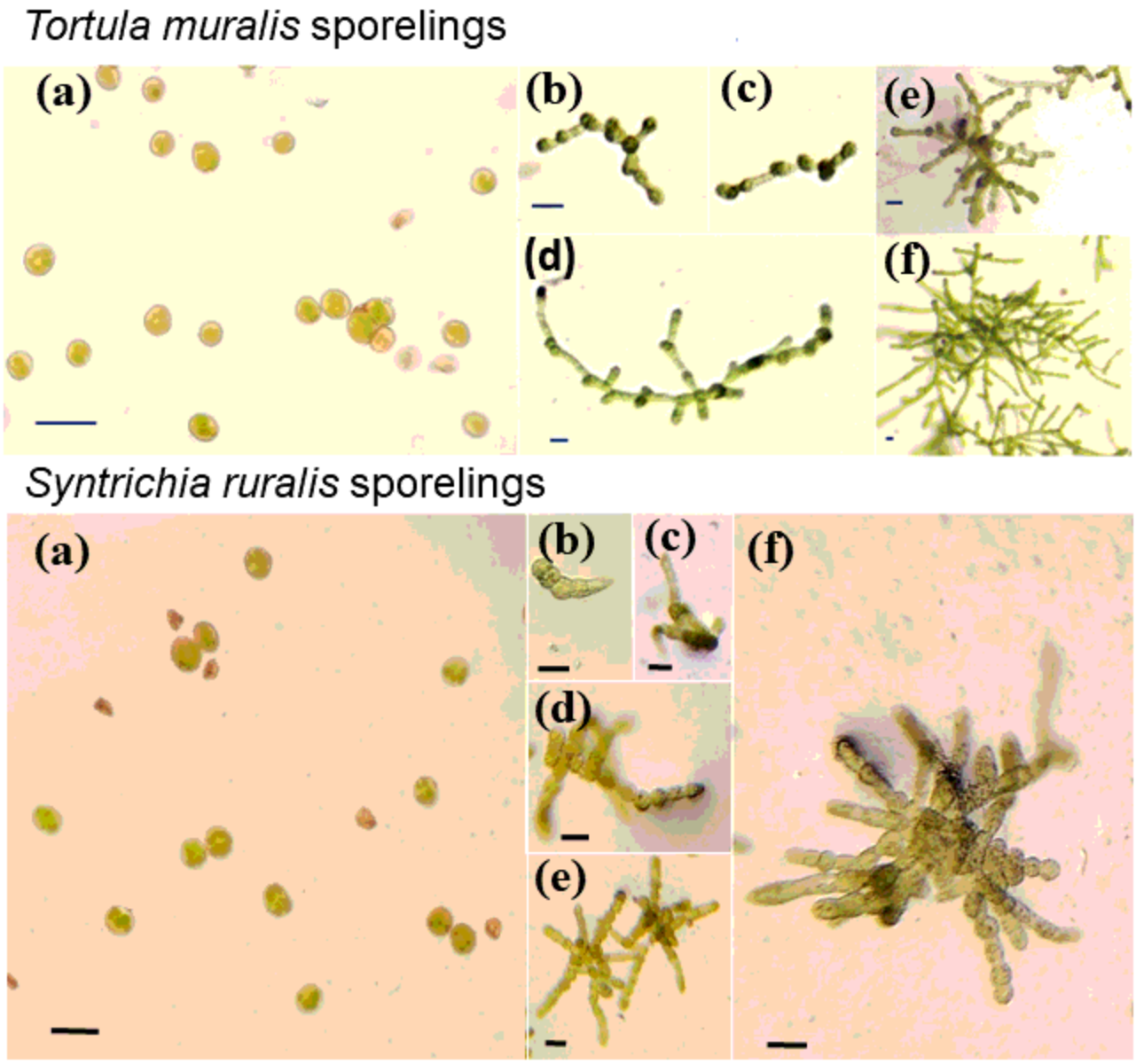

Figure 3

Sporelings of *Tortula muralis* (top) and *Syntrichia ruralis* (bottom). a: uncultured spores; b-f: sporelings growing on top of the sandwich (as the target species), b, c: 2–20-celled protonemata; d: 21–40-celled protonema (ca. 35 cells); e: 41–100-celled protonemata; f: > 100-celled protonema. (Bar = 25 μm)

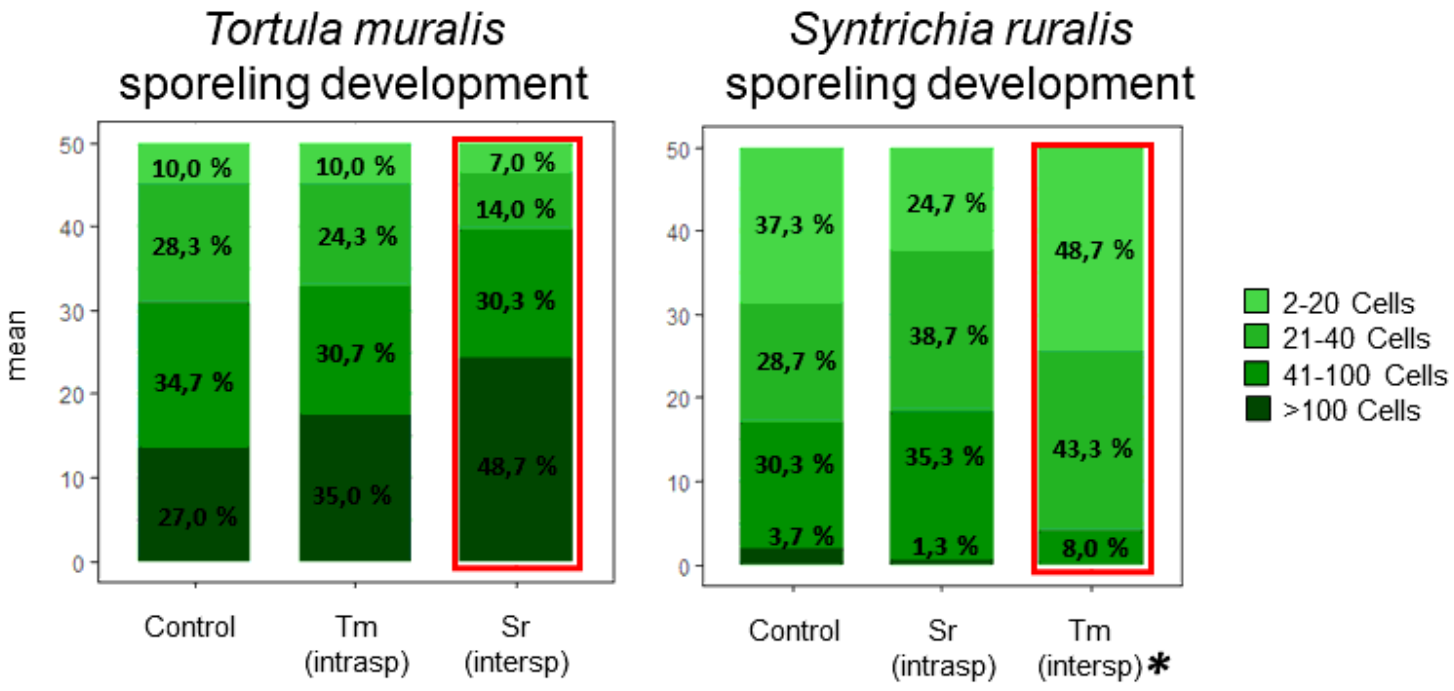

Figure 4

Average proportion of sporelings in each developmental category. Columns with a greater proportion of dark colours show more developed stages. Columns with statistically significant differences are framed in red. Tm, *Tortula muralis*; Sr *Syntrichia ruralis*; 2–20, protonemata with 2–20 cells; 21–40, protonemata with 21–40 cells; 41–100, protonemata with 41–100 cells; > 100 protonemata with more than 100 cells

Supplementary Files

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