

APPLICATION ARTICLE

Accelerating gametophytic growth in the model hornwort *Anthoceros agrestis*

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Abstract

Premise: Hornworts belong to a unique lineage of bryophytes with critical traits for elucidating the evolution of land plants; however, the development of functional genetic tools for hornworts has been hampered by their relatively slow gametophytic growth.

Methods: To identify the external factors that influence the development of hornwort gametophytes and potentially augment their growth, we evaluated the contributions of several culture medium components on the axenic gametophytic growth of *Anthoceros agrestis*, a model hornwort. A streamlined growth assay utilizing semiautomated image analysis was developed to rapidly quantify and compare tissue development spanning four weeks of culture on solidified medium.

Results: The addition of sucrose, ammonium nitrate, activated charcoal, pH buffering, and growth regulators (2,4-dichlorophenoxyacetic acid, 6-benzylaminopurine, and thidiazuron) affected gametophyte tissue survival, growth patterns, and the rate of thalli growth. Subsequently, an optimized medium composition and growth regimen for accelerating *A. agrestis* gametophytic growth were formulated, which at four weeks of culture increased the tissue wet weight by 2.1- to 8.5-fold compared with other previously utilized hornwort growth media.

Discussion: Our protocol for generating vigorous starting material and accelerated tissue regeneration is pertinent for advancing gene function characterization and genome editing in hornworts.

KEYWORDS

Anthocerotophyta, growth medium, image analysis, plant growth regulator, plant tissue culture

Akin to other bryophytes, but in stark contrast to vascular plants, the dominant haploid gametophyte of hornworts supports the growth of the sporophyte. *Anthoceros agrestis* Paton, a model hornwort, possesses a simple thalloid gametophyte with many representative physiological features that are unique to hornworts, such as the pyrenoid-based carbon-concentrating mechanism and the capacity for symbiosis with fungi and cyanobacteria (Szövényi et al., 2015; Frangedakis et al., 2021a). The establishment of an in vitro culture of *A. agrestis* gametophytes therefore serves as a crucial foundation for the study of hornwort biology. Culturing *A. agrestis* gametophytes is

relatively straightforward because of its high regenerative potential following explant excision and mechanical wounding; however, we observed (unpublished data) that the rate of biomass accumulation of *A. agrestis* gametophytes grown in vitro is relatively slow compared with that of other model bryophytes, such as the moss *Physcomitrium patens* (Hedw.) Mitt. (Cove et al., 2009) and the liverwort *Marchantia polymorpha* L. (Ishizaki et al., 2015).

With the recent resurgence of scientific interest and ongoing efforts to develop functional genetic tools for *A. agrestis* (Szövényi et al., 2021), several growth media,

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especially BCD and Knop (Szövényi et al., 2015; Frangedakis et al., 2021b), have become routinely used for maintaining *A. agrestis* cultures. However, both BCD and Knop were initially developed for the model moss *Physcomitrium patens* (Reski and Abel, 1985; Cove et al., 2009), and have been only minimally modified for culturing *A. agrestis* gametophytes. Likewise, although Hatcher (Hatcher, 1965) and Hutner (Proskauer, 1969; Wong and Meeks, 2002) media were initially developed for hornworts, these media have not been fully optimized for *A. agrestis*. The resulting slow in vitro growth rates of *A. agrestis* gametophytes using BCD, Knop, Hatcher, or Hutner media present a major bottleneck in advancing functional genetic studies in hornworts.

In this study, using the Hatcher medium as a starting point, we evaluated the contributions of several culture medium components on the axenic growth of *A. agrestis* gametophytes in a concerted effort to identify the external factors that influence hornwort development and to augment its growth. A streamlined growth assay utilizing semiautomated image analysis was developed to rapidly quantify and compare tissue development over four weeks of culture on solidified media. The supplementation of the Hatcher medium with sucrose, activated charcoal, ammonium nitrate, and pH buffering, as well as the growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA), and thidiazuron (TDZ), affected gametophyte tissue survival, growth patterns, and the rate of thalli growth. These findings culminated in the development of the *A. agrestis* gametophyte growth medium, referred to as AG medium, which could be used to increase the wet weight of the gametophyte by 2.1- to 8.5-fold compared with previously utilized growth media for hornworts after four weeks of culture. Our protocol for generating vigorous starting material and accelerated gametophyte tissue recovery is pertinent for advancing gene function characterization and genome editing in hornworts.

METHODS

Plant materials and growth conditions

Axenic stock cultures of *A. agrestis* gametophytes (Oxford and Bonn strains), along with several hornwort species from our collection (*A. fusiformis* Austin, *A. punctatus* L., and *Phaeoceros carolinianus* (Michx.) Prosk.), were initially maintained on solidified Hatcher medium (Hatcher, 1965; Table 1). These gametophytes were subcultured monthly and did not contain visible endosymbionts. Plant materials with visible fungal or bacterial contamination were discarded. For the optimization studies, *A. agrestis* Oxford strain gametophyte tissues were used to determine the effects of various concentrations and combinations of supplements (Table 2) to the basal Hatcher medium.

The components of the optimized *A. agrestis* gametophyte growth medium (AG) are listed in Table 1 alongside the commonly used hornwort growth media:

BCD (Cove et al., 2009), Hatcher (Hatcher, 1965), modified Knop (Reski and Abel, 1985; Frangedakis et al., 2021b), and the Lorbeer-based Hutner (Proskauer, 1969; Wong and Meeks, 2002). Following optimization, the growth of *A. agrestis* gametophytes using the Hatcher-based AG medium was compared with their growth on BCD, Hatcher, modified Knop, and Hutner media. The Hutner micronutrient stock solution was kindly supplied by John Meeks (University of California, Davis, California, USA). The type of gelling agents (gelzan or agar) and their concentration used in the BCD, Hatcher, modified Knop, and Hutner media (Table 1) followed previously published formulations that were used for hornworts (Hatcher, 1965; Wong and Meeks, 2002; Szövényi et al., 2015; Frangedakis et al., 2021b). All growth media were standardized at pH 6.5 (Szövényi et al., 2015) using potassium hydroxide (Thermo Fisher Scientific, Waltham, Massachusetts, USA) prior to autoclave sterilization for 20 min at 121°C and 15 psi (103.42 kPa). Approximately 35 mL of each medium was then poured into 100 × 25-mm Petri plates and allowed to solidify. All hornwort gametophyte stock cultures were subcultured using sterilized forceps and a scalpel.

For more uniform tissue distribution and quality during the growth assays, *A. agrestis* gametophytes were homogenized prior to being distributed onto all of the solidified medium formulations. For the homogenizations, 2–5 g (wet weight) of tissue was immersed in sterile deionized water containing 300 mg · L⁻¹ timentin (Caisson Labs, Smithfield, Utah, USA) and homogenized using a T18 digital ULTRA TURRAX homogenizer (IKA, Staufen, Germany) for approximately 10 s at 4000 rpm. The homogenized material was filtered through a 100-µm cell strainer (MTC Bio, Sayreville, New Jersey, USA), rinsed with 50 mL of sterile deionized water, and 0.15 g of tissue was distributed with a 1-mL Luer-Lok Tip syringe (BD, Franklin Lakes, New Jersey, USA) onto each Petri plate. Petri plates were sealed with a double layer of parafilm “M” (Thermo Fisher Scientific) and maintained at 20–22°C under a 16/8 h light/dark photoperiod and photosynthetic photon flux density of 6–25 µmol · m⁻² · s⁻¹. The light was provided by an Ecolux XL Starcoat F32T8 XL SP30 ECO paired with F32T8 XL SP41 ECO fluorescence bulbs (General Electric, Boston, Massachusetts, USA), and the plant tissues inside the Petri plates were placed (unstacked) approximately 69 cm from the fluorescence bulbs. Cultures maintained in Hatcher-based liquid medium were grown in 45-mL aliquots in autoclaved 100-mL flasks and continuously agitated at 130 rpm under similar light conditions as used for the Petri plate cultures.

Experimental design for growth assays on solidified media

During the growth assay experiments, each medium formulation was assessed using four replicates (Petri plates), with each

TABLE 1 Composition of growth media used to culture hornwort gametophytes. All compositions listed are for a total of 1 L of solidified medium. A dash (—) within a column indicates the absence of this chemical component

Media components	Chemical compositions	AG (Hatcher-based)	BCD	Hatcher	Knop	Hutner (Lorbeer-based)
Macro-nutrients	CaCl ₂	—	111 mg	—	—	105 mg
	Ca(NO ₃) ₂ 4H ₂ O	260 mg	—	260 mg	1000 mg	—
	KCl	65 mg	—	65 mg	250 mg	—
	KH ₂ PO ₄	165 mg	250 mg	165 mg	250 mg	200 mg
	MgSO ₄ 7H ₂ O	735 mg	250 mg	735 mg	250 mg	100 mg
	KNO ₃	—	1010 mg	—	—	—
	NaNO ₃	80 mg	—	80 mg	—	—
	NH ₄ NO ₃	400 mg	—	—	—	200 mg
	Na ₂ SO ₄	200 mg	—	200 mg	—	—
	H ₂ SO ₄ (concentrated)	1.5 µL	—	1.5 µL	—	—
Micro-nutrients	AlK(SO ₄) ₂ 12H ₂ O	—	0.055 mg	—	—	—
	MnSO ₄ 4H ₂ O	9 mg	—	9 mg	—	—
	MnCl ₂ 4H ₂ O	—	0.389 mg	—	—	5 mg
	ZnSO ₄ 7H ₂ O	1.5 mg	0.055 mg	1.5 mg	—	22 mg
	H ₃ BO ₃	1.5 mg	0.614 mg	1.5 mg	—	10 mg
	CuSO ₄ 5H ₂ O	0.075 mg	0.055 mg	0.075 mg	—	1.5 mg
	Na ₂ MoO ₄ 2H ₂ O	0.075 mg	—	0.075 mg	—	—
	(NH ₄) ₆ Mo ₇ O ₂₄ 4H ₂ O	—	—	—	—	1 mg
	CoNO ₃	0.075 mg	—	0.075 mg	—	—
	CoCl ₂ 6H ₂ O	—	0.055 mg	—	—	1.5 mg
	KBr	—	0.028 mg	—	—	—
	KI	—	0.028 mg	—	—	—
	LiCl	—	0.028 mg	—	—	—
	SnCl ₂ 2H ₂ O	—	0.028 mg	—	—	—
	FeSO ₄ 7H ₂ O	—	12.5 mg	—	12.5 mg	5 mg
	FeC ₆ H ₅ O ₇ 7H ₂ O	2.5 mg	—	2.5 mg	—	—
Sugar supplement	Sucrose	2 g	—	—	—	—
	Glucose	—	—	—	—	5 g
Buffering	MES (pH 6.5)	5 mM	—	—	—	5 mM
	pH (using KOH)	6.5	6.5	6.5	6.5	6.5
Solidifying agent	Agar	—	8 g	—	—	6.5 g
	Gelzan	4 g	—	4 g	7.5 g	—
Additional	Activated charcoal	100 mg	—	—	—	—
	EDTA disodium	—	—	—	—	50 mg
	6-Benzyl aminopurine	10 nM	—	—	—	—

replicate containing 0.15 g of starting homogenized tissue (Figure 1C). The optimal concentration of each medium component evaluated in this study (sucrose, activated charcoal, ammonium nitrate, 2-[N-morpholino]ethanesulfonic acid

[MES], 2,4-D, BA, and TDZ) was determined by comparing a medium lacking the component (standard control) with medium formulations spanning a concentration gradient that was previously reported for the in vitro culture of plants (Table 2).

TABLE 2 Medium components evaluated in this study for enhancing hornwort gametophyte growth

Component	Supplier	Rationale	Concentrations compared
Sucrose	Thermo Fisher Scientific, Waltham, Massachusetts, USA	Additional carbon source for boosting plant regeneration and metabolism, and an osmotic agent (Vogelsang et al., 2006; Kubota et al., 2013; Yaseen et al., 2013)	0.02, 0.05, 0.1, 0.2, 0.33, 0.67, 1, 1.33, 1.67, and 2% (0.2–20 g · L ⁻¹)
Activated charcoal	Sigma-Aldrich, St. Louis, Missouri, USA	Promotes plant growth through various functions, such as adsorption of chemicals, pH buffering, and darkening the growth environment (Proskauer and Berman, 1970; Thomas, 2008)	10, 100, 500, and 1000 mg · L ⁻¹
Ammonium nitrate	MilliporeSigma, Burlington, Massachusetts, USA	Provides an alternative source of nitrogen other than nitrate (Basile, 1975; Ono et al., 1994), and known to be compatible for <i>A. punctatus</i> (Meeks et al., 1983)	200, 400, and 800 mg · L ⁻¹
2-(N-morpholino) ethanesulfonic acid	Sigma-Aldrich, St. Louis, Missouri, USA	Buffers the pH of growth medium (de Klerk et al., 2008; Kagenishi et al., 2016), prevents medium acidification by hornwort gametophyte	1, 5, and 25 mM
2,4-Dichloro phenoxyacetic acid	Caisson Laboratories, Smithfield, Utah, USA	An auxin analog. Alters plant cellular metabolism and growth patterns (Gaspar et al., 1996; Ishizaki et al., 2012)	1, 10, 100, 1000, and 10,000 nM
6-Benzyl aminopurine	PhytoTechnology Laboratories, Lenexa, Kansas, USA	A cytokinin analog. Alters plant cellular metabolism and growth patterns (Selkirk, 1980; Gaspar et al., 1996; von Schwartzenberg et al., 2007)	1, 10, 100, 1000, and 10,000 nM
Thidiazuron	PhytoTechnology Laboratories, Lenexa, Kansas, USA	A cytokinin-like compound. Alters plant cellular metabolism and growth patterns (Gaspar et al., 1996; Erland et al., 2020)	1, 10, 100, 1000, and 10,000 nM

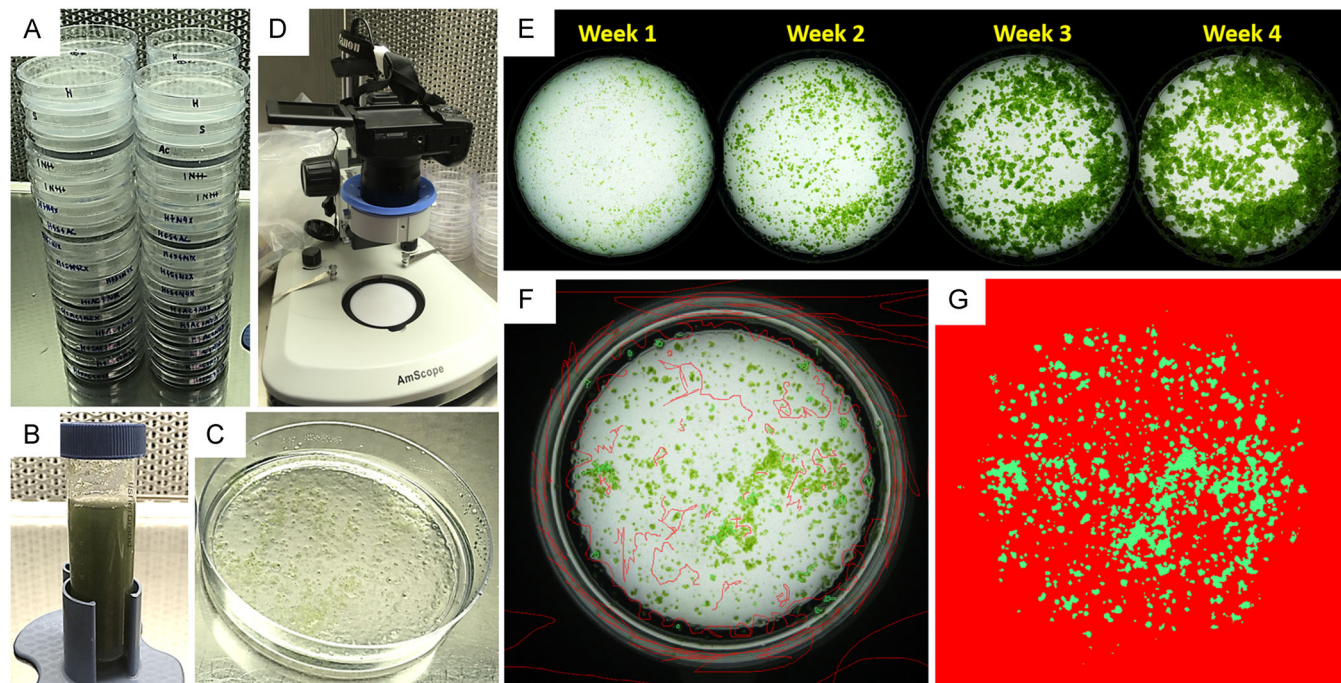


FIGURE 1 Sequential progression of the image analysis pipeline. (A) The medium formulations for each experiment were prepared together. (B) The hornwort gametophyte tissue was homogenized. (C) Equal wet weights of the homogenized plant tissue were then spread onto each Petri plate containing the medium formulations. (D, E) Images of the growing gametophyte tissues on each Petri plate were captured using a combination of a microscope stand and digital camera setup each week for a total of four weeks of culture. (F, G) During image segmentation training for each timepoint, the living gametophyte tissues were manually curated into a class using the green-colored selection, while the non-tissue part of the image of the Petri plate was curated using into a separate class using the red-colored selection. A calibrated classifier was used to estimate the area of the growing gametophyte tissue for each replicate of the same medium formulation treatment

Quantification of tissue growth based on image analysis

Images were taken of each Petri plate every 7 d for four weeks using a PowerShot SX50 HS digital camera (Canon, Tokyo, Japan). Each Petri plate was backlit with an AmScope 7X-45X Dual Lit 6 W LED Trinocular Stereo Zoom microscope stand (SM-2T-6WB-V331; AmScope, Irvine, California, USA) (Figure 1D). The standardized image capture conditions consisted of 1/400 s shutter speed with the following settings: Manual, Macro, ISO 640, and no flash. The images were captured from a standardized camera height of 12 cm from the base of the Petri plate by securing the camera on the microscope stand. Lighting was consistent between images for the same treatment (medium formulation) and timepoint of an experiment, although the lighting between different timepoints and experiments could vary depending on the optimal conditions needed to illuminate the tissue.

The images, in TIF format (1000 × 666 pixels, RGB), were processed using the Trainable WEKA Segmentation (version 3.2.33; Arganda-Carreras et al., 2017) plugin for Fiji software (version 2.0.0-rc-69/1.52n; Schindelin et al., 2012) to differentiate the green hornwort gametophyte tissue from the background (Figure 1F). A unique classifier was developed for each medium treatment at each timepoint using a representative image from one of the four replicates. At least 10 labels were used to define each class, capturing the various colors and shapes in the representative image to form the basis for distinguishing living gametophyte tissue from the background. The classifiers were trained to optimize the detection of the green gametophyte tissue in each representative image. An optimized classifier was applied to all replicates of the treatment at each timepoint, and the area of gametophyte tissue was quantified per replicate using the Fiji histogram function.

Quantification of tissue growth based on wet weight

To complement the image analysis of gametophyte tissue growth, wet weight data were collected for the growth regulator and final growth medium comparison experiments. A day after data collection at the four-week timepoint, the gametophyte tissue in each Petri plate was gently harvested using a Nunc cell scraper (Thermo Fisher Scientific) and weighed in a sterile 50-mL centrifuge tube (Thermo Fisher Scientific).

Microscopy and tissue staining

Gametophyte tissue samples from the growth regulator experiment were immersed in a 0.1% aqueous solution of SYBR Safe DNA gel stain (Thermo Fisher Scientific) for 10 min with gentle agitation. The samples were rinsed three

times with sterile deionized water prior to imaging with a M205 FA Stereomicroscope (Leica Microsystems, Wetzlar, Germany) without filters (white light), as well as with yellow fluorescent protein (YFP) and long-pass green fluorescent protein (GFP) filters. The YFP filter was used to visualize only the SYBR Safe stain, while the long-pass GFP filter was used to visualize both chloroplast autofluorescence and the SYBR Safe stain.

Statistical analysis

Raw data were compiled in Excel (version 16.43; Microsoft, Redmond, Washington, USA). Mean and standard deviation values were calculated for both the tissue area and tissue wet weight. A Dunnett's test was performed on the data from the four-week timepoint for each Hatcher medium formulation experiment to compare the mean tissue values with those from the control or baseline Hatcher medium. Tukey's HSD test was performed on the fourth-week data for the final media comparison data sets using the Oxford and Bonn strains, enabling the simultaneous comparison of all the means of the different medium formulations. All mean separations and their respective analyses of variance were conducted in R (version 4.1.0; R Core Team, 2021). Correlations between the area of gametophyte tissue and the corresponding wet weight were determined using Pearson's correlation (PEARSON function) in Excel.

RESULTS

Sucrose, ammonium nitrate, and activated charcoal synergistically accelerated growth

In this study, the basal Hatcher medium was supplemented with several components in standardized consecutive growth experiments, with the aim of accelerating the growth of *A. agrestis* gametophytes. Following a preliminary experiment in liquid culture using sucrose (Appendix 1), we initially added 2% sucrose to the Hatcher medium. This concentration of sucrose was found to induce rapid tissue growth, but also causes tissue stress with prolonged exposure. We hypothesized that the medium is lacking other components necessary to offset the stress. We initially also assessed the effects of activated charcoal on *A. agrestis* and observed a slight improvement in tissue growth and greener coloration when the basal Hatcher medium was supplemented with 100 mg · L⁻¹ activated charcoal (Appendix 2). In addition, we observed that the basic Hatcher medium only contains nitrate as the supplemental nitrogen source (Table 1). Plants may more readily take up ammonium over nitrate or vice versa in a species- and genotype-dependent manner (Basile, 1975). Ammonium has been previously identified as a preferred nitrogen source in another hornwort species, *A. punctatus* (Meeks et al., 1983), as well as in

another bryophyte, *Marchantia paleacea* Bertol. (Ono et al., 1994).

We compared the effects of 2% sucrose, 100 mg · L⁻¹ activated charcoal, and three concentrations of ammonium nitrate separately and in combination to determine which of these medium components best improved gametophytic growth compared with the basic Hatcher medium (Figure 2). Synergistic effects from the three components were observed, with the best growth (2.7-fold more tissue area after four weeks of culture compared with basal Hatcher) and darker green tissues achieved through the supplementation of 2% sucrose, 0.4 g · L⁻¹ ammonium nitrate, and 100 mg · L⁻¹ activated charcoal in the Hatcher medium. In this experiment, we also observed that the supplementation of ammonium nitrate was responsible for enhancing tissue greenness, although it does not increase the area of tissue as greatly as 2% sucrose. Of the three ammonium nitrate concentrations, 0.4 g · L⁻¹ was optimal, while 0.8 g · L⁻¹ resulted in a slightly reduced amount of tissue compared with 0.4 g · L⁻¹ when combined with activated charcoal in the presence or absence of 2% sucrose.

Improved tissue quality through MES buffering and reduced sucrose

Although we achieved statistically significant gains in the amount of gametophyte tissue produced, we observed that our best medium formulation resulted in the overproduction of rhizoids, as well as large sectors of necrotic tissue by four weeks of culture (Figure 2B). We hypothesized that the stress response caused by the addition of sucrose could arise from the acidification of the medium. In a related hornwort species, *A. punctatus*, acidification of the Hutner basal medium has been known to cause cell death (John Meeks, University of California, Davis, personal communication). To offset the acidification, MES buffer was incorporated into the Hutner medium (Table 1). When we assessed the pH of the *A. agrestis* gametophyte grown in liquid Hatcher medium over time (Appendix 3), we observed a steep reduction in pH in the basal Hatcher liquid medium (down to pH 4.6 after two weeks), which was further accentuated by the addition of 2% sucrose (down to pH 3.3 after two weeks).

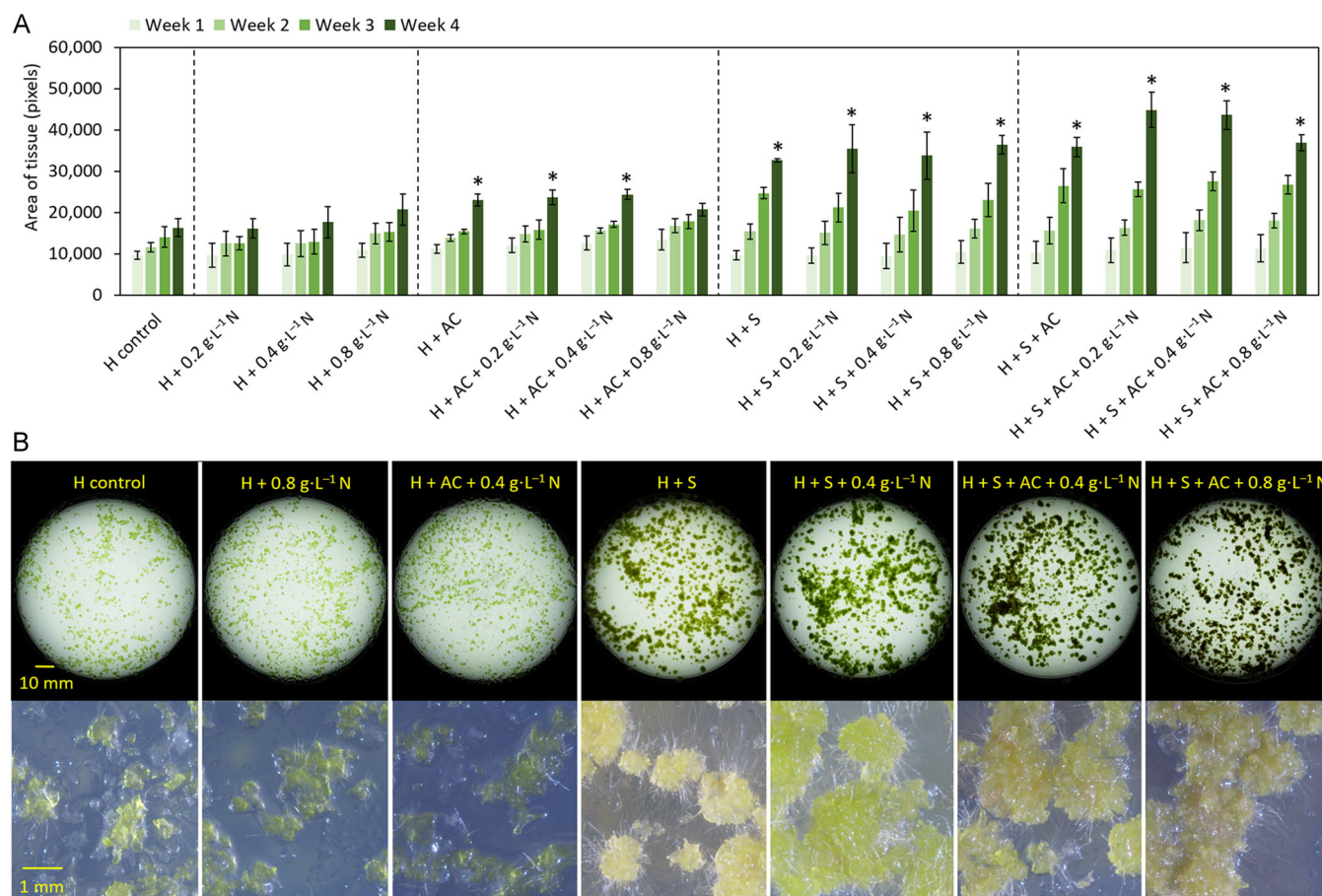


FIGURE 2 Effect of baseline Hatcher (H) medium supplementation with 2% sucrose (S), 100 mg · L⁻¹ activated charcoal (AC), and ammonium nitrate (N) on *Anthoceros agrestis* gametophytic growth. (A) Quantification of tissue area produced by gametophytes grown on the different media. Error bars indicate standard deviation. Asterisks denote a medium formulation with a significantly higher average area of tissue than the plants in the Hatcher control medium (Dunnett's test, $P < 0.05$) after four weeks of culture. (B) Representative images captured at the fourth week of culture

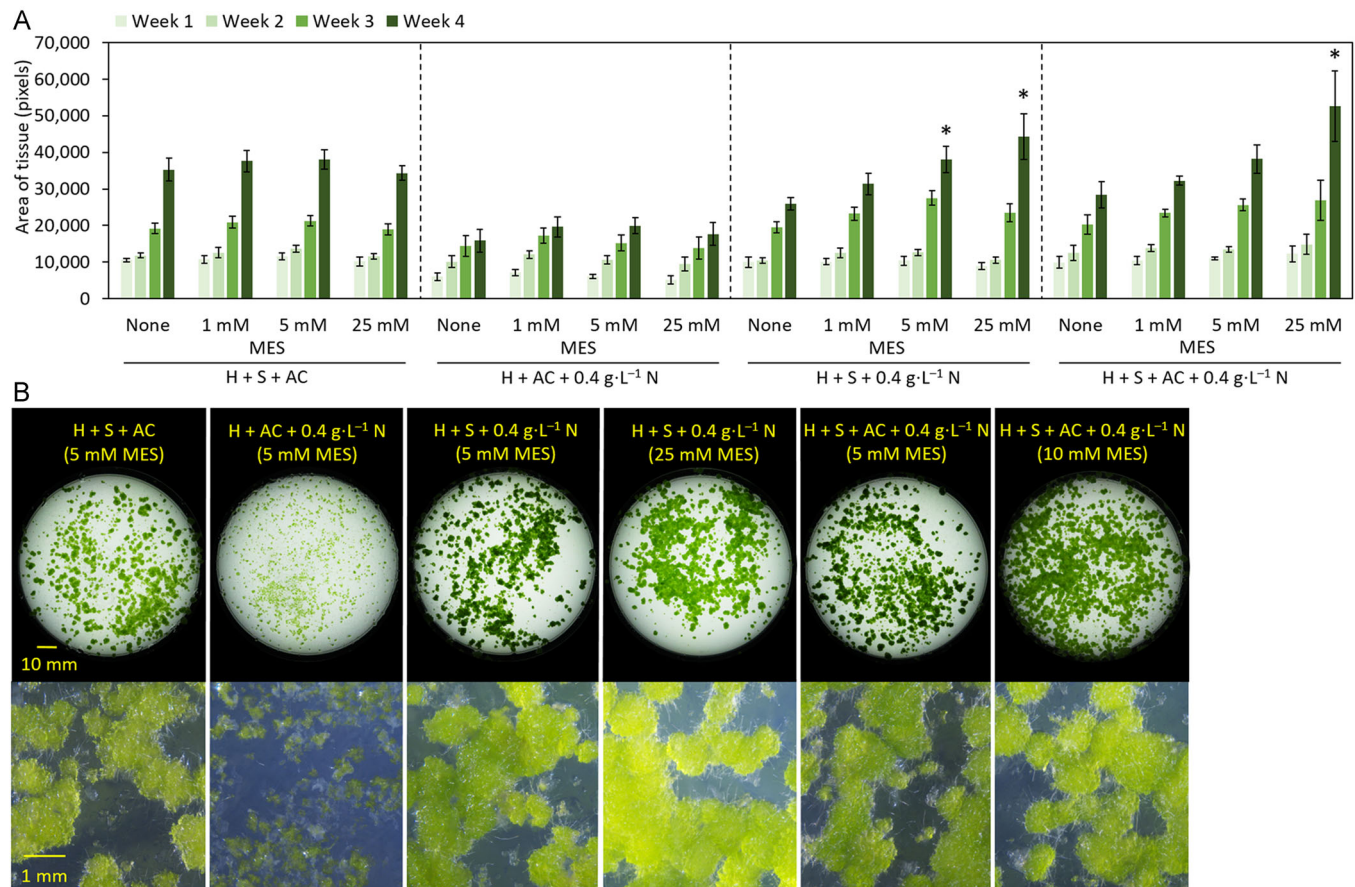


FIGURE 3 Effects of four types of modified Hatcher media supplemented with 0, 1, 5, and 25 mM MES buffer (pH 6.5) on *Anthoceros agrestis* gametophytic growth. For the treatment description, “S” indicates supplementation with 2% sucrose, “AC” indicates supplementation with 100 mg · L⁻¹ activated charcoal, and “N” indicates supplementation with ammonium nitrate. (A) Quantification of tissue area produced by gametophytes grown on the different media. Error bars indicate standard deviation. Asterisks denote a medium formulation with a significantly higher average area of tissue than the plants in the control medium lacking the MES buffer (Dunnett’s test, $P < 0.05$) after four weeks of culture. (B) Representative images captured at the fourth week of culture

This observation led to the next growth assay experiment, which compared the four best medium compositions from the previous growth experiment in combination with supplemental MES buffer (pH 6.5) at 0, 1, 5, and 25 mM (Figure 3). The area of tissue was further increased by the supplementation of 5 and 25 mM of MES, with 5 mM resulting in greener tissue than 25 mM (Figure 3B); however, some tissue chlorosis and necrosis continued to develop after four weeks of culture under all assessed MES concentrations.

We hypothesized that this persistent stress response may be further alleviated by reducing the sucrose concentration. Two consecutive experiments were conducted to more finely determine the minimal amount of sucrose required for optimal gametophyte growth (Figure 4). The first experiment was carried out to compare the addition of 2%, 1.67%, 1.33%, 1%, 0.67%, 0.33%, and 0% sucrose into the Hatcher medium, which was also supplemented with 100 mg · L⁻¹ activated charcoal, 0.4 g · L⁻¹ ammonium nitrate, and 5 mM MES (pH 6.5). These lower concentrations of sucrose had a significant effect

(Dunnett’s, $P < 0.05$) in further increasing tissue growth (Figure 4A, left graph). Because 0.33% sucrose resulted in the greatest amount of tissue, a follow-up experiment was conducted to compare the effects of 0.33%, 0.2%, 0.1%, 0.05%, 0.02%, and 0% sucrose (Figure 4A, right graph). In this latter experiment, we observed that just 0.2% sucrose, rather than the original 2% sucrose, resulted in optimal tissue growth, much reduced rhizoid formation, and a more vigorous horizontal gametophytic growth pattern (Figure 4B). Moreover, the tissues continued to grow rapidly beyond four weeks of culture with no chlorosis and necrosis.

Fine-tuning cellular growth patterns using exogenous growth regulators

Next, using Hatcher medium supplemented with 100 mg · L⁻¹ activated charcoal, 0.4 g · L⁻¹ ammonium nitrate, 5 mM MES (pH 6.5), and 0.2% sucrose as the basal medium, we explored the effects of three growth regulators: 2,4-D, BA,

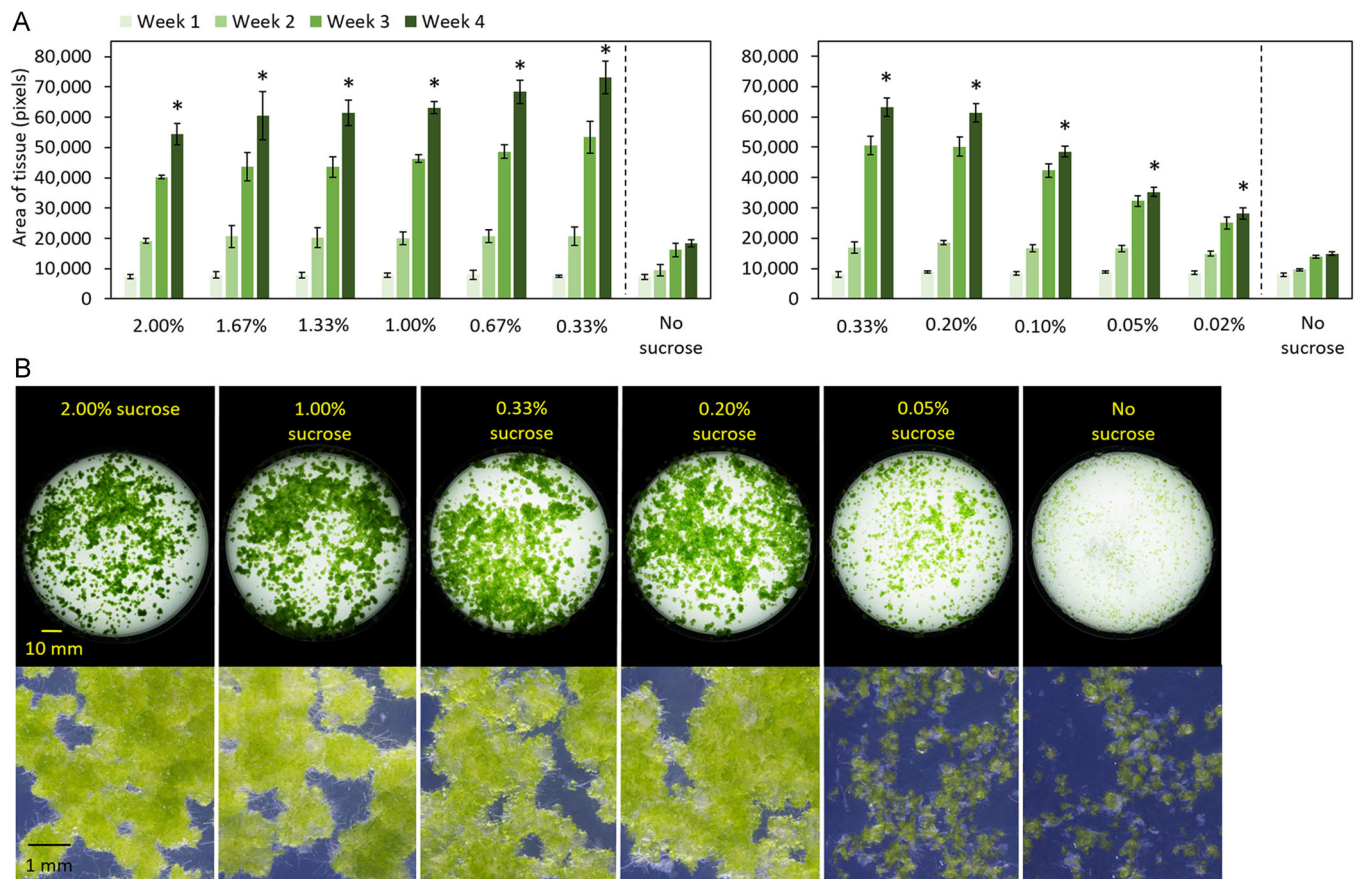


FIGURE 4 Two consecutive experiments were used to optimize the reduced sucrose concentration. (A) Quantification of tissue area produced by gametophytes grown on the different media. The first experiment (left) involved media supplemented with 2% to 0.33% sucrose, while the second experiment (right) investigated the effects of reduced sucrose concentrations (0.33% to 0.02%). Error bars indicate standard deviation. For both experiments, asterisks denote a medium formulation with a significantly higher average area of tissue than the plants grown in the control medium lacking sucrose (Dunnett's test, $P < 0.05$) after four weeks of culture. (B) Representative images captured at the fourth week of culture

and TDZ (Table 2, Figure 5). There was a statistically significant (Dunnett's, $P < 0.05$) increase in the area of gametophyte tissue at four weeks of culture on media supplemented with 100 or 1000 nM 2,4-D, as well as 1 to 10 nM BA (Figures 5A, B). The tissues were observed under a stereomicroscope, as well as through the staining of the nucleic acids, which allowed the visualization of cell size (Figure 5C). As the concentration of 2,4-D (an auxin analog) increased, there was a consistent increase in cell size, as evidenced by the larger surface area of the cell walls and more space between the nuclei and the chloroplasts (Figure 5C). On the other hand, as the BA (a cytokinin analog) concentration increased, there was an increase in cell division, as evidenced by smaller cell sizes and less space between the nuclei and the chloroplasts, especially at the growth tips of the gametophyte thalli (Figure 5C). The addition of TDZ (a cytokinin-like compound) did not result in significant phenotypic changes at any of the concentrations tested, although the general patterns of cellular growth seemed to be more similar to that of BA supplementation (Figure 5C).

AG medium accelerates hornwort growth

After identifying the factors that synergistically improved the growth of *A. agrestis* Oxford strain gametophytes, we investigated the utility of our optimized AG medium (Table 1) through several comparisons. First, we used the Oxford strain for comparisons of the AG medium with other growth media (BCD, basal Hatcher, Knop, and Hutner) previously used to cultivate hornworts (Figure 6). Additionally, we compared the growth of *A. agrestis* Bonn strain on AG medium with their growth on BCD and basal Hatcher (Figure 6). As with the growth regulator experiment (Figure 7A), the tissue area and wet weight correlated well for the Oxford and Bonn strain media comparison experiment, with a Pearson's correlation coefficient of $r(16) = 0.83$, $P < 0.005$ (Figure 7A) and $r(8) = 0.91$, $P < 0.005$ (Figure 7B), respectively. For both the Oxford and Bonn strains, gametophyte tissues grown in AG significantly outperformed the other media in both tissue area and wet weight, maintaining vigorous tissue material after four weeks of culture (Figures 6, 7B). Lastly,

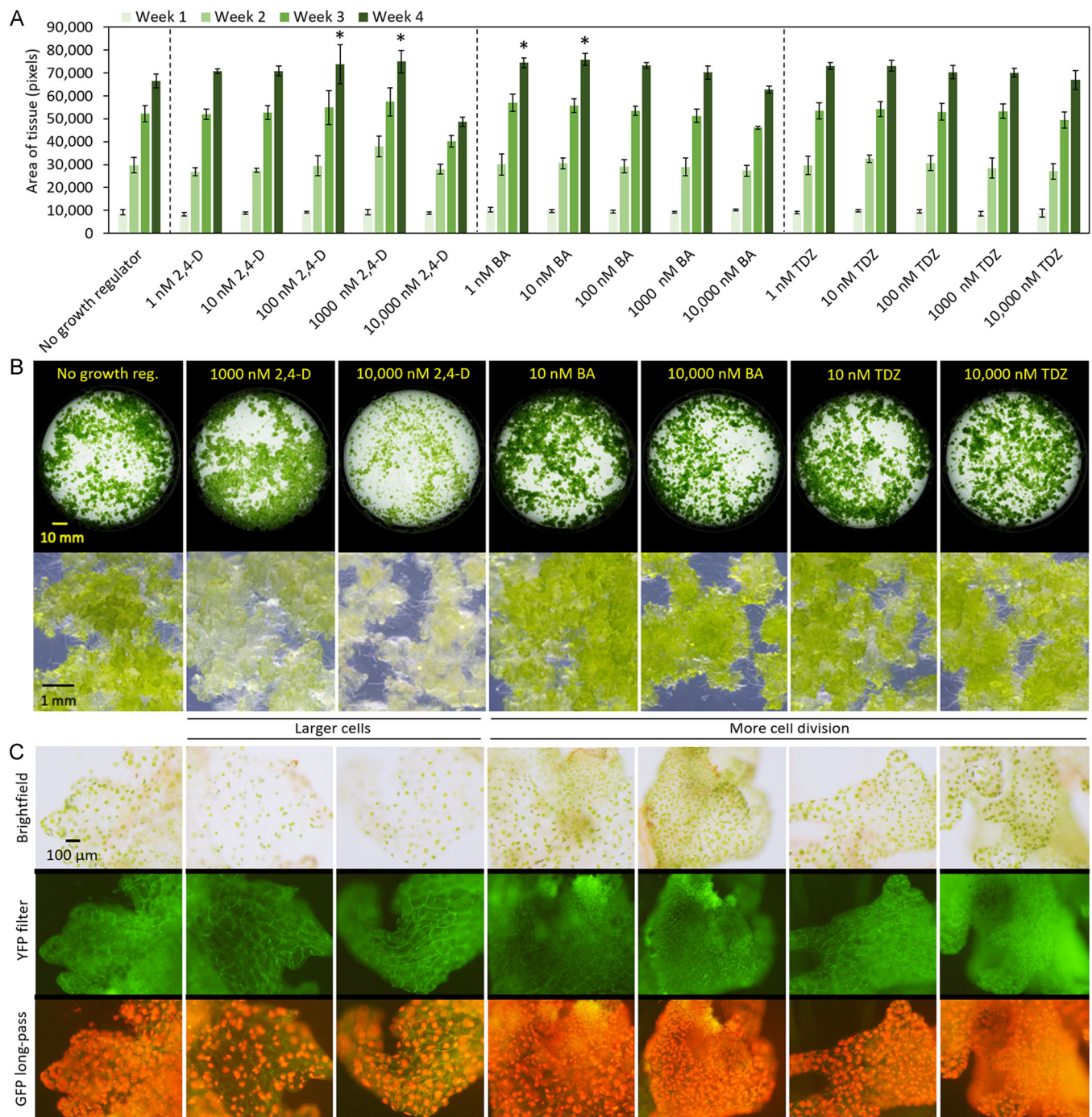


FIGURE 5 The effects of media supplemented with the growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA), and thidiazuron (TDZ) on *Anthoceros agrestis* gametophyte growth. Concentrations of 1, 10, 100, 1000, and 10,000 nM were assessed for each growth regulator. (A) Quantification of tissue area produced by gametophytes grown on the different media. Error bars indicate standard deviation. Asterisks denote a medium formulation with a significantly higher average area of tissue than the plants grown in the control medium lacking the growth regulator (Dunnnett's test, $P < 0.05$) after four weeks of culture. (B, C) Representative images and SYBR Safe tissue staining captured at the fourth week of culture

a preliminary experiment was also conducted with other hornwort species (Appendix 4), in which we demonstrated that AG could also accelerate the gametophyte growth of *A. fusiformis*, *A. punctatus*, and *Phaeoceros carolinianus*.

DISCUSSION

Within the past decade, our understanding of hornwort biology has experienced a renaissance following the establishment of *A. agrestis* as the model hornwort species

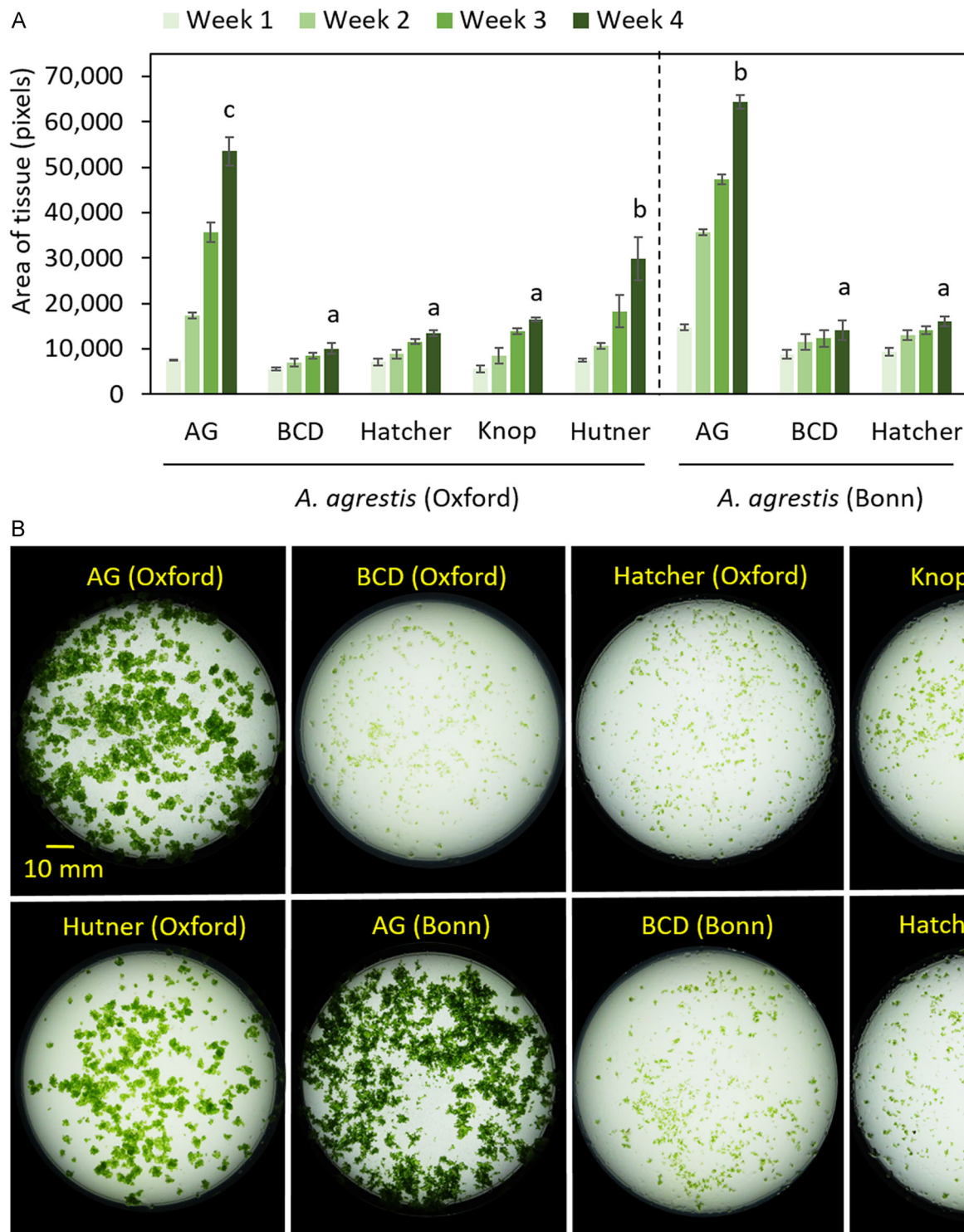


FIGURE 6 Comparison of the gametophytic growth of *Anthoceros agrestis* Oxford and Bonn strains on different media. The AG, BCD, Hatcher, Knop, and Hutner media were compared using the Oxford strain, while the AG, BCD, and Hatcher media were also compared using the Bonn strain. (A) Quantification of tissue area produced by gametophytes grown on the different media. Error bars indicate standard deviation. For each strain, different letters denote statistically different means (Tukey's HSD test, $P < 0.005$) at four weeks of culture. (B) Representative images captured at the fourth week of culture

(Szövényi et al., 2015; Frangedakis et al., 2021a), including the development of protocols for the genetic transformation of *A. agrestis* gametophytes (Frangedakis et al., 2021b) and the increasing availability of high-quality hornwort genome

assemblies (Li et al., 2020; Zhang et al., 2020). Because the hornwort gametophyte stage is the dominant phase of its life cycle, ongoing and future efforts to elucidate the biology of these bryophytes depend on establishing reliable and

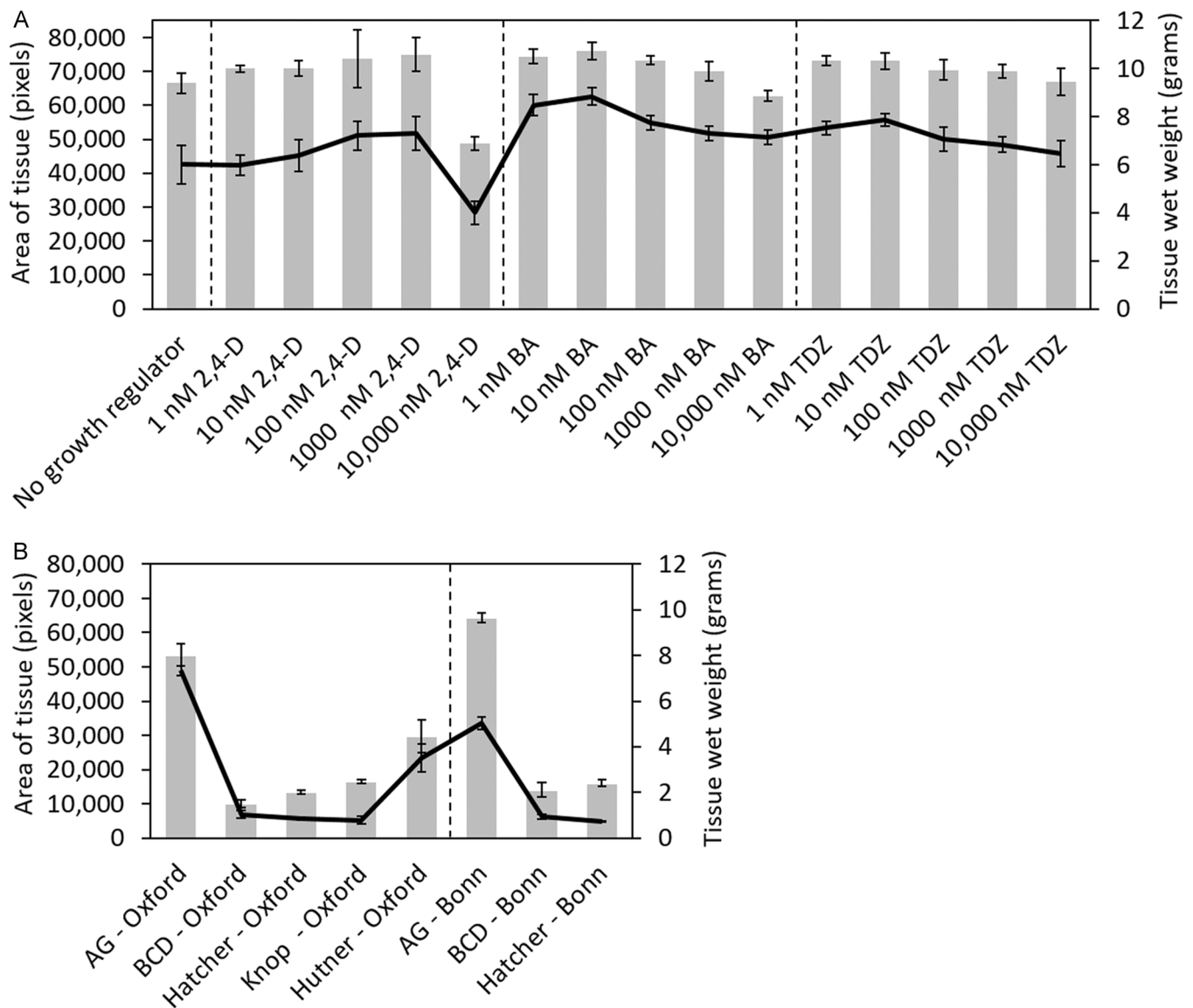


FIGURE 7 Correlations between the tissue area and wet weight in the experiments of this study. (A) Correlations between the results of the growth regulator experiment. (B) Correlations between the results of the media comparison experiment using Oxford and Bonn strains. The bar graph represents the tissue area, while the line graph represents the tissue wet weight. Error bars indicate standard deviation. The tissue area and wet weight correlate (using Pearson's correlation) at $r(16) = 0.83$, $P < 0.005$ in (A) and $r(8) = 0.91$, $P < 0.005$ in (B)

expedient methods to grow and assess hornwort gametophytes. To this end, we developed a user-friendly imaging pipeline for quantifying hornwort tissue growth, identified factors that affect the growth of *A. agrestis* gametophytes, and established a superior growth medium that is effective for accelerating the growth of hornwort gametophyte tissues.

Effective measurements of hornwort gametophytic growth using an imaging pipeline

Our imaging pipeline for determining the area of gametophyte tissue provides a rapid and reproducible method

for quantifying hornwort gametophytic growth. This method takes advantage of image segmentation and machine learning to recognize complex patterns within images and reduce the time required for analyzing multiple images (Arganda-Carreras et al., 2017). However, our method quantifies horizontal growth much more accurately than the vertical growth of the gametophyte tissue (Figure 1), which presents an important caveat for future applications. Nevertheless, this method correlates well with the more direct quantification of tissue growth via tissue wet weight (Figure 7), while at the same time offering a noninvasive alternative for estimating the overall hornwort gametophyte tissue growth and quality. Our use of microscopy and tissue staining provided

another layer of quality data regarding the various effects of the medium components on the growth of hornwort gametophyte tissues. The staining results (Figure 5C) indicate that SYBR Safe can be successfully adapted for staining nuclei and estimating the cellular dimensions of live hornwort gametophyte tissues. According to its chemical structure, SYBR Safe belongs to the cyanine dye family, which has been previously utilized to stain nucleic acids within live animal tissues (Nygren et al., 1998).

Fine-tuning hornwort gametophyte growth by medium supplementation

The initial basal Hatcher growth medium for the in vitro culture of hornworts lacked sucrose and ammonium nitrogen (Table 1). Building upon the Hatcher medium, several factors for inducing predictable phenotypic responses in hornwort gametophyte growth have been identified in this study. Medium supplementation with sucrose as a carbon source was identified as a major accelerator of biomass accumulation in hornwort gametophytes. A sucrose concentration of 0.2% was optimal for *A. agrestis* tissue growth, as higher concentrations consistently induced excessive rhizoid formation, more vertical “bushy” gametophyte growth, as well as tissue chlorosis and necrosis (Figures 2B, 4B). These trends are in alignment with previous studies on the effects of sucrose supplementation on other land plants (Yaseen et al., 2013); however, *A. agrestis* gametophyte tissues uniquely tolerate much less sucrose than many other land plants before the onset of stress responses, such as chlorosis and necrosis. It is possible that the low threshold for achieving a beneficial response to sucrose may be related to the activity of the pyrenoid-based carbon-concentrating mechanism in *A. agrestis*, but further studies are needed to determine this relationship.

We also identified the synergistic combination of sucrose with activated charcoal and ammonium nitrate (Figure 2; Appendices 1, 2). Unlike sucrose and the ambient atmospheric carbon, activated charcoal is composed of carbon-carbon bonds in the form of graphite. Although not readily available for plant uptake, activated charcoal may promote plant tissue growth through a combination of its high adsorption potential, buffering potential, and medium-darkening properties (Thomas, 2008). In our growth experiments, both activated charcoal and ammonium nitrate (an alternative nitrogen source) supplementation generally increased tissue greenness while only slightly accelerating tissue growth (Appendix 2, Figure 3); however, the combinatory effect of sucrose, activated charcoal, and ammonium nitrate improved the gametophyte tissue growth rate and quality beyond sucrose supplementation alone.

Next, MES (pH 6.5) supplementation was used to counter medium acidification, and it improved the tissue quality of hornwort gametophytes. MES buffering has been used in other plant tissue culture systems to stabilize the pH of the medium, although higher concentrations have been documented to adversely affect normal root growth patterns in vascular plants (de Klerk et al., 2008; Kagenishi et al., 2016). In *A. agrestis*, accelerated gametophyte tissue growth could be sustained beyond four weeks of culture using 5 mM MES buffer, which was more effective than the other MES concentrations tested (Figure 3).

The last category of media amendments assessed were the plant growth regulators, which have been routinely leveraged in a wide range of plant tissue culture techniques (mostly in vascular plants) to both induce optimal tissue growth patterns and to accelerate the recovery of genome-edited plants (Altpeter et al., 2016). For this purpose, auxins, cytokinins, and TDZ (Dewir et al., 2018) have proven especially useful for vascular plants. Endogenous auxin and cytokinin biosynthesis genes are present in nonvascular plants, including hornworts (Mutte et al., 2018; Li et al., 2020; Rashotte, 2021); thus, our evaluation of the response of *A. agrestis* to the exogenous supplementation of these growth regulators provides a valuable opportunity to compare their effects in vascular and nonvascular plants. Pertinently, our imaging results indicated that *A. agrestis* gametophyte responses align well with some classical vascular plant responses to these growth regulators, with a general enlargement of cell size in response to 2,4-D (an auxin analog), as well as a general increase in cell division and a smaller cell size in response to both BA (a cytokinin) and TDZ (a cytokinin-like compound) compared with the control without growth regulators (Figure 5C). We also observed that the assessed range of 2,4-D, BA, and TDZ concentrations, which was based on reports for vascular plants, was able to capture the effective concentrations that are suitable for altering cellular growth patterns in *A. agrestis* gametophytes.

Applications for AG growth medium

The AG growth medium, containing reduced sucrose (0.2%), activated charcoal ($100 \text{ mg} \cdot \text{L}^{-1}$), ammonium nitrate ($0.4 \text{ g} \cdot \text{L}^{-1}$), MES (pH 6.5) buffering (5 mM), and BA (10 nM) supplementations to the basal Hatcher medium, generated vigorous green *A. agrestis* thalli with minimal rhizoid formation and improved horizontal growth (Figure 5). The gametophyte growth rate on AG medium is significantly greater than on other media that have been commonly used for hornworts (Figure 6). Our initial experimentation also suggests that AG is useful for accelerating the gametophyte growth of other hornwort species (Appendix 4), although further fine-tuning of the medium components for each species is recommended for

optimal growth. We also observed that the level of gametophyte tolerance to sucrose varies between hornwort species (unpublished data).

Recently, *Agrobacterium*- and biolistic-mediated transformation methods were developed for *A. agrestis* gametophytes (Frangedakis et al., 2021b; Gunadi et al., unpublished data). These transformation methods pave the way for genome-modification experiments, which could tease out the genetic underpinnings of the multifaceted unique traits that distinguish hornworts from other land plants. Previously, using Hatcher medium supplemented with only 2% sucrose, we generated more than 10 independent stably-transformed lines from our initial attempt at biolistic-mediated transformation, but our transformation efficiency was low due to the lengthy process of recovering stable events (up to four months) and the low recovery of transformed tissue (Gunadi et al., unpublished data). A similar timeframe for transgenic event recovery was also observed in the *Agrobacterium*-mediated transformation protocol, which used Knop medium (Frangedakis et al., 2021b). The relatively slow growth of the gametophyte tissue using these unoptimized hornwort growth media lengthened the time required for transformation experiments and likely contributed to the low transgenic event recovery. The implementation of the optimized AG medium described here promises to further enhance the preexisting transformation methods by generating vigorous starting material and accelerating tissue recovery. Moreover, highly proliferative tissue material is also an ideal prerequisite for the development of more advanced functional genetic tools such as RNAi and genome editing in hornworts. Efforts to incorporate AG medium in developing these pipelines using *A. agrestis* are already underway.

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AUTHOR CONTRIBUTIONS

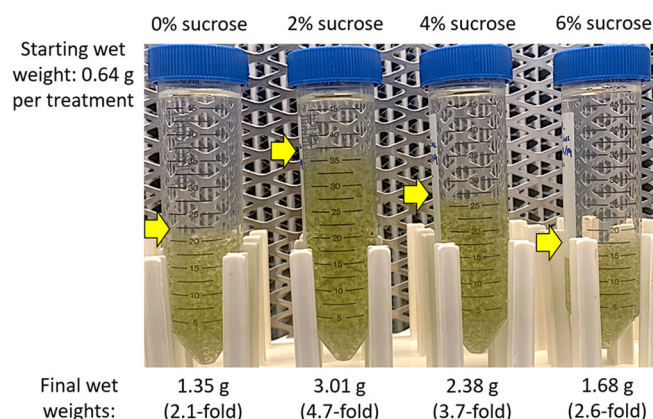
A.G. designed and performed the experiments, with input from F.W.L. and J.V.E. A.G., F.W.L., and J.V.E. analyzed the data. A.G. prepared the first draft of the manuscript, with input from F.W.L. and J.V.E. All authors approved the final version of the manuscript.

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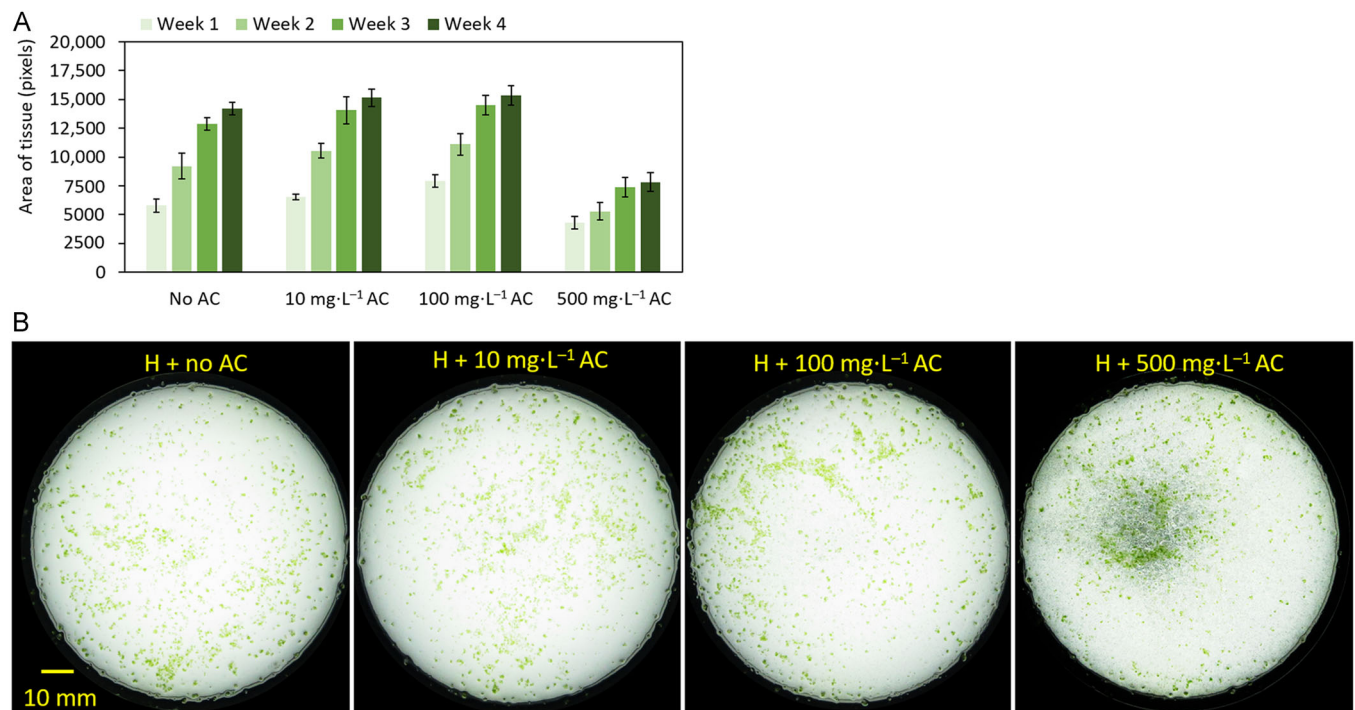
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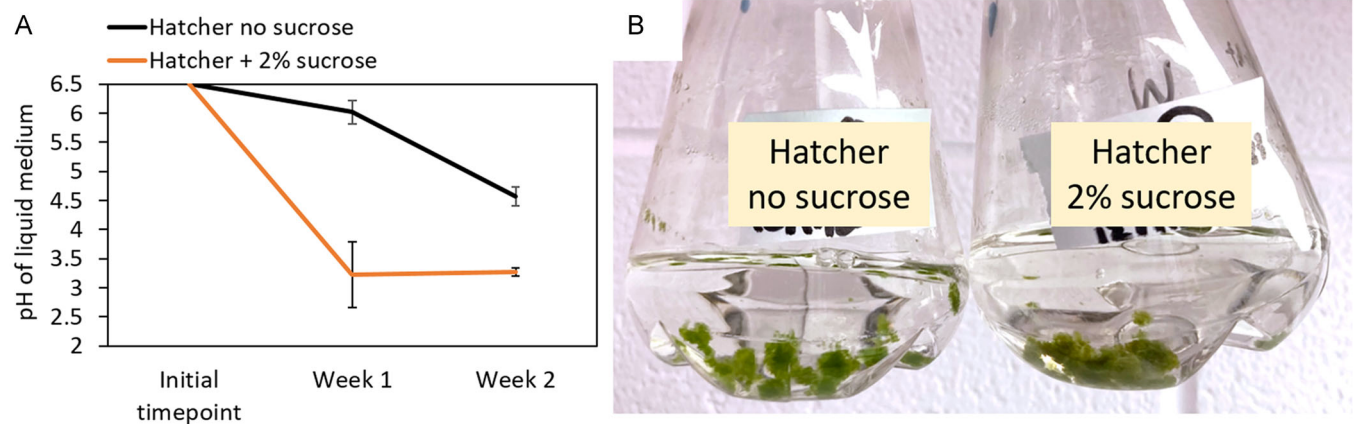
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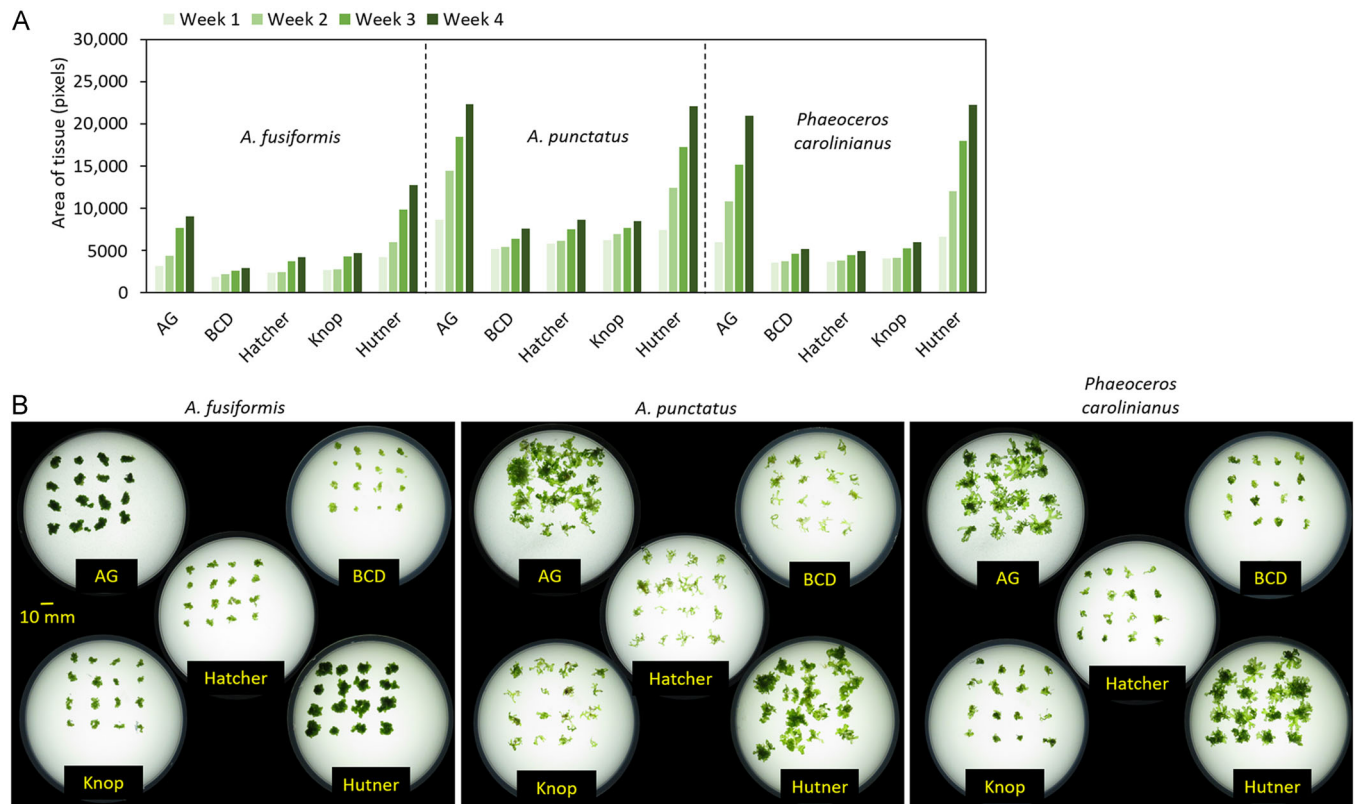
APPENDIX 1 Preliminary liquid medium supplementation with sucrose. This experiment was replicated once. The volume difference was measured using 50-mL centrifuge tubes. After two weeks of culture, the tissues were collected using a cell strainer and their final wet weights were measured



APPENDIX 2 Medium supplementation with activated charcoal (AC). (A, B) Both 10 and 100 mg · L⁻¹ AC resulted in a slightly higher average area of tissue than the Hatcher (no AC) control, albeit not statistically significant (Dunnett's test, $P > 0.05$) using the fourth week data. By contrast, 100 mg · L⁻¹ AC supplementation resulted in a statistically higher area of tissue in a separate experiment (Figure 2A)



APPENDIX 3 Liquid medium acidification during the culture of *Anthoceros agrestis* (Oxford strain). Approximately 0.3 g of tissue was cultured in the liquid Hatcher medium (without sucrose) and Hatcher medium supplemented with 2% sucrose for three replicates (flasks) each. (A) Both the 0% and 2% sucrose treatments acidified the medium over two weeks of culture. (B) Gametophyte tissues without sucrose supplementation displayed greener coloration and more pronounced thalloid growth than those grown with a 2% sucrose supplementation



APPENDIX 4 Preliminary growth comparison of *Anthoceros fusiformis*, *A. punctatus*, and *Phaeoceros carolinianus* in AG, BCD, Hatcher, Knop, and Hutner growth media. Initial starting tissue (unhomogenized 0.5 g gametophyte) for each hornwort species was placed into each Petri plate (media type) and allowed to grow for four weeks. (A) Images were collected every week and area of gametophytic growth was quantified. (B) The fourth-week images were displayed for comparison