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Tunable plant-based materials via *in vitro* cell culture using a *Zinnia elegans* model

Ashley L. Beckwith [a, b], Jeffrey T. Borenstein [b], Luis F. Velásquez-García [c, *]

[a] Mechanical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave, Cambridge, MA, 02139, USA
[b] Charles Stark Draper Laboratory, 555 Technology Square, Cambridge, MA, 02139, USA
[c] Microsystems Technology Laboratories, Massachusetts Institute of Technology, 60 Vassar St, Cambridge, MA, 02139, USA

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ABSTRACT

Current systems for plant-based biomaterial production are inefficient and place unsustainable demands on environmental resources. This work proposes a novel solution to these shortcomings based on selective cultivation of tunable plant tissues using scalable, land-free techniques unconstrained by seasonality, climate, or local resource availability. By limiting biomass cultivation to only desirable plant tissues, *ex planta* farming promises to improve yields while reducing plant waste and competition for arable land. Employing a *Zinnia elegans* model system, this work provides the first proof-of-concept demonstration of isolated, tissue-like plant material production *in vitro* by way of gel-mediated cell culture. Parameters governing cell development and morphology including hormone concentrations, medium pH, and initial cell density are optimized and implemented to demonstrate the tunability of cultured biomaterials at cellular and macroscopic scales. Targeted deposition of cell-doped, nutrient-rich gel scaffolds via casting and 3D bioprinting enable biomaterial growth in near-final form, reducing downstream processing requirements. These investigations demonstrate the implementation of plant cell culture in a new application space, propose novel methods for quantification and evaluation of cell development, and characterize morphological developments in response to critical culture parameters—illustrating the feasibility and potential of the proposed techniques.

1. Introduction

Plant-based materials are ubiquitous in modern life. Wood and other plant feedstocks are key resources (Devillard and Walter, 2014) for global infrastructure development, manufacture of consumer goods, and energy production (Energy Information Mon, 2020; Simmons et al., 2008; Plomion et al., 2001). Unlike fossil fuel-derived alternatives, plant-based products are often lauded for their renewability, but current rates of consumption are unsustainable. Strain on agricultural resources in response to the ever-increasing demands for both food and non-food crops has generated lasting environmental consequences. For example, consumption of wood and the clearing of wooded areas to access arable lands resulted in the global loss of over 500,000 square miles of natural forests between 1990 and 2016 (Nunez, 2019)—an area roughly two and half times the size of France. Continued deforestation contributes to the endangerment of species and the reduction of natural carbon stores.

From a high-level perspective, the process of plant-based feedstock production has changed little in centuries: whole plants are cultivated, useful portions are harvested, and the remains are discarded or burned for energy (van Dam, 2009). Useful fractions of the original biomass are then mechanically or chemically restructured into functional forms or isolated chemical compounds (e.g., cellulose). With surging demands for plant-based feedstocks (Simmons et al., 2008), efforts have been made, on a process-specific level, to reduce inefficiencies of biomass manipulation within the industrial setting. Nevertheless, the greatest costs and resource consumption in the supply chain often precede these steps, e.g., consider the time, land, water, fertilizers, and pesticides dedicated to cultivation of whole plants. In addition, harvest and transportation of biomass to processing locations can require significant investment of financial capital and energy (e.g., in harvesting woody biomass, logging and transportation expenses make up a sizeable fraction of gate costs (Stasko et al., 2011)). Despite

* Corresponding author.
E-mail addresses: ashbeck@mit.edu (A.L. Beckwith), jborenstein@draper.com (J.T. Borenstein), Velasquez@alum.mit.edu (L.F. Velásquez-García).

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considerable and early resource investment, only a small fraction of the cultivated crop may be economically valuable at harvest (for the production of some natural fibers, as little as 2%–4% of the harvested plant matter comprises useful material; for other crops, just one third of the stem dry weight may be characterized as such (van Dam, 2009)). As long as cultivation practices remain fundamentally unchanged, little can be done to address these wasteful process steps.

Biomass generation via plant cell culture has the potential to alleviate persistent challenges in standard agricultural methods. This work aims to demonstrate plant material production through the directed growth of specific plant tissues, free of unwanted components, with modifiable properties and in controllable architectures. Living plant cells exhibit impressive developmental potency (Fehér, 2019), i.e., they can give rise to plant cells of nearly any type—or even an entire plant under the right environmental conditions (Ikeuchi et al., 2013). Analogous to human stem cells, plant cells retain the ability to differentiate in response to local environmental cues (Loyola-Vargas and Vazquez-Flota, 2006). This property motivated and enabled the establishment of plant cell culture techniques that support efforts on both academic and industrial scales. *In vitro* plant models not only facilitate greater understanding of the underlying mechanisms of plant growth and development (Loyola-Vargas and Vazquez-Flota, 2006), but have also guided the generation of more robust crops and more easily processable biomass for biofuel production (Kapp et al., 2015). Plant cell cultures can be employed to produce natural products (Ochoa-Villarreal et al., 2016), biopharmaceuticals (Xu and Zhang, 2014), and even nanoparticles (Mohammadinejad et al., 2019). Additionally, micropropagation practices, whereby small plant samples are cultured to generate thousands or millions of genetically-identical seedlings, are readily used in the agriculture industry to rapidly increase plant populations with desirable properties (e.g., pathogen resistance) at any time of the year, even when faced with limited space requirements (Bhatia et al., 2015). Micropropagation is particularly valuable in plant species that are endangered, slow to reproduce (Isikawa, 1984), or cannot otherwise be propagated by traditional methods (e.g., seeds, cuttings, division) (Chu, 1992). As in micropropagation, this study proposes methods of biomass generation capitalizing on large-scale, laboratory-mediated expansion of plant matter. However, rather than encouraging growth of complete new organisms, techniques developed for this work intend to promote the selective growth of only desirable plant tissues.

As a specific example: in a tree, a sub-category of vascular tissue known as *xylem* comprises the wood—an important feedstock for a range of industries, e.g., infrastructure, paper, clothing, consumer products (Plomion et al., 2001). In a natural setting, wood production is a slow process; even fast-growing trees such as poplars may take as long as 20 years to acquire a profitable volume of timber (Bajaj, 1989), at which point a large portion of the tree's available resources and energy have been expended producing industrially unworkable materials such as leaves, bark, and small limbs. As such, the potential to recapitulate isolated xylem tissue independent of the economically undesirable plant constituents is an intriguing production concept. This type of cultivation scheme could improve upon currently unavoidable inefficiencies in the agricultural process and also reduce total cultivation time.

This study proposes a new approach to plant-based biomaterial production that substantially lessens or altogether eliminates inefficiencies in the agricultural and pre-processing stages. A proof-of-concept demonstration using a model *Zinnia elegans* (*Z. elegans*) system illustrates the feasibility of targeted cultivation methods. Tunable plant-based constructs, intended to emulate isolated plant tissues, are grown in pre-defined architectures by way of gel-mediated cell culture. Materials produced via isolated tissue-like generation require only small, non-sacrificial donations from a parent plant. From these biological starters, cells are extracted, maintained, and expanded many times to yield large volumes of plant matter. The proposed isolated plant tissue-like generation promises several key advantages over existing practices. For example, the production of only useful plant components (e.g., wood or secondary xylem) without unwanted or unusable plant anatomy (e.g., bark, small twigs, roots, leaves) reduces waste associated with the biomaterial production process. Additionally, *in vitro* plant substrate growth allows for greater control over cellular-level composition, enhancing tunability of the product's material properties and chemical make-up. Finally, macroscopic substrate configuration can be adjusted to meet specific application requirements through the controlled deposition of gel-scaffold material, enabling the growth of structures in near-final form via casting or 3D bioprinting—further reducing waste. *Z. elegans* is a practical model species for this demonstration because the plant is well-studied, grows rapidly, and certain cells of the xylem family (tracheary elements) can be produced reliably in suspension cultures at frequencies as high as 60% (Domingo et al., 1998; Fukuda and Komamine, 1980; Milioni et al., 2002).

The proposed concept of selectively-grown, tunable plant materials *via* gel-mediated cell culture is believed to be the first of its kind. This work uniquely quantifies and modulates cell development of cultured primary plant products to optimize and direct growth of plant materials. Section 3.4 reviews current and historical efforts pertaining to gel-mediated cell growth.

2. Materials and methods

2.1. Media preparation

To prepare media, all listed ingredients (Table 1 or Table 2), except for BAP, were dissolved in 1 L of deionized water. The pH of the solution was adjusted to a value between 5.5 and 6.5 prior to autoclave sterilization at 121 °C for 20 min. The assigned, sterile quantity of BAP was then added to autoclaved medium in an aseptic environment. To prepare gel media, Gelzan CM (Sigma Aldrich) was also added to the media mixture at 4 g L^{-1} prior to autoclaving. When gel cultures were plated, final gelling agent concentration was reduced to 3 g L^{-1} by dilution with liquid medium at a ratio of 3:1 (v/v). Media recipes presented in Tables 1 and 2 represent a simplified mixture of ingredients with a final formulation closely related to those presented by Fukuda and Komamine (1980) and Domingo et al. (1998).

2.2. Cell isolation and experiment preparation

Inspired by methods of Fukuda and Komamine (1980), *Z. elegans* cells were isolated by the maceration of young zinnia leaves. Leaves collected from approximately 14 day-old zinnia plants were rinsed under running tap water for 5 min. Leaves were sterilized in a solution of 5 ml commercial bleach (Clorox), 95 ml of deionized water, and 200 μl of Tween 20 (Sigma Aldrich) for 5 min. Subsequently, leaves were rinsed thoroughly with sterile distilled water, sliced into strips, and gently ground between the surfaces of a small sieve and a stainless-steel spoon. The leaf matter was intermittently rinsed with ZE-M media and the rinsate was collected in a small bowl positioned beneath the supporting sieve. After completion of grinding, the rinsate was collected and filtered through a 70 μm cell strainer (Fisher Scientific) to remove large debris. The filtered solution was centrifuged at 100 g for 8 min and resulting supernatant removed if more concentrated cell solutions were needed. Liquid cultures were maintained at concentrations between 250,000 and

Table 1
Recipe for *Z. elegans* maintenance medium (ZE-M).

Product name	Supplier	Quantity [per liter of medium]
N616 Nitsch medium	Phytotech Laboratories	2.21 g
Sucrose	Sigma Aldrich	10 g
Mannitol	Sigma Aldrich	36.4 g
α-Naphthaleneacetic acid (NAA)	Sigma Aldrich	0.001 mg[a]
6-Benzylaminopurine solution (BAP)	Sigma Aldrich	1 μl

[a] 1 μl of 1 mg ml^{-1} NAA stock solution.

Table 2
Recipe for *Z. elegans* induction medium (ZE-I) for differentiation.

Product name	Supplier	Quantity [per liter of medium]
N616 Nitsch medium	Phytotech Laboratories	2.21 g
Sucrose	Sigma Aldrich	10 g
Mannitol	Sigma Aldrich	36.4 g
α-Naphthaleneacetic acid (NAA)	Sigma Aldrich	1 mg[a]
6-Benzylaminopurine solution (BAP)	Sigma Aldrich	1 ml

[a] 1 ml of 1 mg ml^{-1} NAA stock solution.

500,000 cells ml^{-1} at 3 ml per well in a 6-well plate wrapped in parafilm. Cultures were maintained at 22 °C in the dark on an orbital shaker operating at 80 rpm. Cells were cultured in this manner for 48 h in the low-hormone media before transference to specialized experimental media. An incubation period improved differentiation rates when cells were later exposed to elevated levels of auxin and cytokinin.

For dispersed gel cultures, cells were extracted and maintained in low-hormone, liquid medium for a 48 h acclimation period, as previously described, prior to transference to a gel medium solidified by the addition of Gelzan CM (Sigma Aldrich) at a final concentration of 3 g L^{-1}. Cultures were sealed off with parafilm and maintained at 22 °C in the dark.

2.3. Ex vivo generation of plant tissue-like substrates

From a small volume of plant material, cells were isolated to establish a liquid suspension culture. Early process steps are subject to variation depending on the plant species in use: for *Z. elegans*, cells are readily acquired through maceration of young leaves, but other species require an intermediate callus culture step, whereby a cell mass is initiated through prolonged culture atop a gelled, nutrient-rich medium (Mustafa et al., 2011). In both cases, collected cells were transferred to a liquid culture where they may be cultivated, sub-cultured and utilized as a long-term feedstock for the subsequent culture steps. To initiate construct growth, cell suspension stock was mixed with a thermosetting gel medium at a 1:3 ratio (v/v). The resulting mixture solidified when cooled to room temperature to yield a culture of single cells dispersed with a gelled, nutrient-rich scaffold (Fig. 1). With time, the dispersed gel cultures grew to generate confluent cellular material. In gel-mediated culture, cells survived for several weeks and, by tuning local biochemical and mechanical properties, cells were directed to develop into desirable cell types or morphologies. The shape of the cultivated materials was controlled via casting or bioprinting (Tissue Scribe Gen.3, 3D Cultures) of cell-doped scaffolds.

2.4. Fluorescence microscopy and image analysis

For liquid cultures, well-mixed 250 μl aliquots of cell suspension were transferred to 48 well plates for imaging. A 5 μl volume of a fluorescein diacetate (FDA) stock solution (prepared at 2 mg ml^{-1} acetone) was added to the cell suspension and incubated in the dark for 20 min. After the incubation period, 63 μl of calcofluor white (CW) was added and the solution rested for an additional 5 min prior to imaging. A Zeiss LSM780 confocal microscope was set to excitation/emission wavelengths of 265 nm/440 nm (DAPI filter) for CW and 490 nm/526 nm for FDA for imaging. Gel cultures were stained through a similar dual-step process after wetting the gel surface with a small amount of liquid medium. Relative staining volumes were the same, but increased incubation times were required (e.g., incubation times were approximately 45 min for FDA incubation, 45 min for CW incubation). Image analysis was performed using Image-J. The thresholding tool was used to select the relevant cell areas. To ensure robustness of the analyzed data ranges, the images corresponding to the highest and lowest values for a single sample were analyzed a total of 3 times, with the repeated results averaged to yield a final value for the given image.

2.5. Measurement metrics for monitoring culture growth and development

In plants, cellular make-up of a plant tissue affects the corresponding macroscopic mechanical properties. For example, elevated proportions of highly aligned, stiffened (i.e., with a lignified secondary cell wall) vascular cells in plant stems contribute to increased rigidity of the tissue. Therefore, understanding and controlling cellular composition of cultured biomaterials is critical to producing useful substances for a wide range of applications. The study of cellular development in response to culture parameters allows for growth optimization yielding substrates with desirable compositions of cellular constituents and associated macro-scale material properties. Numerous environmental factors impact plant cell development *in vitro*. Based on extensive literature review and preliminary experimentation, this work focuses on three particularly influential and controllable parameters: the interactive effects of two hormone concentrations, medium pH, and initial cell density. These parameters were ultimately selected because they allow for measurable, largely independent manipulation, and preliminary experiments indicated cell responsiveness to their adjustment. Although hormone concentration (Fukuda and Komamine, 1980; Pesquet et al., 2005), pH (Ślesak et al., 2007), cell density (Turner et al., 2007) and other factors (Ivakov and Persson, 2013; Zaban et al., 2014) have been independently investigated to various extents, thorough re-evaluation of these variables is required in order to: (a) verify relevant cell behavior in spite of

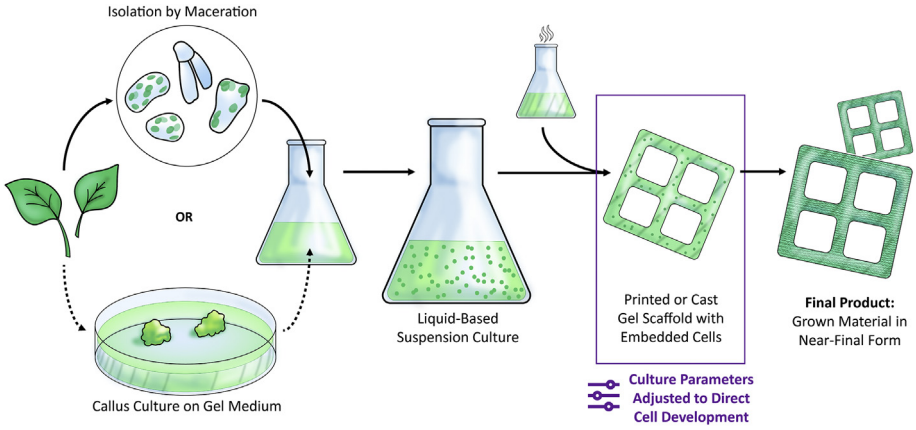

Fig. 1. Process flow of selective tissue-like growth from non-destructive plant sample. From a small volume of plant material, *ex vivo* tissue-like constructs can be grown in the lab. The process of cell isolation from plant samples varies with plant species. In some species, cells can be obtained directly through maceration of leaves, while others require an additional callus culture step as depicted by the dashed arrows.

new, simplified media recipes (Tables 1 and 2), (b) characterize previously unreported developmental traits such as enlargement and elongation, (c) demonstrate the use of new metrics to quantify cell development, and (d) contribute to the limited information available on cell growth and development in dispersed gel culture. Four measurement metrics, i.e., *live fraction, lignification metric, enlargement metric,* and *elongation metric*, quantify collective culture development in a measurable way and allow for methodical selection of culture parameters to suit output requirements. The metrics are tabulated from micrographs and reflect live fraction, tracheary element differentiation (i.e., lignification), cell enlargement, and cell elongation in response to applied culture conditions:

i. The live fraction metric is the ratio between the percentage of the micrograph occupied by cells marked with a viability probe (fluorescein diacetate), A_L, and the percentage of the micrograph occupied by all cells marked with a cell wall stain (calcofluor white), A_T, i.e.,

$$Live\ \ Fraction[\%] = 100\%*\frac{A_L}{A_T}; \tag{1}$$

Live fraction provides insights into cell health and can act as a secondary indicator of tracheary element differentiation as cells undergo programmed cell death at late stages of development.

ii. The lignification metric is the ratio between C_L—the number of lignified cells in the micrograph, and the corresponding A_T, i.e.,

$$Lignification\ \ Metric[\%^{-1}] = \frac{C_L}{A_T}; \tag{2}$$

Lignification metric quantifies the extent of culture differentiation into tracheary elements possessing a rigid, lignified cell wall—the presence of which may increase stiffness of the overall grown material.

iii. The cell enlargement metric is the ratio between C_{En}—the number of cells in the micrograph with a maximum dimension greater than a certain threshold, and the corresponding A_T, i.e.,

$$Cell\ \ Enlargement\ \ Metric[\%^{-1}] = \frac{C_{En}}{A_T}; \tag{3}$$

the threshold value for cell enlargement, l_α, represents the maximum dimension of cultured cells at 48 h after cell isolation (l_α is approximately equal to 84 μm in this case). The cell enlargement metric provides an indicator of average cell-level growth or swelling.

iv. The cell elongation metric is the ratio between C_{El}—the number of cells in the micrograph with a maximum dimension greater than a certain threshold, and the corresponding A_T, i.e.,

$$Cell\ \ Elongation\ \ Metric[\%^{-1}] = \frac{C_{El}}{A_T}; \tag{4}$$

the threshold value for cell elongation, l_β, represents the maximum dimension of cells grown in low-hormone media for 12 days that exhibit multi-directional enlargement without pronounced uniaxial elongation (l_β is approximately equal to 119 μm). Greater proportions of elongated cells may increase prevalence of cell-to-cell entanglement in confluent cultures, potentially influencing grown material properties.

For viability metrics and the calculation of percent cell area (A_T) in all cases, two-channel images visualizing FDA and CW were taken with focal plane adjusted to resolve FDA features. Lignified cell counts, enlargement, and elongation measurements were made on a corresponding single-channel CW image, with identical field of view but focus adjusted slightly to resolve CW features.

2.6. Statistical methods

Generally, data reported for a specific timepoint and treatment were averaged across all images evaluated for that treatment on that day. Error bars on provided data plots represent one sample standard deviation above and below the mean. Two sample t-tests were performed at a confidence level of 95% to establish P-values between pairs of datasets (Matlab). In the case of hormone response experiments, in which cell response to two factors were investigated simultaneously, a full factorial design was performed at 4, near-equally-incremented levels of each hormone concentration (i.e. amounting to 16 individual hormone combinations). Factorial approaches are generally preferred to one-factor-at-a-time experimentation because they enable the detection of interaction effects between variables. The resulting response metrics were mapped using the Matlab contour function.

3. Results and discussion

Cell development is characterized in response to changes in three, independently controllable parameters: concentrations of auxin and cytokinin in the prepared medium, medium pH, and initial cell concentration. Quantification and visualization of development trends using the proposed measurement metrics enable purposeful adjustment to culture growth. Findings from characterization studies are put to use cultivating grown materials with distinct cellular make-ups. The physical form of cultivated materials is controlled by the casting or bioprinting of cell-doped scaffolds.

3.1. Effects of hormone concentrations on cell development

Two hormone classes, i.e., auxin and cytokinin, are drivers of plant cell development (Ikeuchi et al., 2013) and are critical to vascular tissue development in particular (Milioni et al., 2002). Both auxin and cytokinin independently control a wide range of cell behaviors (Taiz and Zeiger, 2003). Considered together, the hormones elicit complex, interactive effects. For example, elevated levels of both auxin and cytokinin can induce the differentiation of *Z. elegans* cells into lignified tracheary elements (Fukuda, 1997), while collectively low hormone concentrations can be provided to *Z. elegans* cultures to encourage maintenance and proliferation without further differentiation. Cell morphology and development at unbalanced ratios of auxin and cytokinin concentrations have not been as well-characterized, particularly in relation to cell enlargement and elongation. In this work, a full factorial experiment was performed to evaluate cellular development at a range of hormone concentrations over a total 12 day culture period, using the previously described metrics (see Section 2.5). After a 48 h acclimation phase in which isolated cells were cultured in low-hormone media, samples were transferred to treatment media and imaged periodically over the course of the subsequent 10 days. The two hormones selected for evaluation are commonly employed in plant cell culture: a synthetic auxin—α-naphthaleneacetic acid (NAA), and a synthetic cytokinin—6-benzylaminopurine solution (BAP). Hormone concentrations ranged from 0.001 mg ml^{-1}, as recommended for culture maintenance, to 1.5 mg ml^{-1}—one and a half times the concentration regularly cited for tracheary element induction (Domingo et al., 1998), evaluated at approximately 0.5 mg ml^{-1} intervals. Contour plots of live fraction, lignification, enlargement, and elongation metrics map the cellular responses to the varied hormone levels (Fig. 2a–d). The experimental results demonstrate that tuning hormone concentrations allows for control over final cellular composition of the treated culture. In low-hormone media, cells exhibit high levels of viability (>70% live fraction) after ten days in treatment media and the corresponding levels of lignification remain at or near zero. Cells in low-hormone treatment media tend to enlarge over time, but experience limited uniaxial elongation. The highest cumulative levels of lignification occurred with NAA at 0.5 mg ml^{-1} (0.5 ml L^{-1} of stock solution) and BAP at 1 mg ml^{-1} (1 ml L^{-1} of stock solution); correspondingly, these hormone levels exhibit the lowest live fraction at day 12—a reasonable result as lignifying tracheary elements undergo programmed cell death in the final stages of differentiation (Plomion et al., 2001). For this reason, the lignification metric should trend inversely with live fraction. Both the day-to-day **(S.1 in the Supplementary Information document)** and cumulative lignification values (Fig. 2b) tend to align with this projected behavior. Results also indicate that with the selected base media formulation, BAP plays an important role in determining the elongation fate of cells, although this control is commonly attributed to auxin specifically (Domingo et al., 1998); (Zaban et al., 2014); (Taiz and Zeiger, 2003). Elongation metric plotted across hormone levels shows that when BAP is elevated, elongation tends to be reduced across most NAA concentrations (Fig. 2d).

3.2. Effects of pH on cell development

Auxin-mediated cell elongation is believed to act, at least in part, by encouraging the release of cell wall-loosening factors, which may include hydrogen ions (Taiz and Zeiger, 2003). Wall-loosening factors are believed to promote wall compliance, enabling cell expansion and restructuring (Taiz and Zeiger, 2003). Therefore, hydrogen ion concentration in the growth medium, as reflected by pH, was similarly suspected to influence cellular development. To quantify the effects of pH on cell development, *Z. elegans* cells were cultured in either maintenance media (ZE-M) or induction media (ZE-I) at one of 3 pH values (i.e., prepared at pH 5.22, 5.75, or 6.4 prior to a final autoclave sterilization). After a 48 h acclimation period in which all isolated cells were cultured in low-hormone medium at pH 5.75, samples were transferred to treatment media and imaged periodically over the course of the subsequent 10 days. From the analysis of fluorescence micrographs, live fraction, lignification, enlargement, and elongation metrics were evaluated for each of the culture treatments. In maintenance media cultures, pH had negligible effect on live fraction or lignification metrics. For all pH levels, live fraction for ZE-M cultures increased from a starting point of 36.4% to a final value close to 80% (Fig. 3). These results trend similarly with those seen in the factorial hormone experiment where low-hormone cultures experienced a shift in live fraction from 36.8% to an excess of 70% at the final time-point. For the pH evaluation, the lack of significant differences between live fraction of maintenance media (ZE-M) groups suggests that the selected pH values were not independently detrimental to cell viability. At low hormone levels and low pH levels, measured cells tended to be larger in size as quantified by enlargement and elongation metrics. Low pH ZE-M samples were significantly larger than medium or high pH samples ($P = 0.03$ and 0.016, respectively). Low pH ZE-M samples exhibited greater elongation than moderate pH samples ($P = 0.018$) which in turn, experienced significantly greater elongation than high pH samples ($P = 0.0095$) by day 12. For cell cultures grown in high-hormone media (ZE-I), pH proved to be influential in lignification (Fig. 4) and live fraction metrics. Low pH, ZE-I cultures presented significantly elevated lignification metrics for days 6, 8, and 12 when compared to high pH samples ($P = 0.00072$, 0.045 and 0.0013, respectively). Inversely, live fraction for low pH groups were significantly lower than those in high pH samples on the final culture day ($P = 0.00062$). These results align with the hypothesized inverse relationship between differentiation and live fraction. While pH strongly influenced enlargement and elongation in the low-hormone maintenance media, pH adjustments did not generate significant trends in morphological development for high-hormone induction cultures (Fig. 5).

3.3. Effects of initial cell density on cell enlargement and elongation

Cell concentration is reported to influence cellular development in liquid cultures (Turner et al., 2007). Thus, the examination of cell density effects on cell morphologies in gel-media is also necessary to achieve effective control over gel-based culture development. Effects of cell density on development were quantified through the image analysis of gel cultures established at four starting cell densities and then monitored for a 14 day incubation period. Gel cultures were prepared at initial cell densities of 5×10^4, 1×10^5, 2×10^5, and 4×10^5 cells ml^{-1} (i.e., 1x, 2x, 4x, and 8x multiples of 5×10^4 cells ml^{-1}). For every time-point, two replicate gel cultures were prepared at each concentration and at least three

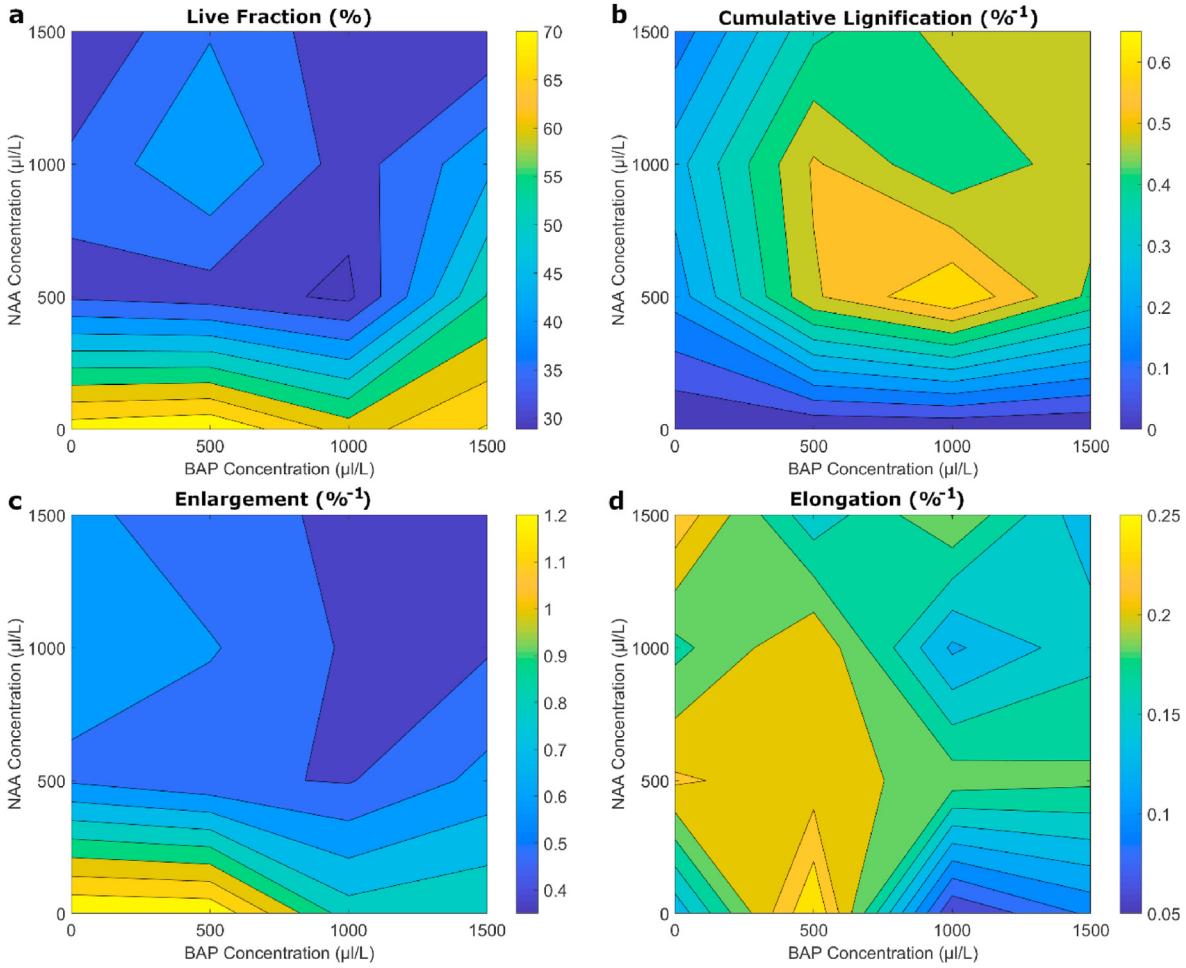

Fig. 2. Characterization of cell response to varied hormone levels. Live fraction (a), cumulative lignification metric (b), enlargement metric (c), and elongation metric (d) on the twelfth day in culture are mapped for cells exposed to varied levels of NAA and BAP. N = 4–5 images per datum point. For (a), (b), (c), and (d), the average standard deviations for analyzed data points across each map are 6.63, 0.034, 0.095, and 0.055, respectively.

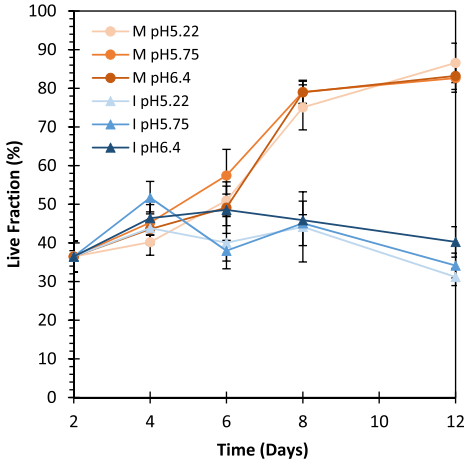

Fig. 3. Live fraction versus time in pH-adjusted culture. Live fraction increases for cultures in maintenance media (M) for all pH levels, whereas live fraction remains low when cells are supplied with induction media (I). When cultured in induction medium, cultures yield lower live fraction for lower pH values in later culture stages. Error bars represent ±1 SD. N = 4–5 images per datum point.

independent images were captured and evaluated per replicate. After 14 days in culture, cells seeded at higher initial cell densities exhibited increased cell size as quantified by both enlargement and elongation metrics (Fig. 6). While differences between enlargement metrics at low cell densities (i.e., 5×10^4 cells ml^{-1} (1x) and 1×10^5 cells ml^{-1} (2x) cultures) were not significant, metrics for 2×10^5 cells ml^{-1} (4x) cultures were higher than 1×10^5 cells ml^{-1} (2x) cultures ($P = 0.0097$), and cells of 4×10^5 cells ml^{-1} (8x) cultures were larger than 2×10^5 cells ml^{-1} (4x) cultures ($P = 0.0014$). Similarly, with respect to elongation, 5×10^4 cells ml^{-1} (1x) and 1×10^5 cells ml^{-1} (2x) values were not significantly different, but 2×10^5 cells ml^{-1} (4x) and 4×10^5 cells ml^{-1} (8x) cultures contained longer cells than the 1×10^5 cells ml^{-1} (2x) cultures as measured by elongation metrics (with $P = 0.0241$ and 0.011, respectively). To confirm that the imaging methods provided a representative snapshot of the gel cultures in spite of their three-dimensional nature, total evaluated percent area (A_T) was plotted against concentration factor to check for linearity (**S.2 in the Supplementary Information document**). The relationship between cell concentration and evaluated area was confirmed to be linear with R-squared values for both time-points exceeding 0.98 when linear trendline intercepts were set to zero; setting the intercept as

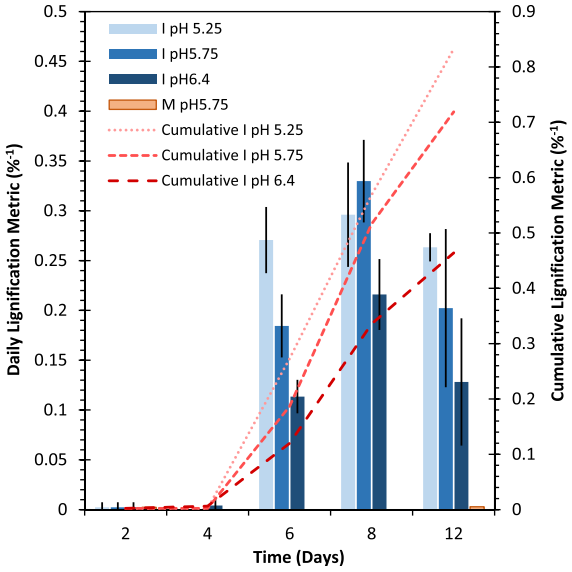

Fig. 4. Lignification versus time in pH-adjusted culture. Daily lignification metric and a cumulative lignification metric (i.e., summed data of a given timepoint and all previous timepoints for a selected treatment group) versus time in culture. Daily and cumulative lignification metrics are highest for induction media (I) with the lowest pH while culture sustained in maintenance media (M) retains a lignification metric close to zero throughout the experiment. Error bars represent ±1 SD. N = 4–5 images per datum point.

such reflects the state at which no cells are present and, therefore, the total percent cell area, A_T, is zero.

3.4. Growing plant-based materials in vitro

Insights from the experiments on hormone concentration, medium pH, and cell density guided the development of lab-grown, plant-based materials. Gel media parameters were selected based upon the ultimate desired cellular constituents. Macroscopic culture architectures were controlled either through casting (Fig. 7) or through 3D bioprinting of a cell-doped gel media solution (Fig. 8). Because required nutrients and hormones are incorporated within the scaffold itself, this fully contained setup requires little intervention after deposition. The scaffold can sustain growth through differentiation and to confluency without requiring supporting perfusion systems. Because viability is not required beyond the point of confluency, this approach provides a simple, low energy means of cultured plant material production. The employment of nutrient-rich gel as a medium for tissue-like growth is inspired by culture methods put forth by Ludwig Bergmann in the 1960's. Bergmann demonstrated the immobilization of single plant cells in a thin layer of gel media for the purpose of monitoring cell division and colony development (Bergmann, 1960). Through modifications to foundational techniques, advancements in this work allow for not only the prolonged immobilization and survival of cells in gel, but the directed growth and development of gel-based cultures to produce specific, tunable, tissue-like materials.

Plants and plant products are of growing interest to the bioprinting community. Increasingly, plant products (e.g., cellulose, pectin) are employed as scaffold materials to support animal cell culture (Jovic et al., 2019), but bioprinting of plant cells themselves has only been explored in a limited capacity to date (Mehrotra et al., 2020). Preliminary investigations into the field have focused on the development of suitable bioprinting strategies and the application of immobilized cell culture techniques to investigate phenomena such as metabolite production (Seidel et al., 2017) or single plant

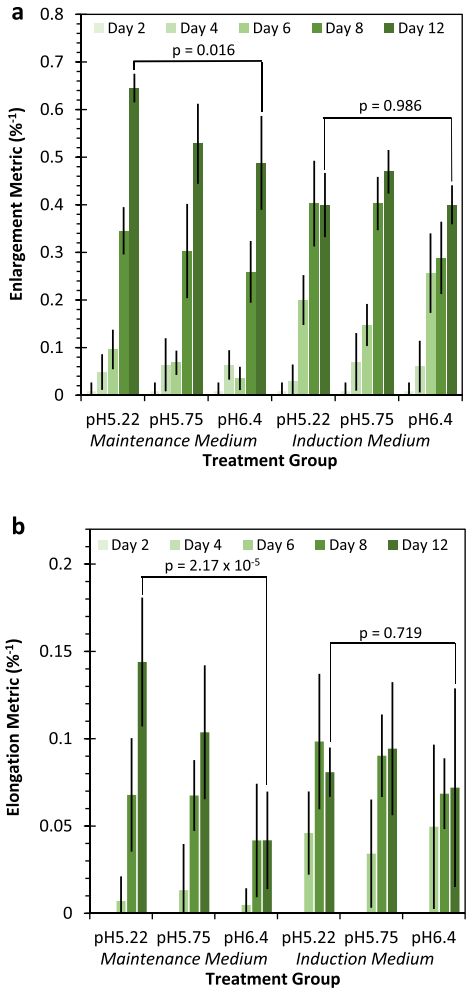

Fig. 5. Cell enlargement (a) and elongation (b) metrics versus time in pH-adjusted culture. Cell enlargement and elongation increase with culture time and are influenced by pH when hormone levels are low. Error bars represent ±1 SD. N = 4–5 images per datum point.

cell behavior in response to physical microenvironments (Wightman and Luo, 2016). Others have sought to employ gel-mediated culture of callus as a potential food production technique (Park et al., 2020). These published efforts, promising steps into a young research space, are furthered by the detailed investigations of cell development and tissue-like generation included in this work. The demonstration of tunable biomaterial production as allowed by the characterization of controllable culture inputs marks significant progress towards the production of much-desired (Wightman and Luo, 2016) *ex vivo* plant tissues. Together with the newly proposed applications for plant cell culture technologies, this work escalates explorations on plant cell bioprinting to high-impact, real-world solutions for biomass-driven industries.

4. Research implications

Current plant-based material production practices are limited by inefficiencies in cultivation, harvest, transport, and early processing. Without fundamental changes to existing process flows, increasing biomass demand (i.e., for consumer products, infrastructure, biofuels, etc.) exacerbates the insurmountable challenges of declining land availability, strained local resources, seasonal and

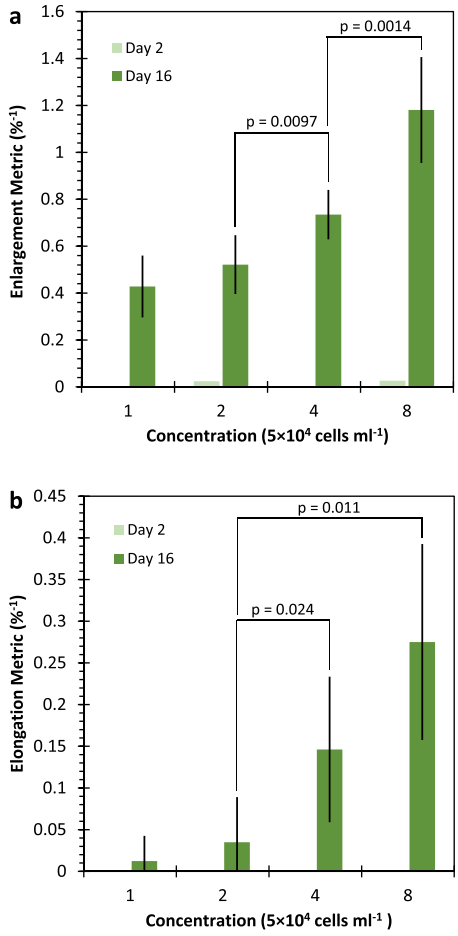

Fig. 6. Enlargement and elongation metrics versus initial cell concentration. Enlargement metric (a) and elongation metric (b) increase with time in culture and with increasing initial cell density. Error bars represent ±1 SD. N = 6 images per datum point.

climatic susceptibility, as well as proportionally low yields of useful plant materials.

This work proposes an alternative cultivation strategy to the large-scale monoculture operations that presently support the biomass industry. The model of selectively grown materials proposed in this work has the potential to address many of the failings of modern plant-material production efforts. Advantageously, the direct growth of materials:

(i) does not require access to arable land and allows for compact production—with potential for high yields per unit of land area,
(ii) enables local, pesticide-free production with precise control over water usage—reducing run-off and evaporation,
(iii) allows continuous cultivation irrespective of season or climate, and
(iv) facilitates growth of only desirable materials in convenient architectures thereby reducing waste, processing, and harvest cycle duration.

Land-free production strategies have the potential to share in the fulfillment of biomass needs, reducing overall competition for arable and forested acreage and enabling increased preservation of natural lands—an effort crucial to the maintenance of the planet's biodiversity and capacity for carbon sequestration.

This investigation indicates the feasibility of producing grown

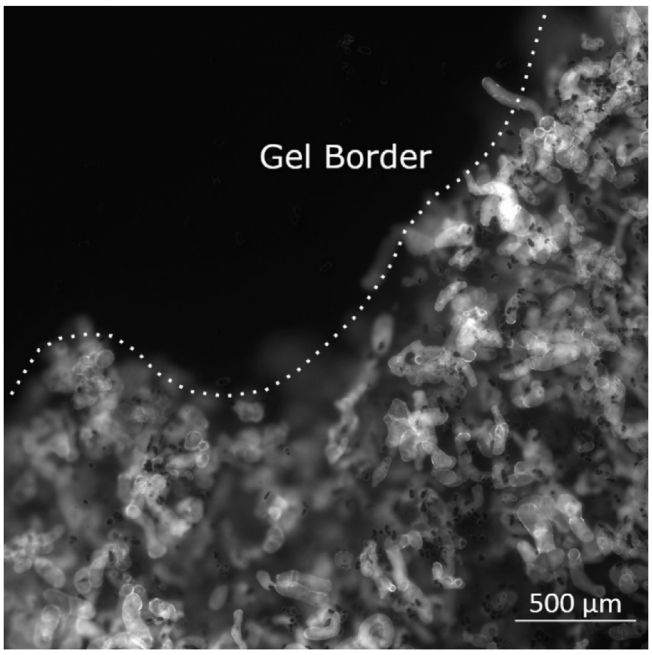

Fig. 7. Confluent, casted gel-based culture with elongated cells. The border of a confluent gel sample comprised of elongated cells and without lignified cells is indicated with a dotted line. Cell walls are stained with calcofluor white and visualized using a fluorescence microscope.

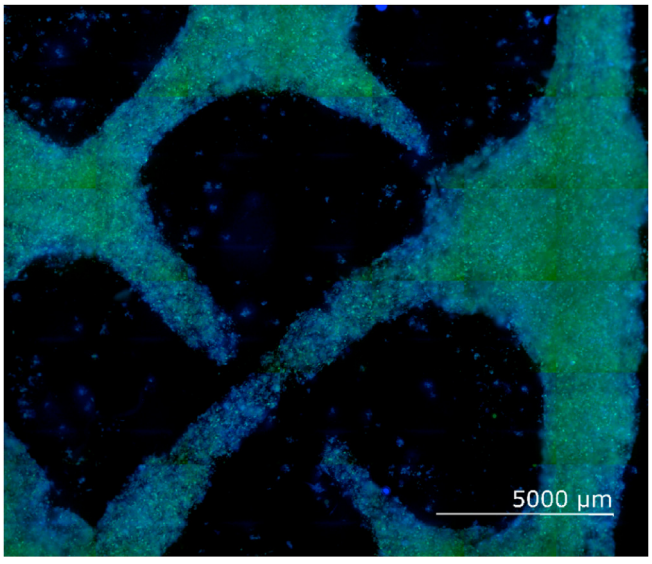

Fig. 8. Confluent, bioprinted culture with lignified cells. A stitched collection of images depicting a thin film of bioprinted *Z. elegans* cells in ZE-I gel medium grown to confluency and visualized non-destructively using natural autofluorescence at two sets of wavelengths (355/488 nm and 455/535 nm). The culture shown in the figure comprises a mixture of lignified and un-lignified cell types, as would be expected in the vascular tissue of the zinnia. Lignin fluoresces at both of these excitation/emission pairs (Donaldson and Williams, 2018), therefore lignified cells appear as teal foci.

materials and of modulating growth to achieve tunable material compositions. Several controlling parameters are identified, and cell development trends in response to parameter adjustments are characterized using new quantification methods. Thus, future cultures can now be thoughtfully prepared to yield desired material make-ups. With this established groundwork, the developed strategy may be translated to higher-value species at larger scales

with the hope of eventually achieving outputs competitive with standard production techniques.

Further investigations on parameters impacting cell growth and development (e.g., gas exchange, mechanical forces within the gel matrix, cell-to-cell biochemical signaling, interactions between gelling agent and media constituents, etc.) would enhance understanding of and capabilities in directing *ex planta* tissue production. These and other candidates for investigation present unique challenges in controllability and measurability and were therefore excluded from these preliminary investigations. Elucidating the role of gelling agent chemistry and the resulting mechanical environment on cell development, as well as the impact of culture geometries on survival are necessary next steps. Additional directions for future efforts include the extension of the proposed techniques to other, higher-value species, in addition to the assessment of cultured material properties as they relate to cellular-level compositions. When considering future implementations of lab-based biomaterial generation, a production system will require thoughtful design to ensure that the waste- and energy-saving potential of this method is not undermined by supporting infrastructure.

5. Conclusions

This work details a new method for the production of tissue-like biomaterials through the gel-mediated culture of plant cells in 3D scaffolds and provides a proof-of-concept demonstration using a *Z. elegans* model. These early experiments demonstrate the feasibility of growing confluent materials *in vitro* in controlled macroscopic architectures. Investigations also illustrate that making simple modifications to culture parameters allows for refined control over cell growth and development, thereby facilitating the creation of customized plant materials. New characterization methods and results provide a measurable understanding of cell development through the simultaneous evaluation of viability, differentiation, enlargement, and elongation as a result of changes to hormone levels, pH, and initial cell density.

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CRediT authorship contribution statement

Ashley L. Beckwith: Conceptualization, Methodology, Investigation, Project administration, Formal analysis, Visualization, Writing - original draft. **Jeffrey T. Borenstein:** Supervision, Resources, Funding acquisition, Writing - review & editing. **Luis F. Velásquez-García:** Supervision, Resources, Funding acquisition, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2020.125571.

References

Bajaj, Y.P.S. (Ed.), 1989. Biotechnology in Agriculture and Forestry 5: Trees II. Springer-Verlag, Berlin. https://doi.org/10.1007/978-3-642-61535-1.
Bergmann, L., 1960. Growth and division of single cells of higher plants in vitro. J. Gen. Physiol. 43, 841–851. https://doi.org/10.1085/jgp.43.4.841.
Bhatia, S., Sharma, K., Dahiya, R., Bera, T., 2015. Modern Applications of Plant Biotechnology in Pharmaceutical Sciences. Academic Press.
Chu, I.Y.E., 1992. Perspective of Micropropagation Industry, Transplant Production Systems. https://doi.org/10.1007/978-94-011-2785-1_8.
Devillard, C., Walter, C., 2014. Formation of plant tracheary elements in vitro – a review. New Zeal. J. For. Sci. https://doi.org/10.1186/s40490-014-0022-7.
Domingo, C., Roberts, K., Stacey, N.J., Connerton, I., Ruíz-Teran, F., McCann, M.C., 1998. A pectate lyase from Zinnia elegans is auxin inducible. Plant J. 13, 17–28. https://doi.org/10.1046/j.1365-313X.1998.00002.x.
Donaldson, L., Williams, N., 2018. Imaging and spectroscopy of natural fluorophores in pine needles. Plants 7, 1–16. https://doi.org/10.3390/plants7010010.
US Energy Information Monthly Energy Review, 2020. Renewable Energy Production and Consumption by Source.
Fehér, A., 2019. Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? Front. Plant Sci. 10, 1–11. https://doi.org/10.3389/fpls.2019.00536.
Fukuda, H., 1997. Tracheary element differentiation. Plant Cell 9, 1147–1156. https://doi.org/10.1105/tpc.9.7.1147.
Fukuda, H., Komamine, A., 1980. Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of Zinnia elegans. Plant Physiol. 65, 57–60. https://doi.org/10.1104/pp.65.1.57.
Ikeuchi, M., Sugimoto, K., Iwase, A., 2013. Plant callus: mechanisms of induction and repression. Plant Cell 25, 3159–3173. https://doi.org/10.1105/tpc.113.116053.
Isikawa, H., 1984. In vitro culture of forest tree calluses and organs. JARQ (Jpn. Agric. Res. Q.) 18, 131–141.
Ivakov, A., Persson, S., 2013. Plant cell shape: modulators and measurements. Front. Plant Sci. 4, 1–13. https://doi.org/10.3389/fpls.2013.00439.
Jovic, T.H., Kungwengwe, G., Mills, A.C., Whitaker, I.S., 2019. Plant-derived biomaterials: a review of 3D bioprinting and biomedical applications. Front. Mech. Eng. 5, 1–18. https://doi.org/10.3389/fmech.2019.00019.
Kapp, N., Barnes, W.J., Richard, T.L., Anderson, C.T., 2015. Imaging with the fluorogenic dye Basic Fuchsin reveals subcellular patterning and ecotype variation of lignification in Brachypodium distachyon. J. Exp. Bot. 66, 4295–4304. https://doi.org/10.1093/jxb/erv158.
Loyola-Vargas, V.M., Vazquez-Flota, F. (Eds.), 2006. Plant Cell Culture Protocols. Humana Press, Totawa.
Mehrotra, S., Kumar, S., Srivastava, V., Mishra, T., Mishra, B.N., 2020. 3D Bioprinting in plant science: an interdisciplinary approach. Trends Plant Sci. 25, 9–13. https://doi.org/10.1016/j.tplants.2019.10.014.
Milioni, D., Sado, P.E., Stacey, N.J., Roberts, K., McCann, M.C., 2002. Early gene expression associated with the commitment and differentiation of a plant tracheary element is revealed by cDNA-amplified fragment length polymorphism analysis. Plant Cell 14, 2813–2824. https://doi.org/10.1105/tpc.005231.
Mohammadinejad, R., Shavandi, A., Raie, D.S., Sangeetha, J., Soleimani, M., Shokrian Hajibehzad, S., Thangadurai, D., Hospet, R., Popoola, J.O., Arzani, A., Gómez-Lim, M.A., Iravani, S., Varma, R.S., 2019. Plant molecular farming: production of metallic nanoparticles and therapeutic proteins using green factories. Green Chem. 21, 1845–1865. https://doi.org/10.1039/c9gc00335e.
Mustafa, N.R., de Winter, W., Van Iren, F., Verpoorte, R., 2011. Initiation, growth and cryopreservation of plant cell suspension cultures. Nat. Protoc. 6, 715–742. https://doi.org/10.1038/nprot.2010.144.
Nunez, C., 2019. Climate 101: deforestation [WWW document]. Natl. Geogr. Mag. URL https://www.nationalgeographic.com/environment/global-warming/deforestation/ accessed 7.13.20.
Ochoa-Villarreal, M., Howat, S., Hong, S., Jang, M.O., Jin, Y.W., Lee, E.K., Loake, G.J., 2016. Plant cell culture strategies for the production of natural products. BMB Rep 49, 149–158. https://doi.org/10.5483/BMBRep.2016.49.3.264.
Park, S.M., Kim, H.W., Park, H.J., 2020. Callus-based 3D printing for food exemplified with carrot tissues and its potential for innovative food production. J. Food Eng. 271 https://doi.org/10.1016/j.jfoodeng.2019.109781.
Pesquet, E., Ranocha, P., Legay, S., Digonnet, C., Barbier, O., Pichon, M., Goffner, D., 2005. Novel markers of xylogenesis in Zinnia are differentially regulated by auxin and cytokinin. Plant Physiol. 139, 1821–1839. https://doi.org/10.1104/pp.105.064337.
Plomion, C., Leprovost, G., Stokes, A., 2001. Wood formation in trees. Plant Physiol. 127, 1513–1523. https://doi.org/10.1104/pp.010816.
Seidel, J., Ahlfeld, T., Adolph, M., Kümmritz, S., Steingroewer, J., Krujatz, F., Bley, T., Gelinsky, M., Lode, A., 2017. Green bioprinting: extrusion-based fabrication of plant cell-laden biopolymer hydrogel scaffolds. Biofabrication 9. https://doi.org/10.1088/1758-5090/aa8854.

Simmons, B.A., Loque, D., Blanch, H.W., 2008. Next-generation biomass feedstocks for biofuel production. Genome Biol. 9, 242. https://doi.org/10.1186/gb-2008-9-12-242.

Ślesak, I., Libik, M., Karpinska, B., Karpinski, S., Miszalski, Z., 2007. The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. Acta Biochim. Pol. 54, 39–50. https://doi.org/10.18388/abp.2007_3267.

Stasko, T.H., Conrado, R.J., Wankerl, A., Labatut, R., Tasseff, R., Mannion, J.T., Gao, H.O., Sanborn, S.D., Knott, G., 2011. Mapping woody-biomass supply costs using forest inventory and competing industry data. Biomass Bioenergy 35, 263–271. https://doi.org/10.1016/j.biombioe.2010.08.044.

Taiz, L., Zeiger, E., 2003. Plant Physiology. Benjamin-Cummings Publishing Co., Toledo.

Turner, S., Gallois, P., Brown, D., 2007. Tracheary element differentiation. Annu. Rev. Plant Biol. 58, 407–433. https://doi.org/10.1146/annurev.arplant.57.032905.105236.

van Dam, J.E.G., 2009. Environmental benefits of natural fibre production and use. Proc. Symp. Nat. Fibres 3–17.

Wightman, R., Luo, C.J., 2016. From mammalian tissue engineering to 3D plant cell culture. Biochemistry 38, 32–35. https://doi.org/10.1042/bio03804032.

Xu, J., Zhang, N., 2014. On the way to commercializing plant cell culture platform for biopharmaceuticals: present status and prospect. Pharm. Bioprocess. 2, 499–518. https://doi.org/10.4155/pbp.14.32.

Zaban, B., Liu, W., Jiang, X., Nick, P., 2014. Plant cells use auxin efflux to explore geometry. Sci. Rep. 4, 1–8. https://doi.org/10.1038/srep05852.