ORIGINAL ARTICLE

CO_2 supplementation eliminates sugar-rich media requirement for plant propagation using a simple inexpensive temporary immersion photobioreactor

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Abstract

In vitro plant propagation systems such as temporary immersion bioreactors (TIBs) are valuable tools that enable production of disease-free plants with improved traits. However, TIB systems can be expensive, difficult to implement, and prone to contamination due to sugar rich propagation media. Using rapidly growing chicory root cultures to expedite design-build-test cycles, we report here an improved, low-cost version of a previously reported Hydrostatically-driven TIB (Hy-TIB) that facilitates economical use of gas mixtures. Bioreactor improvements include decreased material costs, expanded modes of operation, and a horizontal orientation of a plastic film plant growth chambers that increase propagule light exposure. To take advantage of these improvements, we describe here experiments that evaluate the impacts of elevated CO_2 on propagation of cacao (*Theobroma cacao*) secondary embryos and nodal cultures of yam (*Dioscorea* spp.) during both phototrophic and photomixotrophic growth. Our experiments show that elevated CO_2 during plant propagation significantly improved both cacao and yam propagule development and eliminated the need for supplemental sugars in tissue culture growth media. Thus, our improved Hy-TIB shows potential as a simple, low-cost, and scalable propagation platform with cost-effective gas composition control and reduced risk of contamination overgrowth. We provide detailed instructions for assembly of this Hy-TIB design and discuss the implications of its adoption in food-insecure regions of the world.

Key message

Elevated CO_2 in a temporary immersion bioreactor facilitated removal of sugar from the propagation media, resulting in decreased contamination issues and improved plant root development in agriculturally relevant plant species.

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Abbreviations

DBT	Design-Build-Test
FAO	Food and Agriculture Organization of the United Nations
Hy-TIB	Hydrostatically-driven temporary immersion bioreactor
H	Horizontal orientation (describing Hy-TIB vessel)
LA	Labview-based automated operation
M	Manual operation
McA	Microcontroller-based automated operation
NO	Nitric oxide
PAR	Photosynthetically active radiation
PTC	Plant tissue culture
PP	Polypropylene
RI	Refractive index
SE	Somatic embryogenesis
SS	Stainless steel
TIB	Temporary immersion bioreactor
V	Vertical orientation (describing Hy-TIB vessel)
WUB	Wave and undertow bioreactor

Introduction

The propagation of plants with desirable traits has been a cornerstone of societal development throughout history. Its current technological application spans from luxury crops (e.g. ornamental flowers and plants, coffee, chocolate) to industrial agriculture. Commodity crops like seedless watermelon and grape illustrate our ability to take advantage of the non-obvious characteristics of plant propagation. Further, virus-indexed crops such as seed potatoes provide a disease-free supply chain that can otherwise result from traditional vegetative propagation. In less developed agricultural cropping systems, the serial propagation of plants via seeds, tubers, and cuttings is part of the fabric of everyday life. As human population grows and climate change worsens, plant propagation will invariably remain a critical component of modern agriculture. A report by the Food and Agriculture Organization of the United Nations (FAO) estimates that 10% of the world population has severe food insecurity exposure, affecting 750 million people globally. This trend will likely be exacerbated by climate change, limited arable land and water, and increasing global population, particularly for developing nations (FAO et al. 2020). Food production is not only being affected by biotic and abiotic stresses and climate change but contributes 19% of global greenhouse gas emissions. Even in Nigeria, despite having the continent's highest GDP, food security remains a struggle (Matemilola and Elegbede 2017). Nigeria, the most prolific exporter of yam, has suffered 60% deforestation since 1990 (Gates 2021), a trend that is unlikely to reverse course without technological intervention.

Indeed, modern and sustainable agriculture across Africa and elsewhere would benefit from more robust and affordable plant propagation systems. In many developing countries, cacao and yam both represent economic security crops. Cacao is a small woody tree and a luxury crop that constitutes a major component of the GDP for several tropical countries. Similarly, yam is a carbohydrate-rich staple crop supporting 60 million West African livelihoods at current production levels, with 70% of global supply produced in Nigeria (Nanbol and Namo 2019). Despite the enormous economic importance, yam has not shown progressive productivity gain in recent decades due to various production constraints. These constraints include the shortage of quality seed yam of popular landraces and improved varieties, as well as the high cost of planting materials. Robust agricultural production via a decentralized supply chain from farmers is imperative to mitigate adverse climate impacts and enable widespread prosperity by supporting both Nigeria's domestic demand and income security by increasing production for foreign supply.

To pragmatically address food security challenges, the plant propagation industry must always carefully consider advances in technology relative to the cost of implementation. To that end, superior plant varieties (e.g. high yield, stress-tolerant, disease-free) can be clonally propagated in aseptic tissue culture, which is particularly useful for plants with long maturation cycles. Temporary immersion bioreactor (TIB) systems facilitate aseptic plant propagation until plants are sufficiently mature for transition to soil. To date, plant propagation systems include single-use rocking reactors (e.g. Southern Sun Biosystems® Rocker (Kämäräinen-Karppinen et al. 2010), WAVE, Biowave, BIOSTAT® Cultibag), slug bubble (Valdiani et al. 2019), Wave and Undertow (WUB), Appliflex, CELL-tainer (Eibl et al. 2009), rotating wall vessels (Valdiani et al. 2019) or rotating drum reactors (e.g. roller bottles (Shetty 2005)), mist bioreactors or nutrient sprinkle reactors (Steingroewer et al. 2013), and Temporary Immersion Bioreactors (TIBs). Existing TIBs include Temporary Root zone Immersion (TRI) bioreactors (Neumann et al. 2020a), Periodic Immersion Bioreactor (PIB), Plantform™ (Ruta et al. 2020), and the twin flask bioreactor system commercialized as the RITA®/SETIS™ (Georgiev et al. 2013). However, due to design constraints that limit economic feasibility (Eibl et al. 2018) these solutions have generally been relegated to research environments (Balogun et al. 2017) and commercial production of high-margin plant

medicinal products and luxury crops (Ducos et al. 2010). Thus, to achieve cost viability for low-margin fiber and food staples, further economical advancements are required.

Our laboratory previously presented a novel hydrostatically-driven TIB (Hy-TIB) system that decouples gas and liquid flows thereby facilitating economical use of gas phase mixtures that would otherwise be cost-prohibitive compared to air (Florez et al. 2016). This work demonstrated enhanced growth in tobacco (*Nicotiana benthamiana*) hairy root tissue cultures by elevating oxygen levels to 40%, and triploid seedless watermelon (*Citrullus lanatus*) with elevated CO_2, likely due to changes in oxygen partial pressure that drive mass transfer and suppression of ethylene signaling, respectively. Our previous Hy-TIB design also implemented typical engineering automation software to control the height of media reservoirs and fault monitoring. While effective, this design was unnecessarily complicated for the operational requirements of a TIB bioreactor.

After extensive design-build-test (DBT) cycles, we present here a newly designed low-cost Hy-TIB that functions either manually or automatically via low-cost, open-source platforms (e.g. Arduino). As shown in Florez et al 2016, we demonstrate that our improved Hy-TIB design can be implemented using 40% elevated oxygen to improve chicory (*Cichorium* sp.) heterotrophic root growth. We further show that CO_2 supplementation significantly enhances root development in cacao (*Theobroma cacao)* and survival rates in yam (*Dioscorea* spp.). Finally, we also demonstrate that elevated CO_2 during propagule development eliminates the need for sugar in the plant growth media, resulting in both carbon capture and decreased contamination risk. We provide detailed instructions for assembly of the Hy-TIB designs to facilitate its adoption as an FTO (freedom to operate) and open-source technology.

Materials and methods

Plant tissue culture

Cichorium intybus (chicory) *Agrobacterium*-transformed root cultures were kindly provided from Premier Tech (https://www.premiertech.com) and grown on solid B5 media without plant hormones. Serial cultures were maintained on liquid B5 medium (Gamborg et al. 1968) with 25 g L^{-1} sucrose and subcultured bi-weekly. Subculture involved transferring ~ 0.5 g fresh weight (FW) of tissue into 50 mL of fresh B5 media in wide-mouth 125 mL Erlenmeyer flask on a 120 RPM orbital shaker with a 2.5 cm stroke. Root culture maintenance and bioreactor experiments were conducted at 25 °C in the dark. *Theobroma cacao* secondary somatic embryo tissue cultures of PSU Scavina 6–1 were established from greenhouse grown floral buds as previously described (Li et al. 1998; Maximova et al. 2002). Tissue was kept at 25 °C in the dark on solid media per protocol utilizing secondary callus growth medium (SCG / E5B) and embryo development (ED) media (Shires et al. 2017) for ~ 6–8 weeks. *Dioscorea cayenensis* (yam) plantlets were provided by Morufat Balogun, University of Ibadan, Nigeria; *D. rotundata* (IITA accession no. Tdr2436) were provided by Leena Tripathi, IITA-Nairobi, Kenya. Both species were maintained based on the protocol optimized by Manoharan et al. (2016). Briefly, nodal cuttings of approximately 25 mm in length were subcultured on solid Yam Callus Proliferation Media (YCPM), a MS-based media supplemented with picloram, casein hydrolysate, and proline (see Online Resource S1A-1) for three weeks. Thereafter, they were subcultured every 2–3 weeks onto fresh solid Yam Basic Media (YBM) (see Online Resource S1A-2) for 2 months, in air, without light, and at 25 °C. Next, explants were subcultured on the same YBM media into larger Magenta™ GA-7 culture vessels and placed in a growth chamber with 16:8 h diurnal cycle, measured as PAR of 77 ± 2 µE m^{-2} s^{-1}) for approximately 6–8 weeks or until plants reached ~ 95 mm and had substantial node growth. After several months of meristem proliferation, the process was re-initiated to ensure propagules maintained embryogenicity.

Horizontal (H) Hy-TIB vessel component fabrication

H Hy-TIB version 3 (v.3)

The cost analysis spreadsheet (Online Resource 2) outlines the source and costs of materials for both previously reported vertical orientation (V) of Hy-TIB and horizontal orientation (H) Hy-TIBs of the current work. Intermediate materials, including high durometer PP tubing, larger inner and outer diameter (ID/OD) SS tubing in fluid distributor, that were used only in versions 1 (v.1) and 2 (v.2) have been excluded. Several of the components of the reactor were custom machined at Penn State's Learning Factory including (1) stainless steel (SS) insert that had to be cut to size, sanded, perforated, and bent, (2) SS fluid distributor that had to be cut to size, perforated, and epoxied to insert, and (3) SS gas connectors that had to be cut to size and lathed to specification. CAD component drawings, PDFs, and machining specifications are included in Online Resource S3,1B, and S1C, respectively.

H Hy-TIB infrastructure, assembly and inoculation

Pre-sterilization assembly

Online Resource S1D and S4 provide instructions on the assembly of H Hy-TIB vessel via step-by-step pictorial instructions and video. In brief, the insert is placed inside

the polypropylene (PP) plastic bag (VWR 95-42-564) with protruding fluid distributor-end inserted first (towards closed end). Just below (~½") the seam and in the center of the bag, the distributor is pushed through the PP bag, and the bag is punctured to ensure flow. Similarly, gas tubing is attached to the bag with the press-fit swage as described in the Discussion. A growth matrix (e.g. filter paper, cheesecloth, fabric) is added onto the insert; this prevents propagules from flowing through perforations during operation. For in-line sampling (i.e. refractive index), a Luer tee connector (Cole Parmer, EW-45500-56) and syringe port (MediDose, IV2004) were installed into tubing connected between the fluid distributor and media reservoir.

Post-sterilization inoculation

H Hy-TIB reactors were pre-assembled without tissue and autoclaved. Explants (e.g. root fragments, cotyledons, nodal cuttings) were aseptically distributed into the bag, sealed in a laminar flow hood using a commercial heat sealer and inflated via the gas inlet filter with a Drummond pipettor to ensure asepsis (or 'sterility'). Media was introduced post-autoclaving by replacing the empty bottle with a sterile media-filled counterpart, then clamping all tubing lines to avoid liquid getting into the air lines when the media bottle was inverted into operational position (noting that air lines must be above media level).

Bioreactor operation and monitoring

Gas composition and flow monitoring

The gas delivery infrastructure including a low-cost humidification setup and manifold was described previously (Florez et al. 2016). Gas flow rates were targeted at 10 mL per minute per vessel. Oxygen and Carbon Dioxide Analyzer (Illinois Instruments, Model 3750) was used to confirm composition of supplemented gases. Because we required extremely low overall gas flow rates, particularly for supplementation gases (O_2 and CO_2), even the lowest-flow rotameters (Brooks Sho-Rate model 1355E, tube: R-2-15-AAA, spherical glass floats, nominal 47.1 mL min^{-1}) were insufficient for maintaining flow. Maintaining low gas flows that more closely match consumption through the TIB reactor is desirable but challenging—particularly for inexpensive multiplexed gas delivery. Inlet gas flow resistance was maintained based on back-pressure filters that were preceded by a metal gas manifold with a small resistance heater (Florez et al., 2016). To prevent condensate from causing variable gas flow resistance in exhaust filters, we describe alternative solutions in Online Resource S1E. This issue has important

implications for cost and performance and is particularly challenging for evaporation under enhanced light flux.

Manual infrastructure and operation

For manual operation, the pulley system was replaced by a two-tiered stand, each with two 'arms', to provide high and low positions for the shelf of inverted media reservoirs. Other configurations could employ a manual hoist like the automated (LA and McA) systems for scaled-up reservoirs. Online Resource S1F contains a diagram of Hy-TIB infrastructure setup, noting the height recommendations for raising and lowering of reservoir relative to Hy-TIB vessel to ensure immersion without propagule displacement.

Microcontroller-based infrastructure and operation

Raising and lowering of the media reservoirs was accomplished using the same infrastructure described by Florez et al. (2016) (adapted into CAD drawings; see (see Online Resources S1B, S3), with the exception that operation of the pulley system was accomplished via a microcontroller rather than computer-based LabView software. In brief, the inverted media reservoir shelf is raised and lowered by a pulley system. In this case, the process was controlled by an Arduino Uno (www.arduino.cc) with the program provided in Online Resource S5. This program sent the stepper motor controller a (0/1) logic output to specify stepper motor direction and a pulsing signal for rotational steps that generated a linear translation rate of roughly 0.5 cm per second. The stepper motor is only powered on during the cycle, with the down position as default to minimize energy use.

Hy-TIB experimental conditions and data analysis

Refractive index/sugar consumption

Refractive index (RI) was measured with a high-precision refractometer (Leica, Model AR600) to determine sugar consumption by the explants. For chicory roots, a syringe port (Cole Parmer, EW-45503-04) was installed in the flow path of fluid distributor such that 0.3 mL media samples could be taken via sterile syringe every 1 to 3 days.

Root tensile strength

The tensile strength of propagule roots impacts ease of manipulation and explant survival. To evaluate differences in root tensile strength, a simple tensometer was fabricated using two Hoffman tubing clamps (Online Resource S1I). Both clamps were covered with a soft silicone tubing to provide for a gentle clamping of both ends of a segment of root with the bottom clamp fixed to a spring nut and weight. The

force (weight) necessary to break the root was assessed by gently pulling upward on the upper clamp and video-taping the balance weight to observe the maximum weight loss on the balance at the time of root rupture. Only roots which ruptured away from the clamp position were considered a valid measurement. All plants with roots removed for tensile strength measurement thrived after potting and did not affect plant survival measurements.

Chicory root elevated oxygen study

To assure actively growing roots, explant tissue was obtained by combining several 1-week-old 125 mL flask subcultures, aseptically blotted and weighed, resulting in ~2 g fresh weight (FW) tissue. The inoculum was incubated for three-days in several flasks containing 50 mL of B5 media to overcome stress from recently cut tissue, reduce the lag growth phase of roots, and identify any contamination. After incubation, the inoculum was distributed onto filter paper (Sigma-Aldrich, WHA1001150) in the assembled autoclave sterilized H Hy-TIB reactors with additional 175 mL of fresh B5 media (total 225 mL liquid inoculum). Reactors were operated with either air or elevated (40%) oxygen in air. Both the incubation periods and the reactor run were carried out at 25 °C under dark conditions. The reactor was manually cycled every 4 h (6 times a day), submerging plant tissue for ~10 min each cycle.

Per design-build-test (DBT) cycle (see Results and Discussion), Hy-TIBs were initially operated manually but once shown to be comparable with LA-V and Mc-A, different modes were used interchangeably as dictated by experimentation. The heterotrophic root reactors were harvested after approximately 2 weeks post-inoculation, or once sugar consumption plateaued for either treatment. Prior to harvesting, a small sample of media was used to test for contamination based on growth on highly permissive R2A media (van der Linde 1999). Roots were carefully removed from the reactor and blotted on paper towels and weighed. To allow mass balance closure due to evaporation, final media volume was meticulously assessed by weighing reactors, roots, and paper towels used to blot. For dry weight measurements, a 2 g fresh weight (FW) sample of the root tissue was placed on pre-tared aluminum foil packets and dried at 70 °C until consistent dry weight.

Cacao elevated CO_2 study

Growth of cacao in M-H Hy-TIBs v.1 were operated with either air or elevated 2% CO_2 in air, each with two replicates. Experimentation was conducted with 12 secondary somatic embryos per reactor (< 1 cm length, without root or leaf development). The total fresh weight of inoculum per TIB was 1.53 ± 0.13 g. The growth matrix used was polyester fabric (JoAnn fabrics, Thermolam Pellon TP980) that required manual submersion in media during inoculation due to hydrophobic properties. All reactors were maintained on the same media schedule over a period of 4.5 weeks (31 days). Reactors were operated with 350 mL of media: 250 mL of ED media with an additional 100 mL containing 30 g L^{-1} sucrose and 1 g L^{-1} glucose.

The photosynthetically active radiation (PAR) light intensity was measured to be 77 ± 2 μE m^{-2} s^{-1} using a light sensor (LICOR, LI-1400, Quantum PAR sensor) where uniformity was achieved by surrounding the TIB growth area with reflective mylar film. The lighting was maintained 24/7 for the first 2 weeks post-inoculation followed by a 16:8 light:dark photoperiod thereafter. The reactors were cycled twice daily (every 12 h) to submerge the tissue for ten minutes each cycle. Reactors were harvested after 4.5 weeks (31 days) in a fashion similar to chicory roots to obtain fresh and dry weight data. In addition, propagules were photographed, and image analysis was conducted with open-source Semi-Automated Root Image Analysis (saRIA) program to determine root volume (Narisetti et al. 2019).

Yam trophism study

A study of yam in McA-H Hy-TIB (v.3) involved growth conditions as described above. The explants were established through serial nodal cutting and propagated within Magenta™ GA-7 vessels. From these plantlets, approximately 1-inch nodes were excised, all appearing of comparable health (i.e. no visible overcrowding, excessively dry tissue, or necrosis) to provide 25 nodal explants for each of eight bioreactor vessels. Twenty-five nodal explants were also inoculated into the GA-7 vessel for the agar control; notably, in a prior study of *D. cayenensis* (see Online Resource S1H), growth based on equal area distribution (i.e. 25 nodes among 4 GA-7) showed comparable growth to air/sugar Hy-TIB. The nodal explants were initially transferred to solid YBM plates and maintained at 25 °C in 16:8 light:dark cycles for three days to check for contamination prior bioreactor inoculations.

All McA-H Hy-TIB (v.3) vessels were assembled and sterilized with cheesecloth (grade 10) inoculated with nodal cuttings and 250 mL liquid YBM (Online Resource 1A-2) with and without 30 g L^{-1} sucrose based on experimental design of two reactor replicates for each treatment. Treatments consisted of air without sucrose, air with sucrose, 5% CO_2 supplementation without sucrose and 5% CO_2 supplementation with sucrose. The agar control containing 50 mL solid YBM containing sugar was placed on the same shelf as the reactors to ensure comparable experimental conditions. Lighting setup and intensity was the same as cacao study above. Reactors were harvested 6 weeks after inoculation. Fresh weights were obtained

by removing each plantlet from its reactor, blotting on a paper towel, and weighing. In addition, propagules were photographed, and image analysis was conducted with saRIA for determination of the fibrous root surface area. Dry weights were not taken since these explants continued in a potted survival study.

Yam survival studies

Plantlets from harvested reactors were transferred to soil in 4 × 9 well plastic potting trays on 1020 greenhouse flat trays with plastic domes for a high-humidity environment under LED lighting. Plants were treated as needed with Gnatrol (Valent, WDG, *Bacillus thuringiensis*, subsp. israelensis, strain AM 65-52 fermentation solids) to suppress fungus gnats. These plants were monitored and watered when soil appeared dry for 26 days. Photos were taken of these explants at 2-day intervals to monitor their progress. Survival fraction was calculated relative to the 25 nodes initially inoculated.

Results

Enhanced Hy-TIB design and operational modes for reduced cost

Figure 1 provides a diagram of the improved horizontal M-H Hy-TIB as well as a photo of M-H Hy-TIB in active operation (Fig. 1A) highlighting the design changes between previously reported vertical LA-V Hy-TIB (Fig. 1B). Areas of modification of vessel orientation and mode of operation were motivated by the goal to (1) reduce component and operation costs, (2) increase light flux to the tissue to improve phototrophic growth, and (3) improve reliability by eliminating risk of breaking the media reservoir siphon. The re-configuration from vertical (V) Hy-TIB to horizontal (H) eliminated both the headplate and use of glass beads (Fig. 1B). The horizontal orientation nearly doubled the lighted growth area for each reactor as outlined in Table 1 with an associated 90% increase in photon flux for the same 8″ × 10″ polypropylene (PP) growth bag. Despite increased growth area, media requirements were comparable (see Table 1). The change to a horizontal delivery allowed for reliable liquid fill and drain cycles by avoiding the siphon
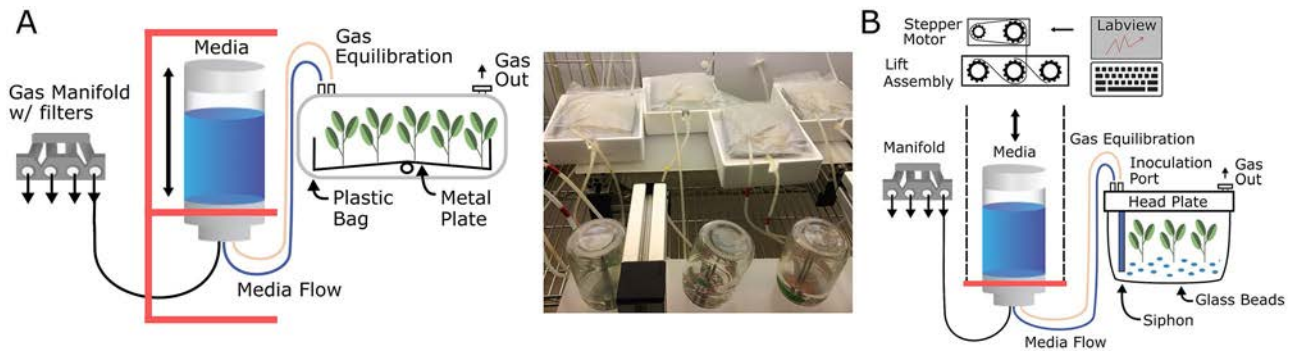

Fig. 1 Cartoon representation juxtaposing the designs of the (manual) M-H and (automated) LA-V Hy-TIB designs. **A** The improved of H Hy-TIB design incorporates a pre-sterilized horizontally oriented plastic bag to house plant tissues to increase propagule light exposure to tissues grown on a stainless-steel plate (**A**, right). **B** The previously reported LA-V Hy-TIB incorporates unnecessarily complicated and costly reactor automation. Further, the LA-V Hy-TIB requires the use of a headplate that reduces propagule light exposure. Both designs use a shelf (orange) to hold the media reservoir. In the manual M-H Hy-TIB operation, the media reservoir is physically moved between low and high shelf locations to cycle media in and out of the plastic bag. The newly developed automated McA-H Hy-TIB uses open-source Arduino to drive the previously described stepper motor and pully lift assembly

Table 1 Hy-TIB cost and design specifications

Hy-TIB bioreactor	LA-V Hy-TIB	M-H Hy-TIB v.3	McA-H Hy-TIB v.3
Cost per reactor	$117.98	$27.80	$53.03
Min. media volume	220 mL	250 mL	
Lighted growth area	122 cm^2	232 cm^2	

Cost estimates per reactor assumes 8 reactors operating simultaneously. The minimum required media and lighted growth area are included to highlight McA- and M-H Hy-TIB design's cost effectiveness relative to previously reported LA-V Hy-TIBs

which would periodically break as experienced using the vertical configuration (Fig. 1B). The cycle time of 10 min remained the same and was empirically arrived at based on the flow resistance of the liquid delivery tubing and hydrostatic head of 7.6 cm. This cycle period is small relative to the 4–12 h drained cycles.

Inexpensive not disposable as a design goal

In developing plant propagation systems, the criticality of cost conservatism cannot be understated due to the low value of plant propagules ranging between $0.20-$1 (Kozai and Xiao 2006; Kozai 2008). A key aspect of Hy-TIB design is enabling economical use of gas composition control by avoiding pneumatically-driven media flows. Previously achieved cost reduction for mixed-gas use (Florez et al. 2016) is retained in the present designs. The focus for the improved design is overall implementation reliability and bioreactor component cost reduction. Table 1 outlines the overall cost per bioreactor for its components control modules for the M-H and McA-H Hy-TIBs v.3 relative to LA-V Hy-TIB (complete cost breakdowns for each scenario are provided in Online Resource 2). Capital costs (e.g. heat sealer to seal PP bag, shelving, basic laboratory infrastructure, gas delivery) that would be equivalent for all three reactor types were excluded. The use of epoxy in H Hy-TIB v.3 was considered negligible. All calculations assume eight bioreactors would run in tandem to maximize use of more expensive components (e.g. humidification train, 4-way gas manifold). The previously described LA-V Hy-TIB's requisite computer and LabView software were omitted due to cost variability but pragmatically would be fiscally significant whereas the M-H Hy-TIB's shelving would be comparatively minimal. Therefore, these projected costs are conservative estimates. Relative to the previously reported La-V Hy-TIB, these design modifications resulted in a 55% and 76% cost reduction for McA-H v.3 and M-H v.3, respectively. Notably, the use of a plastic bag to house propagules suggests the H Hy-TIB culture vessel size can be increased with minimal cost increase.

Other cost considerations are consumables that are equivalent in all scenarios. These consisted of only two components, including a PP bag ($0.73) and filter paper ($0.22), where a variety of less expensive tissue matrix can be used. For the optional addition of a syringe port to the flow-path to provide greater environmental control and/or in-line monitoring would cost $1.92. While media requirements are similar for all TIBs, the cost per plant is proportional to lighted surface area within the bioreactor available for plant growth. The horizontal H Hy-TIB utilized 1.0 mL cm^{-2} as compared with 1.8 mL per cm^2 for the previous vertical V Hy-TIB, achieving a roughly 40% cost reduction for media. Based on significant costs of hormones, antibiotics, media additives, and nutrients—even at low concentrations—this would have significant operating cost implications. However, it should be noted that plants are efficient at assimilating nutrients from a dilute environment, and much lower nutrient levels that more closely match stoichiometric utilization are likely feasible.

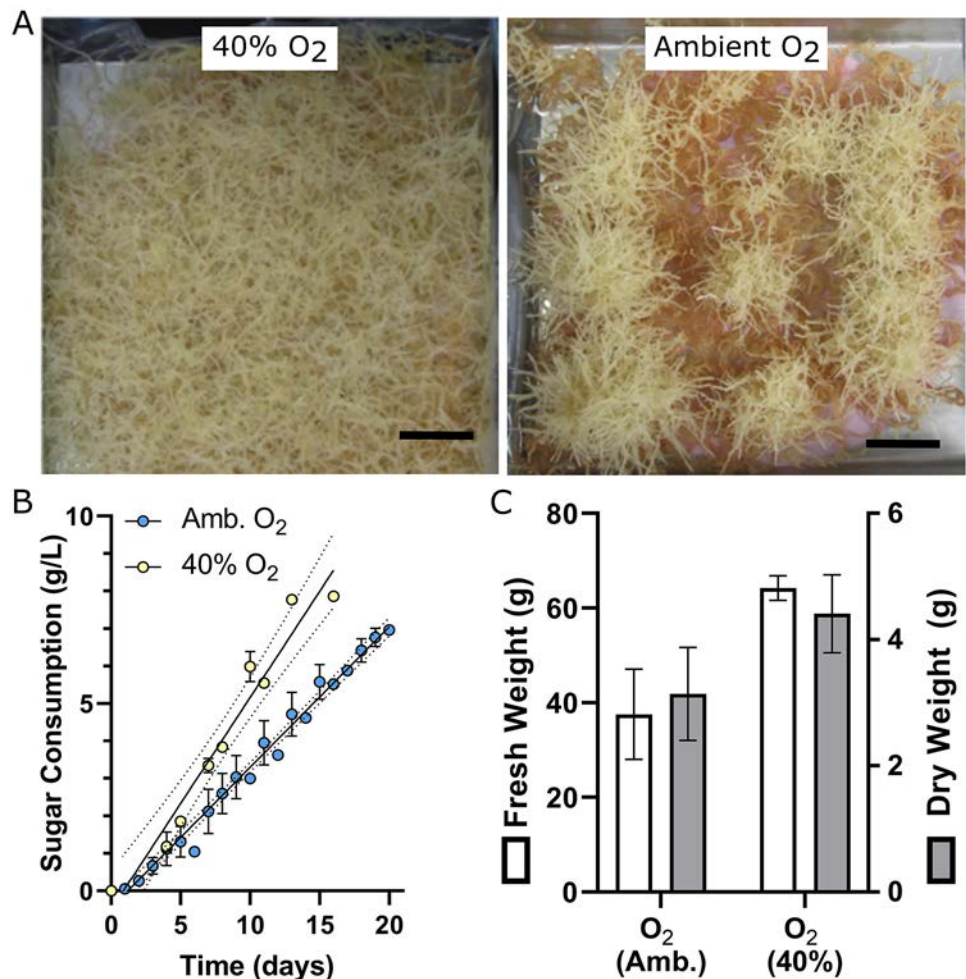## Chicory root culture with elevated oxygen facilitates rapid horizontal Hy-TIB prototyping

To expedite Hy-TIB DBT cycles while evaluating alternative reactor configurations, we chose to use rapidly growing chicory root cultures (*C. intybus*). This allowed us to conduct iterative experiments on the order of weeks, rather than months while taking advantage of the mechanistic similarity gas transport limitations between oxygen-dependent heterotrophic growth and CO_2-dependent phototrophic growth. Throughout DBT cycles of M- and McA-H HyTIB reactor versions 1-3 (v.1-v.3, Table 2), we repeatedly tested the impacts of ambient and elevated oxygen levels on chicory root growth rate. Using the improved H-M Hy-TIB, elevated oxygen resulted in more rapid and uniform root growth that eventually formed a solid mat (Fig. 2A). Further, elevated oxygen also alleviated visible hypoxia and reduced the production of a secreted pink metabolite (Fig. 2A, right).

Sugar consumption was measured at regular intervals by refractive index as a proxy for biomass accumulation (Ramakrishnan et al. 1999). Bioreactors were harvested at 20 days

Table 2 Chronological progression of Hy-TIB designs and modifications

Hy-TIB version	Description
LA-V Hy-TIB (Florez et al. 2016)	Hy-TIB with Labview-based Automated Operation, Vertical vessel with headplate, glass beads
M-H Hy-TIB v.1	1st version of H Hy-TIB (headplate, glass beads removed) with perforated SS plate insert with fabric, perforated PP media distributor, press-fit swage
M-H Hy-TIB v.2	2nd version of H Hy-TIB that modified H1 Hy-TIB media inlet/outlet from PP to SS material, added 'capillary condenser' outlet string, various fabric substitutions
M-H Hy-TIB v.3	3rd version of H Hy-TIB that modified H2 Hy-TIB media inlet/outlet to have smaller diameter SS tubing
McA-H Hy-TIB v.3	Hy-TIB with Microcontroller-based Automated Operation, Horizontal vessel with perforated SS plate insert

Fig. 2 Chicory (*Cichorium intybus*) root tissue culture growth used to facilitate expedited Design-Build-Test (DBT) cycles for gas-phase composition control using the manual M-H Hy-TIB (v.1-3). **A** Elevated oxygen supplemented to 40% resulted in substantially healthier looking roots (left) relative to ambient air (right). **B** As a proxy for root growth, sugar consumption was monitored at regular intervals by refractive index. Chicory roots consumed sugar significantly faster when grown in elevated oxygen (p < 0.001, ANOVA with no left-out terms). **C** At time of harvest (21 days post inoculation into the reactor), chicory roots grown in elevated oxygen reached higher fresh- and dry-weights (represented on the left and right axes, respectively) relative to plants grown in ambient air. Scale bars in (**A**) represent 25 mm

or earlier if sugar consumption plateaued due to depletion. We observed that elevated oxygen resulted in a significant, nearly 50% increase in the rate of sugar consumption, rising from 0.38 g sugar L^{-1} day^{-1} in ambient air to 0.57 g sugar L^{-1} day^{-1} (Fig. 1B)(p-value < 0.001, ANOVA with no significant left-out terms). This enhanced growth is facilitated by increased oxygen availability in the root meristem (Asplund and Curtis 2001), which supports previous observations of linear oxygen-transport limited root growth made using *Agrobacterium rhizogenes*-transformed root cultures (Larsen and Curtis 2012; Florez et al. 2015). After 21 days of growth in ambient air, chicory roots reached a final fresh- and dry-weight of 37.5 g and 3.15 g, respectively (bioreactors n = 2). In contrast, plants grown under elevated oxygen reached a final fresh- and dry-weight of 64.2 and 4.4, respectively (bioreactors n = 3). (Fig. 2B, C).

Cacao somatic embryo development is enhanced by elevated CO_2 in manual horizontal Hy-TIB

To further demonstrate the utility of our improved Hy-TIB designs that take advantage of decoupled gas and media

flows, we executed experiments to evaluate the impacts of elevated CO_2 on cacao (*T. cacao*) propagule development from secondary somatic embryos. Compared to rapidly growing tissues, the slower growth of cacao and a 16:8 photoperiod lends itself to far fewer temporary immersions (twice daily), and thus more practical manual operation. Previous LA-V Hy-TIBs successfully grew cacao cotyledonary embryos for 8 weeks, producing considerable leaf development under photoheterotrophic growth conditions (unpublished data, see Online Resource S1G-1). This suggested Hy-TIBs have the potential to generate potable plants in less than 2 months. To evaluate the benefit of supplemented CO_2 on cacao propagule development, a set of manually cycled M-H Hy-TIBs (v.1) were inoculated with cacao secondary somatic embryos with either ambient air or air supplemented with CO_2 followed by quantification of root growth by image analysis (Fig. 3A) (Narisetti et al. 2019). After 31 days of growth, elevated CO_2 resulted in a significant increase in root volume relative to ambient air (Fig. 3B) (Mann–Whitney U-test, p = 0.01, U = 92). Figure 3A highlights this observation with photos of median roots ranked by volume for each treatment; these were analyzed as 17.7 mm^3 for
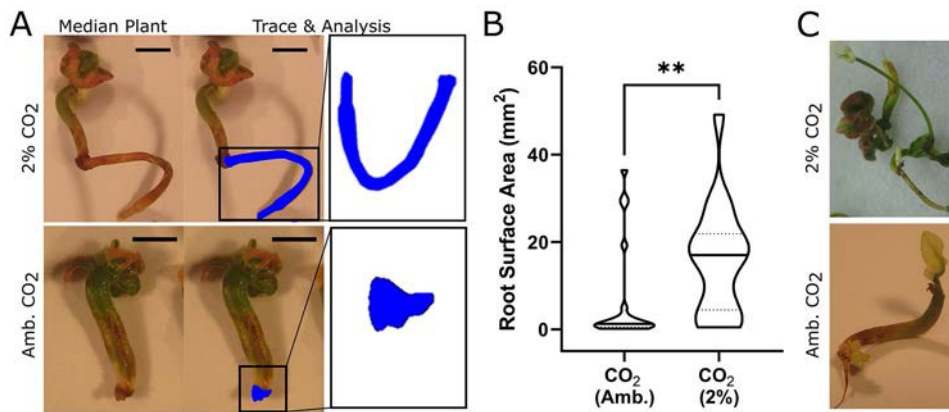
Fig. 3 Evaluation of cacao (*Theobroma cacao*) propagule root development under elevated and ambient CO_2 using M-H Hy-TIB (v.1). **A** After 31 days of growth, root development was quantified by tracing and image analysis using saRIA for both elevated CO_2 (top) and ambient CO_2 (bottom). The example plants shown represent the median of the respective treatments. **B** Elevated CO_2 resulted in a significant increase in cacao root volume (p=0.01, Mann–Whitney U-test). **C** While rare, additional observations included rapid development of floral and leaf tissues in M-H Hy-TIBs using elevated and ambient CO_2, respectively. Scale bars in (**A**) represent 5 mm

CO_2 and 1.2 mm^3 for air. Significant abscission of roots and cotyledons were noted in both treatments, with about half as much abscised tissue observed with CO_2 supplementation. This is consistent with carbon dioxide's role as a plant growth and stress regulator (Huang and Xu 2015). While rare, we also observed accelerated stages of plant development during growth in Hy-TIB reactors, including early leaf emergence and flower formation (Fig. 3C). While standard flowering generally occurs on mature trees over a period of 3–4 weeks (Swanson 2004), this floral development occurred in less than 31 days from the exposure of secondary embryos to light, and is to the authors' knowledge this is the most rapid observation of floral development reported, and notably took place in the absence of exogenous hormone supplementation. Epinastic growth was also noted throughout both treatments but not quantified (see Online Resource S1G-2).

Elevated CO_2 enhances yam propagule development and survival using improved Hy-TIBs

To further evaluate the impacts of controlled gas composition using improved Hy-TIB reactors, experiments were designed to evaluate the impact of elevated CO_2 on yam (*Dioscorea rotunda*) propagule development. Specifically, yam propagules were grown for 42 days in McA-H Hy-TIBs supplied with (1) air, (2) air + sugar, (3) CO_2, and (4) CO_2 + sugar. Providing a supplemental carbon source resulted in substantially healthier plants (Fig. 4A). Growth under elevated CO_2 resulted in a significant increase in propagule fresh weight and root surface area both with and without supplemental sugar (Fig. 4B, C). The greatest accumulation of biomass was observed for the combination

of sugar and elevated CO_2, indicative of photomixotrophic growth. Most importantly, growth with supplemental CO_2 in the absence of sugar was greater than typical heterotrophic growth using sugar and ambient CO_2. This is notable because the elimination of sugar from reactor media drastically reduces the risk for contamination overgrowth. Structural integrity of roots is expected to influence plant survival while transferring explants from reactors to soil). During the course of these experiments, the authors observed a twofold increase in the tensile strength of yam propagule roots grown in Hy-TIB reactors using elevated CO_2 and phototrophic conditions relative to those grown in ambient air (Online Resource S1I). To evaluate explant survival, Hy-TIB-grown yam propagules were transferred to soil and their survival was monitored 25 days post-transplantation. Plants grown in Hy-TIBs with elevated CO_2 and no additional sugar survived as well or better than other conditions (Fig. 4D). These data demonstrate that Hy-TIB reactors can be used to propagate plants using elevated CO_2 without sugar supplementation that survive at high frequency after transfer to soil. The enhanced root development and greater mechanical strength of roots grown in elevated CO_2 are consistent with greater investment of carbon resources to roots when grown in an elevated CO_2.

Discussion

In vitro propagation bioreactors allow control of the environment, nutrient, and hormone conditions of plant tissues. In the development of this type of process system, it is important to recognize that 'design' and 'operation' provide distinctly different opportunities for improvement and cost
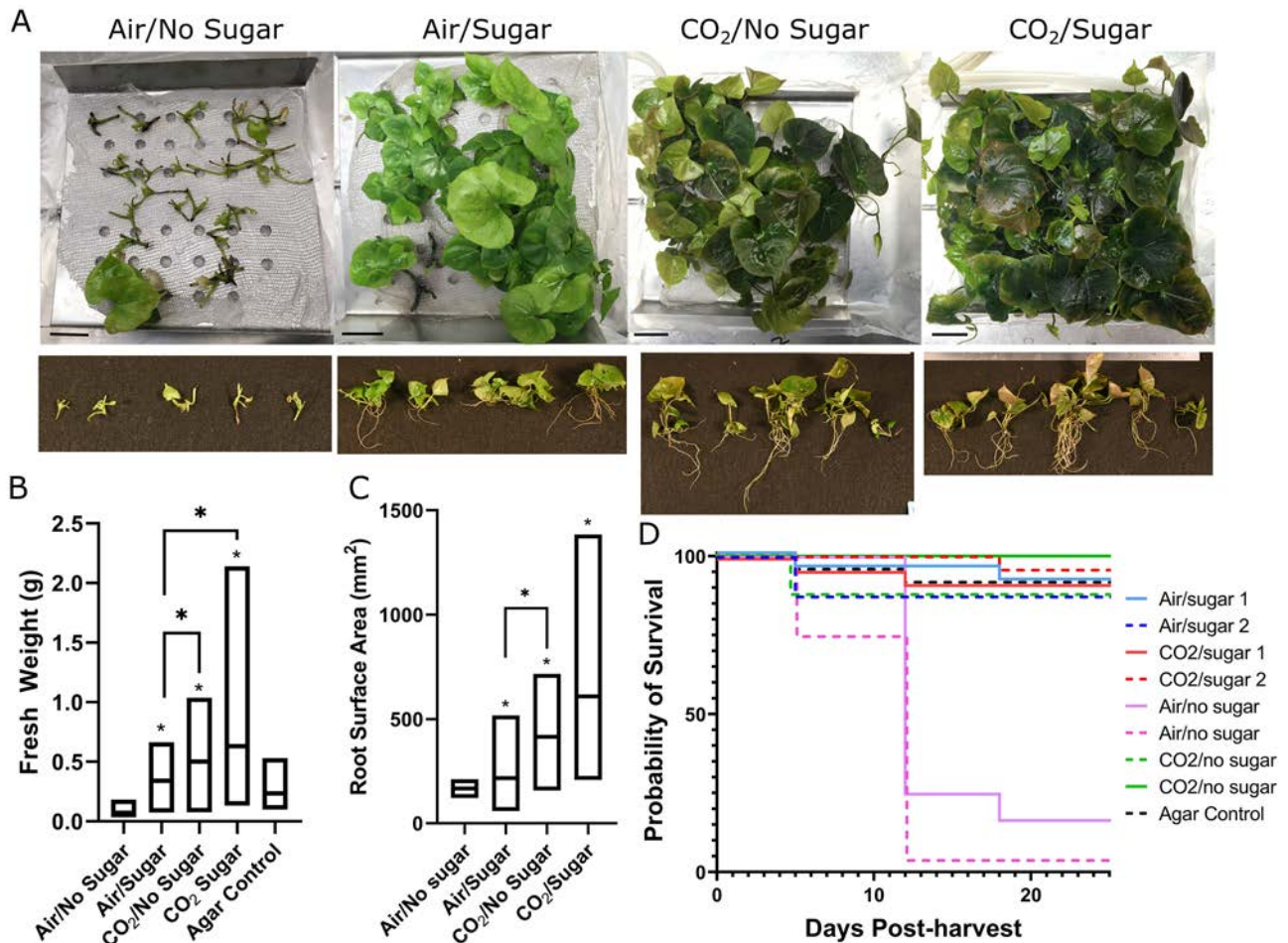
Fig. 4 Yam (*Dioscorea rotundata*) propagule development from axillary buds in the Arduino micro-controller automated McA-H Hy-TIB (v.3) with altered sugar and CO_2 supplementation. **A** *D. rotundata* propagules grown in elevated CO_2 with or without sugar in the growth media resulted in substantially healthier plants compared to ambient air or ambient air with sugar-rich media (top). Elevated CO_2 also resulted in substantially healthier propagule root development (bottom). **B**, **C** Upon harvest after 42 days of growth, elevated CO_2 significantly increased both fresh weight and surface area of propagule roots relative to those grown using typical ambient air and sugar media. **D** To evaluate robustness of horizontal H Hy-TIB grown propagules, the survival of explants was monitored after transfer to soil. Propagules grown using elevated CO_2 without growth media containing sugar survived at similar rates as equivalent plants grown using sugar-rich media. Scale bars in (**A**) represent 25 mm

reduction. Most efforts focus on the design or fabrication of the bioreactor device. Operation relates to how a device is used such as continuous media addition, or in this work control of the gas composition and flow rates. The following discussion emphasizes these differences.

Significance of bioreactor design improvements

Design-build-test

The development of technology is an iterative process commonly referred to as a design-build-test (DBT) cycle. The key to this process is to rapidly achieve prototypes with baseline functionality while overcoming design flaws and refining design for cost, ease-of-use, etc. The Hy-TIB

system developed here makes major strides toward that goal by making most components highly reusable or minimal replacement cost (e.g. plastic bag vessel) as we have developed previously for large scale suspension culture (Curtis 2001). In this phase of DBT, we focused on achieving low-cost, reliable fluid connections, combatting contamination and achieving phototropic growth.

The heterotrophic chicory root growth data of Fig. 2 is the publishable result of the extensive DBT effort. However, this does not reflect the important outcome of incremental improvements and experience from the DBT process. Using the manual cycled bioreactor alleviated simultaneous troubleshooting the technical aspects of automation but resulted in a very demanding effort (cycling 4–8 reactors at 4 h intervals, 6 times per day, 7 days per week). With a

roughly 3-week turn-around on reactors, this effort encompassed about 8 months, which would have taken more than 2 years if implemented with slower growing tissue. Among the issues of leaks, plugged filters, and contamination, was an inability to collect root biomass data due to unanticipated entangled growth into fabric tissue support matrix. These 'rapid failures' are an underappreciated success of the DBT process. Table 2 outlines the chronology of Hy-TIB designs, which were denoted sequentially as versions (v.) 1, 2, and 3. Online Resource S1C has a further detail of each version including specific fabrication details involved.

Achieving reliable low-cost fluid connections

Simplification of the TIB to a plastic bag culture vessel has been enabled by using an inexpensive means of attaching tubing directly to a polymer film. Making simple adhesive-free, autoclavable connections emphasized cost-cutting while preserving reliability. The solution for the tubing connection to the bag is depicted Fig. 5 using a 'press-fit swage', a small SS reducing connector that facilitates reliable connections simply by deforming (i.e. pressing to fit) the PP bag over it, with only snugly fit elastic tubing to hold it in place. The press-fit swage was customized from a SS tube, which was turned on a lathe, cut in short pieces and polished on a buffing wheel (see Online Resource S3, S1B). These connections are surprisingly robust and did not experience failure or leakage in any of the DBT or implementation test efforts. In contrast, failure to sufficiently puncture the stretched plastic resulted in several operational failures. As depicted in Fig. 5, this puncture was reliably achieved by pipette tip insertion through the fitting. The utility of silicone tubing is especially important, where its elasticity and non-thermosetting nature could achieve these connections with only appropriate sizing (i.e. no additional clamps). The connection of the SS insert's fluid distributor was similarly stretched by deforming the bag and connected by silicone tubing. Puncture of the bag

was also crucial for fluid distributor and achieved from outside the bag with wire rather than pipet tip.

Significance of bioreactor operational improvements

Comparison to alternative technology

Given a typical product value of less than a dollar per plant, achieving economic feasibility for axenic tissue culture methods is a tremendous challenge. Since the first report of a Temporary Immersion System, the Auxophyton by Cornell's Steward Lab in 1952, significant productivity gains, and in turn fiscal costs (per plant), have been realized; an analysis of sugarcane in vitro production was reported to reduce production costs by 46% in 2002 (Neumann et al. 2020a, b). Nonetheless, in vitro production of pineapple in a Periodic Immersion Bioreactor (PIB) reported in 2003 was still cost-prohibitive with a 500-fold increase in production despite a mere 35% cost increase. Thus, to move beyond exceptions like ornamentals (e.g. orchids, sterile hybrids) and high-value, long-lived specialty crops (e.g. coffee, chocolate), the contribution of the bioreactor to productions costs must be near-zero as we have achieved with the plastic bag culture vessel.

Gaining an economically feasible basis for using gas mixtures other than air was the driving force behind the design of the LA-V Hy-TIB that decoupled gas and liquid flows (Florez et al. 2016). With the elimination of pneumatic operation of media flow, the Hy-TIB provided an important scale-up advantage over other TIB systems like RITA™, Plantform™, and Temporary Root Zone Immersion system. Estimated costs of 2.5% CO_2 supplementation at scale (depending on scale of operation, i.e. pure or industrial CO_2) were between \$0.01–\$0.10 US per week per bioreactor (Shaw 2012; Florez et al. 2016), made possible by gas usage rates that are orders of magnitude lower than most that operate based on pneumatic movement of media. We
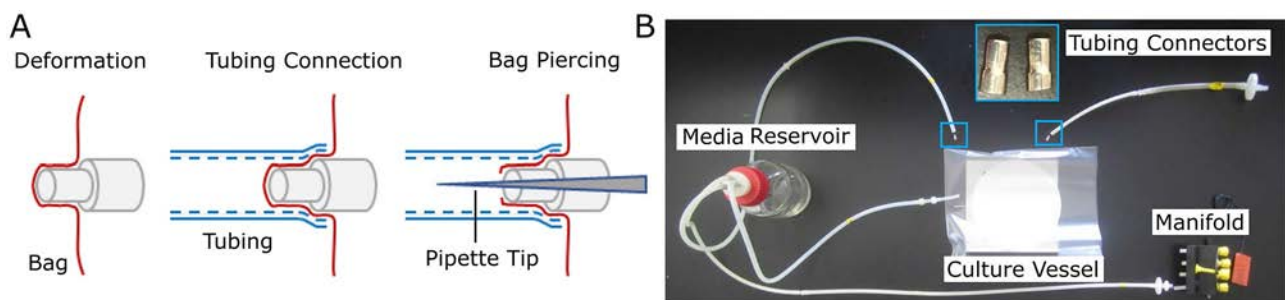

Fig. 5 Achieving simple, reliable adhesive-free silicone tubing connections to the plastic film bioreactor. **A** The press-fit swage connections are formed by first deforming the culture vessel plastic film, pressing the tubing over the deformation, and piercing the bag using a pipette tip. **B** The reusable press-fit swage tubing connectors are used to connect the bag culture vessel to the media reservoir and gas inlet/outlet lines

recognize opportunities for additional minor cost reduction based on materials. 3D printing and the notable success of replacing microfluidic devices with coated, precision-lathed wood (Andar et al. 2019) may provide both lower cost and carbon impact than SS used in insert, manifold, and its Luer-NPT connectors.

Gas phase manipulation

Having now worked with half a dozen plant species in TIB systems over the last decade, decoupling of the gas phase is not only cost-saving, but precision control over gas phase composition enables other advantages of environmental control in manipulating plant growth and development that are consistent with observations of others. Varying concentrations of elevated oxygen were shown in coffee cell suspensions to promote either multiplication or somatic embryogenesis (SE). Notably, Nie et al. (2013) conducted a metanalysis of 110 studies of CO_2 supplementation, showcasing general benefits of root development and carbon sequestration (Nie et al. 2013). Optimization of conditions, however, is often species-dependent and/or developmental stage-dependent with variability also enhanced by complex interactions of stress, hormones, growth conditions, and nutrient availability to name a few (Zhao and Guo 2011; Niu et al. 2016; Lahive et al. 2018). The TIB system lends itself not only to environmental control of the gas phase but also monitoring to elucidate these complex interactions. Ethylene, jasmonate and nitric oxide (NO) are among the volatile compounds known to be indicative of plant stress (Yoo et al. 2009; Zhao and Guo 2011; Kazan 2015; Elhiti et al. 2018) and the TIB system described here provides a means of systematic study of gas-phase plant signaling.

Technology adoption/implementation

Combining low capital cost with considerations of minimal operational costs (CO_2 and light), the remaining hurdle for technological adoption is ease-of-use and reliability. Toward that end, we have initiated efforts with a multidisciplinary "Learning Factory" design team to create both a platform for bioreactor assembly and IoT-based system for remote monitoring (https://bit.ly/3siVY8J) that would be similarly low-cost and open-source. More critically, since the reported scale of propagule production is small (i.e. hundreds of propagules), there is a clear need to scale up the system. The use of phototrophic growth conditions to minimize contamination risk provides a clear path forward for a larger format system and an implementation that can provide a substantial supply chain for disease-free, superior performance plant propagules.

Future improvements/challenges

The target of *Dioscorea* spp. represents a crop that has favorable characteristics of value to smallholder farmers in Africa, while solving issues of propagation of disease and overcoming issues of reliability of year-to-year yam 'seed' propagation. We suggest that scaleup in natural lighting is the next major technical hurdle to advance the Hy-TIB platform's mission to achieve economic feasibility though we also recognize the potential role of microorganisms to enhance field performance, which would require reintroduction after proliferation in an aseptic tissue culture environment.

Natural light

The challenge of increasing light levels to improve phototrophic performance is managing the associated heat load. Those unfamiliar with plant tissue culture are often surprised to learn that they are heterotrophic, and CO_2 accumulates in plant tissue culture (PTC) enclosed vessels (McKelvey et al. 1993). As an example, the root cultures used in this work are oxygen transport-limited—typical of heterotrophic microbial culture. Artificial lighting such as fluorescent and LED provides considerable light for plant physiological functions at minimal heat load. In contrast, natural light doubles the incident energy based on the solar spectrum alone. For example PAR levels at 100 $\mu E\ m^{-2}\ s^{-1}$ corresponds to roughly 22 $W\ m^{-2}$ and natural sunlight can be over 1000 $W\ m^{-2}$. The issue this represents for a TIB system is not only the 'greenhouse effect' heating, but a proportional increase in the water load of the exhaust gas which is an ongoing challenge associated with blockage of the exhaust filters (see Online Resource S1E). Rapid advances in plastic and glass film coatings to exclude solar heat load will invariably be a component of the next generation plant propagation systems, which spans the transition from heterotrophic plant tissue culture to axenic phototrophic propagation and ultimately field introduction.

Microbial reintroduction

The beneficial association of microorganisms with plants is increasingly recognized (Santos et al. 2019) which needs to be taken advantage of in the transition from the plant tissue culture environment to the field. As noted above, phototrophic growth provides an excellent platform to introduce microorganisms with greatly reduced issues of microbial overgrowth. In our work with cultures of *D. cayenensis,* we observed a slow-growing bacterial 'contaminant' which would tend to grow more aggressively during tissue culture stress. These putative endophytes were characterized by 16S ribosomal RNA gene sequencing (sequence in Online

Resource S1J) and were identified as methylotrophs (Shouse 2018)—*Bacillus aryabatthai* (Bhattacharyya et al. 2017), *Bacillus ginsengihumi* (reclassified as *Paenibacillus* species) (Ash et al. 1993; Lee et al. 2007), and *Hyphomicrobium facile* (Fesefeldt et al. 1997). Methylotrophs are common endophytes, as plants produce methanol as a biochemical byproduct of pectin formation, making it an ideal carbon source for endosymbiotic microorganisms. These putative endophytes are also associated with nitrogen-fixing capability which would have obvious benefits to be added back to the plants before field planting.

Role in plant improvement

With the advent of NextGen Sequencing and CRISPR-Cas9 technologies, the barriers to development of transgenics have been lowered significantly. Moving forward, the more prominent barrier will be the recalcitrance of superior cultivars to regeneration (Altpeter et al. 2016; Campos et al. 2017), especially in (non-model) plants critical to food and economic security that are considered highly vulnerable like *Musa* spp. (Ordonez et al. 2015) and *T. cacao* (Evans 2016). Overcoming regeneration recalcitrance includes taking advantage of genetic control via manipulation of embryogenic or morphogenic transcription factors (Florez et al. 2015; Lai 2016; Shires et al. 2017) as a means of expanding those plants amenable to a tissue culture based supply chain for superior plants. The transient delivery of DNA into in vitro grown plant tissues can be implemented in TIB bioreactors—not only as a tool for next generation research of plant development—but also scaled in production systems in support of agronomic production.

Declarations

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