Thin Films of Liquid Media for Heterotrophic Growth and Storage Organ Development: Turmeric (*Curcuma longa*) as a Model Plant

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***Summary*. Geophytes store carbohydrates in modified underground shoot systems protected by a broad array of biologically active chemistry. In vitro formation of storage organs requires months in the lab instead of years in the field, when water and nutrients are correctly supplied. Liquid and agar systems in large and small vessels were compared for sugar and water use with turmeric (*Curcuma longa*) as a model plant. Small jars on a shaker were compared with large, flat-bottomed vessels containing thin films of liquid media, intermittently tilted at slight inclines that allow the advantages of liquid phase transfer with gentle agitation. Liquid culture in small vessels on a shaker yielded the most plants and liquid culture on a thin-film rocker in a large vessel yielded the largest plants. Increased and improved biomass (fresh and dry) in liquid culture compared to agar was based on greater sugar use. When large vessels of liquid media were grown for 5 and 6 months on a rocker, 400 mL of media yielded 150 to 200 g (fresh weight) of plants. Similarly, 13 to 16 g (dry weight) of plant tissue was derived from 24 g of sugar. Plants were about one-third rhizome by fresh mass. Rhizomes had greater dry and fresh weight than leaves or roots, indicating solute actively accumulated in the rhizome. The rhizomes had normal morphology, characteristic pigments and fragrance, and rhizome extracts had strong antioxidant potential. The gentle rocking action of plantlets in sugar-containing liquid medium was demonstrated to produce functional storage organs.**

Turmeric (*Curcuma longa* L.) is valued as a spice, dye, industrial starch, and medicine with virtually all production being in South and Southeast Asia. Its values in traditional and allelopathic medicine are attributed to a mixture of putatively active compounds extracted from ovate (primary) and cylindrical (secondary) rhizomes (Bruneton, 1999). Fresh primary rhizomes are an especially rich source of essential oils, whereas one and two year old rhizomes are the commercial source of the nonvolatile curcuminoids (de Padua et al., 1999). The brightly colored curcuminoids have received most serious attention from the medical research community. The curcuminoids and essential oils are among dozens of phytochemicals found in turmeric that may function as free radical scavengers. There are several antioxidant assays that indicate putative activity of crude plant extracts.

Turmeric has been cultivated in India since time immemorial. It is a sterile triploid and is usually replanted as divisions of cured rhizomes. Disease-free stock plants are micropropagated by enhanced axillary branching of leafy shoot divisions, but more recently microrhizomes were produced in liquid shaker culture (Salvi, et. al. 2002). The biomass of the microrhizomes was related to sugar concentration in medium, and the larger microrhizomes can be directly field planted without an acclimatization treatment (Shirgukar et al., 2001).

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Bioreactor systems that aerate large quantities of liquid tissue culture medium were developed for both micropropation and direct production of phytochemicals (Preil 2005). Many plant species have been tested for secondary metabolite production in tissue cultured plant cell and callus aggregate suspensions, agitated in liquid similar to fermentation technologies. However, after 30 years and hundreds of attempts, there are only four commercially successful applications (Alferman et al., 2003). Two plausible explanations for so many disappointing results are 1) cell culture fermentors are too expensive, and 2) nondifferentiated cells in liquid medium do not accumulate adequate quantities of the desired compounds (Preil, 2005). Cost-effective micropropagation in liquid medium has driven the recent develop-

Fig. 1. Rocker platform used to gently agitate thin-film culture vessels. One rocking motion lasting one minute occurred every 15 min created a wetter (**right**) and dryer (**left**) side of the vessel.

ment of several low-cost bioreactors for plant organ and whole plant culture.

Monocotyledonous plant species are particularly well suited to liquid culture (Takayama and Akita, 2005). Temporary immersion systems (TIS) have optimized gaseous and solute transfer properties resulting in more or larger plants with greater dry weight for many crop species (Berthouly and Etienne, 2005). Rocker systems are simple TIS that aerate plantlets without the requirement of direct connections between the motor and vessels. The Liquid Lab Rocker is one such system that intermittently wets plant tissues by a slow pitching motion of large rectangular vessels on an articulated shelf (Adelberg and Simpson, 2004). On the rocker, monocotyledonous crops, *Alocasia*, *Colocasia,* and *Hosta* had greater rates of multiplication, yielded larger plants, and used greater amounts of sugar when compared to stationary semi-solid agar medium (Adelberg, 2005a; Adelberg and Toler, 2004). Two species of perennial plants valued as functional foods, a monocot, konjak (*Amorphophallus riveri*), and a dicot, wasabi (*Wasabia japonica*), were grown in rocker-type bioreactors (Adelberg et al., 2001). Rhizomes had developed at the bases of large plants in large vessels over several months in vitro. Storage organ formation in vitro was promoted by the gibberellin antagonistic growth regulators, ancymidol and paclobutrazol, in *Allium*, *Colocasia,* and *Solanum* (Kim et al., 2003; Zhou et al., 1999; Ziv and Shemsesh, 1996).

In this current work, biomass production of turmeric, a monocotyledonous crop harvested for chemicals stored in rhizomes, was produced in agar and liquid tissue culture systems. In vitro growth, sugar and media use, in large and small vessels were compared for agar and liquid systems in short-duration experiments. Factors that increased in vitro plant and rhizome size in liquid systems were observed in longer duration experiments. Tissue cultured rhizomes were assayed for antioxidant activity compared to the standard food preservative BHT (butylated hydroxytoluene).

Materials and methods

Plant material. Four accessions of turmeric (*Curcuma longa* L.) were obtained from the University of Arizona Southwest Center for Natural Products Research and Commercialization. Stage I was prepared by dissecting the quiescent shoot tips from rhizomes, immersing in full-strength commercial bleach, and plating on hormone free MS media in petri plates. Stage II cultures were maintained for at least 6 months by subculture every 6 weeks in liquid medium, modified MS (Murashige and Skoog, 1962) that included additional, 170 mg NaH_2PO_4, 100.0 mg myo-inositol, 0.25 mg nicotinic acid, 0.25 mg pyridoxine hydrochloride, 0.05 mg thiamine hydrochloride, 60 g sucrose, and 1 μm benzyladenine (BA) per liter. Medium pH was adjusted to 5.7 before being dispensed. Explants were placed in 180 mL glass jars containing 30 mL of liquid tissue culture medium and cultured on an orbital shaker (100 rpm) with 25 to 35 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PAR provided by cool-white fluorescent tubes with 16 $h \cdot d^{-1}$ photoperiod maintained at 24 ± 2 °C.

Agar vs. liquid in large and small vessels for 26 d. Five explants (*C. longa* accession no. 43-4) per small vessel (180 mL jar) were placed in semi-solid agar or on shaker, six vessels per treatment. Semi-solid agar medium was gelled with 7 $g \cdot L^{-1}$ agar (A296; PhytoTechnology Laboratories, Shawnee Mission, Kan.). Large vessels (about 6 L) (Nalgene Biosafe; Nalge Nunc International, Inc., Rochester N.Y.) received 210 mL liquid or agar medium and 35 explants, three vessels per treatment. Explant density of 6 mL per explant or 167 explants per liter was maintained for both large and small vessels. Large liquid containing vessels were maintained on a thin-film rocker system that produced a 1-cm pitch every 15 min (with a 1 rpm cam). After 26 d, plants were carefully blotted on paper towels and fresh weight (FW) per vessel and the number of plants per vessel were recorded. Residual medium volume was measured in a graduated cylinder, and agar medium was melted in an autoclave and measured as a molten liquid. Plant dry weight (DW) was recorded after drying the contents of each vessel individually in paper envelopes at 60 °C for 48 h. Residual sugar concentration in expended liquid media was determined with a refractometer. Residual sugar was assayed from semi-solid agar by disrupting the matrix with repeated forceful shearing of the medium through a pipette tip. After several pump actions, a bead of liquid slurry was placed on the lens of the refractometer. The experiment was a completely randomized design with vessels being the unit of replication for the four treatments, agar in large and small vessels, and liquid in large vessels on rocker and small vessels on shaker. Statistical analysis was performed using JMP 3.2.6 (SAS Inst., Cary, N.C.).

Long-term growth in large vessels of liquid. A first long-term growth experiment used large (about 2.5 l) Liquid Lab Vessels (Southern Sun Inc., Hodges, S.C.) modified with two, 6 mm diameter holes covered by ventilation patches (Southern Sun Patch). Each vessel received 200 mL of medium and 13 to 20 buds of *C. longa* accession no. 50-3, placed at one end of the vessel. In six of the vessels buds were placed on the wet end of the vessel, and six other vessels were set with the buds on the dry end of the vessel (Fig. 1). Following 90 d in culture, leaf tissue was trimmed from half the vessels on both wet and dry sides, 100 mL medium was aseptically added to all vessels, and they were returned to their original orientations on the rocker. After an additional 42 d, 100 mL of fresh medium was added. Following a total of 168 d, plants were harvested and residual medium volume and sugar concentrations were measured as in first experiments. Plants were dissected into leaves, rhizomes, and roots, before FW and DW determination.

In the second long-term growth experiment, large Liquid Lab vessels received 200 mL media and 11 to 16 buds of *C. longa* accession no. 22-5. Vessels had no ventilation patches, one ventilation patch, or two ventilation patches (as above), and contained media with no ancymidol, 0.32 μM ancymidol or 3.2 μM ancymidol. Plants were grown for 144 d with 100-mL medium supplement after 76 d and again after another 35 d without any cutting. Harvest and processing were as above. The first experiment was a completely randomized design with 2 × 2 factorial treatments (wet and dry, cut and uncut) and the second experiment was completely randomized design with 3 × 3 treatment arrangements (3 ancymidol levels and 3 ventilation levels).

Antioxidant assay. Fresh rhizome preparations of *C. longa* accession nos. 22-5, 30-5, 43-4, and 50-3, were prepared from similarly grown plants in vessels with ventilation for 119 d. Methanol extracts of fresh rhizome tissue were assayed by spectrophotometer for their ability to scavenge free radicals generated by DPPH (2,2-diphenyl-1-picrylhydrazyl) using the method described in Yamaguchi et. al. (1998) with slight modification.

Results and Discussion

Agar vs. liquid in large and small vessels after 26 d. Small vessels of liquid on the shaker produced more plants than agar, or the larger Nalgene Biosafe vessels containing agar, or liquid on the rocker (Table 1). Plants in liquid in large vessels were larger than plants in smaller vessels on a shaker, or either vessel with agar (Fig. 2). However, larger vessels with agar medium did not yield larger plants than smaller vessels. Larger vessels had lost 60% of their medium volume, mostly to evaporation, where smaller jars had lost only 21% and 36% (for agar and liquid, respectively). There was little correlation between final media volume and plant growth (Table 2.) Greater growth in terms of fresh weight, dry weight and multiplication, was related to a greater amount of sugar used. Most of the sugar and medium in the large vessels of

Table 1. Growth and sugar use of *Curcurma longa* 43-4 after 26 d in large (6 L) and small (180 mL) vessels of agar and liquid.

Medium	Vessel size	Multi-rate[z]	FW/ explant(g)	DW/FW (%)	Sugar used (%)[y]	Medium used (%)[x]
Agar	Small	1.1 a	1.0 ab	8 ab	22 a[w]	21 a
	Large	1.1 a	0.6 a	6 a	52 b	60 c
Liquid	Small	2.9 b	1.5 abc	12 bc	58 b	36 b
	Large	1.7 a	2.0 c	14 c	74 b	63 c

[z]Multiplication rate is number of plants harvested/number of initial explants.
[y]Sugar used is 1 – [final sugar concentration (Brix) × final media volume]/[initial sugar concentration (Brix) × initial media volume].
[x]Media used is 1 – (final media volume/initial media volume).
[w]a,b,c designate significantly different means within columns at $P < 0.05$ by Tukey's HSD.

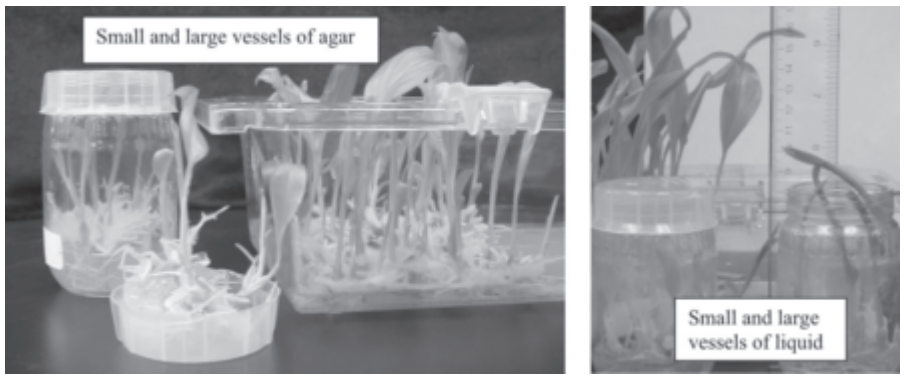

Fig. 2. *Curcurma longa* plantlets on agar (left) were about the same size in small glass jars or large Nalgene Biosafe vessels. Plantlets in liquid (**right**) were larger in the Biosafe vessels than plants in jars, or plants in agar (**left**). Biosafe vessels are shown with the tops removed, allowing the leaves to uncurl along the centimeter scale.

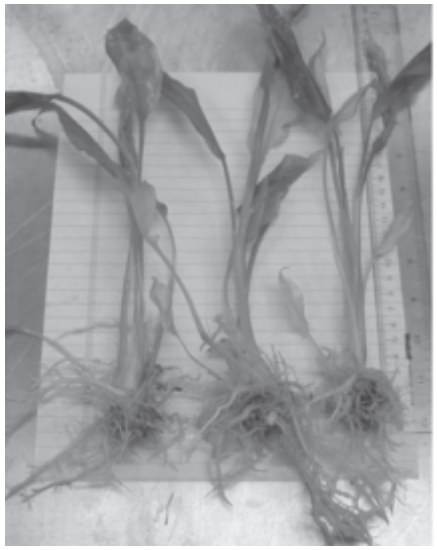

Fig. 3. Plants size often exceeded the vessels 27-cm length of the vessel of the Liquid Lab Rocker vessels.

liquid was used in the 26 d in culture. Longer periods of culture in large boxes of liquid with media supplementation were envisioned.

The Nalgene Biosafe is a two-piece clamshell vessel with large silicone rubber gaskets that needed extensive clamping to maintain asepsis with repeated use. Opening the Biosafe's top lid allows plants in agar cultures to be oriented. Liquid cultures are initiated by a nonoriented dumping motion and do not require such a large opening (Adelberg, 2005b). For convenience, asepsis, and controlling moisture loss, large vessels for liquid culture could use a screw cap closure. The Liquid Lab Rocker vessel was constructed specifically for micropropagation in agitated, thin-films of liquid medium (Adelberg, 2005b). A second set of experiments was conducted to grow plants over longer periods of time with larger volumes of liquid media, supplemented in Liquid Lab Rocker vessels.

Large plants in large vessels, over longer periods of time. After 90 d of growth in Liquid Lab Rocker vessel, leafy shoots had essentially filled the vessel and the media volume was approaching zero. After 168 d of growth, with two 100-mL additions of supplementary medium, rhizome, root and leaf size, were not affected by placing plants on wet or dry side of the vessel (Fig. 1, data not shown). All vessels contained large, leafy plants, many that fully spanned the 27 cm length of the vessel (Fig. 3). Plants that had been trimmed free of leaves at 90 d, and returned for regrowth had the same root and rhizome mass as plants that had not been trimmed before media supplementation (although nontrimmed plants had more leaf mass). Placing plants deliberately at the wet or dry end of the vessel had no effect on growth (treatment means were pooled). Each vessel contained about 154 g FW tissue. After dissecting every plant in this experiment, there was 10.3 g FW per plant, 3.1 g of leaf tissue, 3.9 g FW per plant of root tissue, and 3.3g FW per plant of rhizome, or 0.32 g DW per plant of rhizome. Plants were 32% rhizome by FW, or 40% rhizome on DW basis. The three tissues had significantly different RDW (DW/FW), root tissue – 6.7% ± 0.3%, leaf tissue – 7.6% ± 0.2% RDW, and rhizome – 9.6% ± 0.6%. This demonstrates that plantlets in vitro were actively storing solids in their rhizome.

Table 3. Biomass of *C. longa* 22-5 as affected by ventilation treatment during 144 d in Liquid Lab vessels in media with 0, 0.32, or 3.2 μM ancymidol.

Ventilation patches	FW (g)	Rhizome/ plant mass (FW, %)	Water use efficiency[z] (%)	DW (g)	Sugar use efficiency[y] (%)	DW (g) rhizome
0	202	36	68	16.4	69	9.2
1	193	35	51	14.7	61	7.6
2	159	35	39	13.3	55	6.6
ANOVA $P > F$						
Vents (V)	**	n.s.	***	*	*	*
Ancymidol (A)	**	NS	NS	NS	NS	NS
V × A	NS	NS	NS	NS	NS	NS

[z]Water use efficiency was expressed as (FW – DW)/volume of media used.
[y]Sugar use efficiency was expressed as (g sugar used)/DW.
NS,***,**,*Nonsignificant or significant at $P > F$ at 0.001, 0.01, or 0.05, respectively.

Table 2. Pearson correlation coefficients showing media resources expended by growth of *Curcuma longa* 43-4 after 26 d of culture.

Parameter	Sugar use (% Brix)	Media use (vol, %)
Dry weight per explant	83	24
Fresh weight per explant	82	15
Multiplication rate	79	6

In the second long-term growth study, the presence of 3.2 μM ancymidol reduced FW by 10% (170 g vs. 190 g FW per vessel) with a proportional reduction in rhizome mass. Ventilation of the vessel affected biomass and reduced ventilation increased FW and DW by about 25% (Table 3). Water use was 70%, 85% and 99% of the 400 mL of media for 0, 1 and 2 vents, respectively (Fig. 4). Greater than 99% of the 24 g of sugar was used in all treatments to produce 16.4, 14.7 and 13.3 g DW in the vessels with 0, 1 and 2 vents, respectively. Regarding, water and sugar use efficiency, the proportions of media mass that was incorporated as biomass (FW and DW, respectively) was greatest in vessels without ventilation ports. Roots and leaves were about 6.5% RDW, whereas the rhizomes were 12.7% RDW. Relative dry weight ratios in leaves, rhizomes or roots were not affected by vessel ventilation. Reduced ventilation increased water weight (FW – DW) *in planta* that was permissive to increased accumulation of solids in the rhizome.

Eliminating the ventilation ports increased the DW of the rhizome by about 30% and the rhizome was 35% of the plant's FW or 50% of the plant's DW. Ovate primary rhizomes and cylindrical secondary rhizome fingers are indicative of more mature growth phase and secondary rhizome development was found in some of the vessels (Fig. 5). Many rhizomes had lemon yellow and yellow orange core, indicative of curcuminoid phenolics. A pleasant, aromatic odor emanated from the culture vessels upon opening.

Antioxidant activity. Antioxidant activity was used to assay the potency of rhizome extracts. Rhizomes grown in ventilated vessels were fresh extracted in methanol to quantify the activity of secondary metabolites. A DPPH free radical scavenging assay was performed on extracts of the four clones. The EC_{50} values were 4.5, 3.2, 3.7, and 3.9 mg tissue DW per mL methanol, for the clones 22-5, 35-1, 43-4, and 50-3, respectively. Each clone produced significantly different standard curves (data not shown). The EC_{50} values for the fresh rhizome tissue extracts (expressed on a DW basis) were about 30-fold higher than the standard BHT. We may conclude that the different clones of tissue culture produced rhizomes contained unique mixtures of potent, bioactive chemicals. The 30-fold difference between crude extract and purified chemical preservative BHT shows that the rhizome produced in vitro were highly potent.

In summary, agitated films of liquid medium can be used to produce significant quantities

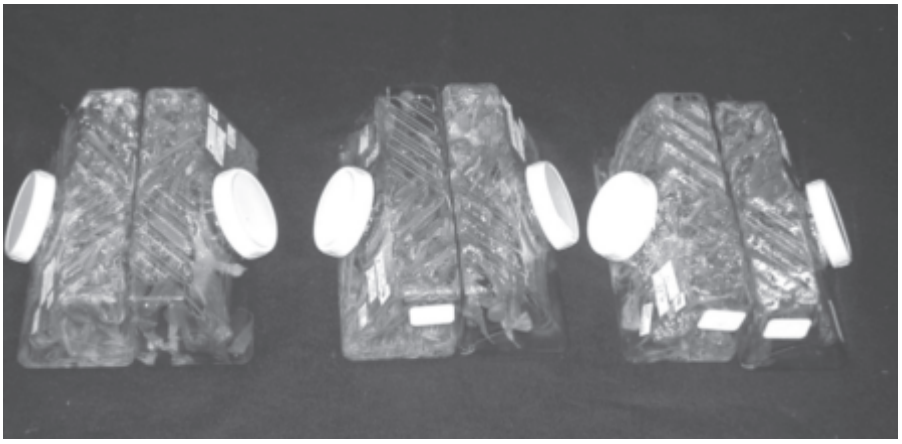

Fig. 4. Liquid lab vessels with 0, 1, and 2 ventilation patches (in pairs, left to right) were shown filled with large leafy plants.

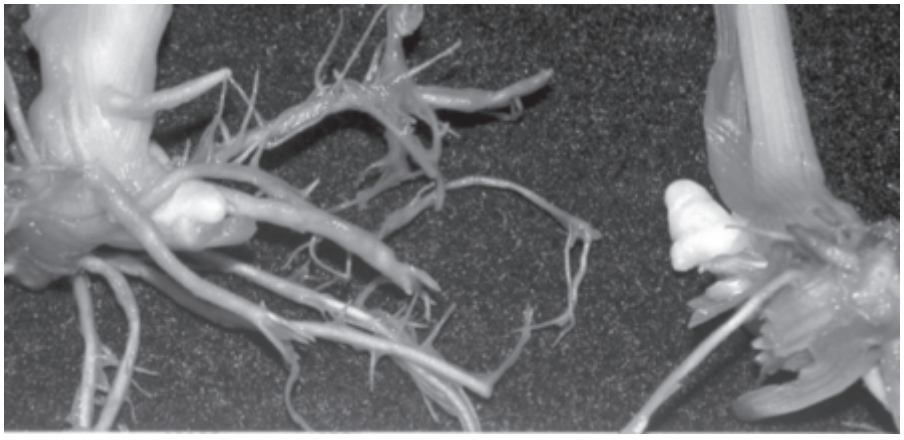

Fig. 5. Primary rhizomes are found beneath the well-rooted base of the large plants from the large rocker vessels. Secondary rhizomes were found to protrude from the primary rhizomes.

of rhizome tissue. Fresh and dry weights of the plants were increased by the availability of water and sugar in large vessels of liquid medium, compared to smaller vessels or vessels of either size containing agar-gelled medium. Long-term culture in large vessels with liquid medium supplements produced rhizomes with greater RDW, than the other portions of the plant, indicating an active storage organ function. Rhizomes of all four genotypes tested in long-term culture experiments contained high levels of antioxidants. Reduced ventilation increased fresh and dry biomass in all organs.

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