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NOVEL TEMPORARY IMMERSION BIOREACTOR ALLOWS THE MANIPULATION OF HEADSPACE COMPOSITION TO IMPROVE PLANT TISSUE PROPAGATION

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ABSTRACT

This thesis describes the iterative development of a bioreactor system to propagate plants. While a large focus of the effort was in instrumentation associated with gravity-driven liquid flows, the work also included extensive trouble-shooting associated with various difficulties of implementation as well as the techniques of maintaining a wide variety of tissue cultures. A temporary immersion bioreactor is tissue culture system in which the liquid nutrient medium is periodically flooded into the culture vessel to provide dissolved nutrients. This is particularly useful for culture plant tissues because constant submergence can lead to being water-logged and difficulties in providing oxygen from the gas phase. In addition to proving for control of liquid contacting, the TIB system allows for changing the nutrients and hormones in the medium. Of particular interest to this project, is the ability to facilitate the proliferation of plant shoots (termed meristem propagation) or the formation of embryos from somatic tissues (termed somatic embryogenesis). The specific work described within this effort involves the design of a stepper motor system to raise and lower media reservoirs that facilitates the desired intermittent exposure to media. This involved writing a LabVIEW computer program that interfaced to a stepper motor through a DIY stepper motor kit that was interfaced to the computer via a simple parallel computer (printer) port. The LabVIEW program included a high level of sophistication including text messaging for operational verification, warnings associated with externally mandated computer resets, and the capture of video images as 'time lapse' files to be monitored over the internet to verify proper bioreactor operation. The thesis also describes the evolution of various designs from basic design and materials of the bioreactor, reservoir, gear and pulley systems, to trouble-shooting of contamination issues where studies of vessel pressure levels using an integrated circuit pressure sensor that demonstrated unexpected internal bioreactor pressure changes that could become sub-ambient and compromise sterility of operation. A solution for gas

delivery from an inexpensive manifold to multiple reactors while maintaining a positive pressure in the vessels is presented as part of the 'Design of Gas Handling' chapter of the thesis. Since the final performance runs are in progress, only brief descriptions of are included of the numerous studies carried out during the period of nearly three years; these include meristem propagation of watermelon, root culture growth rates under elevated oxygen. This work has led to establishing a collaboration with a Nigerian researcher who will be looking to scale up this technology to propagate yam, and the project continues as a bioreactor to be used to deliver transcription factors to manipulate the process of somatic embryo formation as a final illustration of the ability of a bioreactor system to facilitate the manipulation of the gene expression in propagated plant tissues.

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CHAPTER I

INTRODUCTION AND SIGNIFICANCE

The Value of Plants

It is easy to take for granted the critical role that plants play in human existence. Beyond food and shelter, many invest considerable time and money in houseplants and landscaping. The improvements in agriculture associated with the 'green revolution' have made dramatic changes in the production of food in most developed countries around the world. Early improvements were as simple as more efficient mechanized planting of seed, starting with Jethro Tull's Grain drill (1700) or grain separation (thrashers) in the mid 1700's. With the Haber-Bosch (1909) came the dramatic increase in fertilizer, which is still steadily rising to this day[1]. While plant breeding dates back beyond 10,000 BC modern breeding has provided continued improvements in productivity on existing land in recent decades. For example, global grain yields have been rising linearly from 1.3 to 3.3 tons per hectare from 1960 to 2010. This impact of 'modern agriculture' has fundamentally changed society by allowing a great migration to cities and nearly unprecedented increases in human population. While continued population growth is not sustainable, we continue to ask more from our plants despite tyring to reduce the expensive inputs, and utilizing increasingly marginal land.

Barriers to Plant Improvement

The breeding of plants can be very time consuming; for long generation plants such as trees, it literally takes a lifetime. Since plant survival is enhanced through diversity of offspring,

most plants have very complicated genetics, and the crossing of 'superior plants' often results in a very small shift towards the 'desired phenotype'. Early historical breeding was little more than collecting seed from desirable plants. This was very successful for developing 'modern' corn and tomatoes from their ancestors; however, it occurred over a period of thousands of years. 'Modern' genetics[2] greatly accelerated breeding with the improved understanding of gene segregation, which became far more clear with the elucidation of DNA in the 1950s. Unfortunately, the traits we desire from plants are more complex than simply the 'red eyes' of a fruit fly.

The complexities of breeding have been greatly assisted with the tools of genetic markers in 'molecular breeding'. Marker assisted selection provides the ability to use large numbers (hundreds/thousands) of unique DNA sequences that are then correlated to the desired phenotype. In this way, the genetic selection is no longer dependent on individual genes, but simply 'locations' in the chromosome that are 'mapped' for productivity by RFLP (restriction fragment length polymorphism), SNP (single-nucleotide polymorphisms) and many more techniques. While these advanced genetic methods have been extremely useful for improving quantitative traits such as yield (bushels of corn per acre) in a typical agricultural setting, they are not as useful for complex traits such as dealing with stressful environmental conditions. The process of finding exceptional phenotypic plants with traits such as resistance to drought or disease is still often limited to the selection of survivors in the presence of such stress. The challenge then becomes how to propagate that unique plant phenotype – particularly since genetic variability often means that the seeds from that plant will often not have the desired characteristics.

Another interesting dilemma of plant breeding is the issue of quantity versus quality. In a world where agricultural commodities are bought and sold 'by the pound', the nutritional value of the food is often significantly undervalued. In fact, there is evidence that high yield tends to be inversely related to nutritional quality[3]. Unfortunately, plant breeders in the modern age are concerned primarily with creating crops that generate a greater profit. While markets are

beginning to grow for more healthful food goods, the current focus is on ‘organic,’ ‘non-GMO,’ and ‘heirloom’ crops based on the assumption that focused breeding and improvement efforts undermine the healthful qualities of produce. As a result, plant breeders are faced with the effort of considerably changing the image of their trade, along with the evaluation of the true benefits of the products that they generate.

Plant Propagation in Tissue Culture

The culture of plant tissues under aseptic (microbial-free) conditions has provided new routes to plant improvement[4,5]. Most notably, these tissue culture methods provide a way to propagate large numbers of a superior plant that bypasses traditional plant breeding. Micropropagation refers to multiplying plant numbers through stimulating the formation of multiple shoots, and ultimately large numbers of individual plantlets that can be isolated by aseptically cutting the growing plant tissue. The application of plant hormones can cause rapid formation of multiple meristems including phases of elongation (to make them easier to cut up), and rooting. One of the major success stories of micropropagation is the proliferation of inexpensive orchids[6] –a rare specialty hobby only a few decades ago … now available at Walmart (personal observation). Interestingly, the following statement is found in the preface history of this book: “… the development of computerized flow systems for orchid tissue culture, has not gone very far”, noting numerous efforts towards “robotization, bioreactors, and flow systems”. The work in this thesis is precisely trying to develop such technology, but doing so with the objective of achieving commercialization by focusing on reliability and minimization of cost. The Curtis laboratory has had a considerable history in the development of technologies for plant propagation, including various publications and book chapters on bioreactor design for plant propagation systems[7] including specific focus on oxygen transport considerations[8].

CHAPTER II

PLANT PROPAGATION TECHNOLOGY

Plant Propagation using Meristem Culture

Although plants can be very large in size, 'growth' is restricted to localized regions that are often a very small fraction of the actual plant biomass. For plant propagation, this localization of growth, combined with the ability to de-differentiate to 'non-specific' cell types and re-differentiate into organs, is the basis of asexual propagation in plants. The process of rooting the cuttings of plants to produce many new plants from a single plant is often called 'meristemming.' The term 'vegetative propagation' is used more broadly where there might not be a meristem present, but an apical meristem readily develops from pieces of a cut plant such as African violet. Another example of proliferating plants from meristems is the willow, which requires little more than cutting a young branch and pressing it into moist soil to grow a new tree. Many additional plants will naturally root if upper stems come in contact with soil. These roots originating from the stem rather than the primary root are referred to as 'adventitious roots.' While some common plants like the tomatoes can exhibit this behavior, some plants purposely proliferate in this manner including the 'runners' of strawberries. In plants which utilize this behavior to cling to things such as ivy, this same behavior can be utilized in plant tissue culture to rapidly proliferate aseptic plantlets, and can often be manipulated to exaggerate the rate of amplification.

One of the advantages of meristem propagation is that the asexual proliferation of a plant can retain a phenotype that would be lost, or rarely observed from the seeds of that plant. This has proven an important way to preserve flower color, even in annual bedding plants. Combined with the ability to eliminated viruses[9], the meristem propagation of ornamental flowers is a large

industry that provides ‘bed flowers’ with superior phenotype than can’t be obtained from seed. In cases where plants do not naturally root, one can apply ‘rooting hormone’ which is normally a very dilute powder of a synthetic plant hormone such as NAA (Naphthaleneacetamide).

Meristem propagation does have some peculiar problems, since it does not necessarily ‘reboot’ the differentiation patterns of the plant. Rooted cuttings from lateral branches of a conifers such as spruce tend to retain their ‘creeping’ (plagiotropic) growth pattern for as long as 10 years[10]. Many hardwood plants in particular lose their ‘juvenility’ and associated ability to regenerate roots even with hormone application[11]. In some cases, juvenility can be restored when plant tissues are placed in tissue culture.

Somatic Embryogenesis

A fundamental limitation to vegetative meristem propagation is simply the amount of material from the parent plant limits the number of new propagules that can be generated. This leads to the alternative approach of somatic embryogenesis, where in principle, every cell can be induced to produce a plant. By definition, somatic embryogenesis is the formation of embryos from somatic (nonsexual) plant tissues. Achieving somatic embryogenesis is extremely dependent on the plant species, varying even between cultivars of a particular species. The model species for somatic embryogenic in plant cell culture is the carrot, which is able to undergo massive formation of embryos by transferring cultured plant cells into a hormone free medium[12]. This ‘easy’ somatic embryogenic system has been used by many to study and model the dynamics of embryo formation; however, the simplicity of this

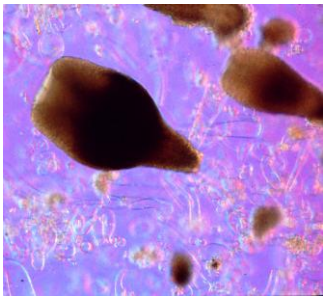

Figure 1 – Carrot cell culture undergoing somatic embryogenesis as a result of hormone reduction.

system has not translated to other plant species where the methods of induction are still largely based on agar based media.

Figure 2 – Loblolly pine embryonic suspensor mass. Dense head is the origin of embryos.

Polyembryogensis or the production of embryonic suspensor masses (ESM) is a special case of somatic embryogenesis that occurs in some plant types where immature embryo structures continuously proliferate in 'flushes' from the original isolated immature embryo[13]. This behavior is prevalent in commercially important species such as loblolly pine and Douglas fir, and the Curtis laboratory undertook a major project to scale up the production of these species for Weyerhaeuser using suspended bioreactor systems[14]. This technology is quite advanced, producing thousands of embryos per liter of culture. By storing the plant materials cryogenically, the various clonal lines can undergo extensive field-testing to evaluate performance since the origin of the material from immature embryos means that the performance phenotype (rate of growth, wood properties etc... in different plantation conditions) is not known at the time of tissue isolation. This example illustrates that different bioreactor designs and configurations are appropriate for different propagated tissues.

To go beyond the stage of immature embryo often requires the use of agar based media, which may result from the need to sustain gradients within the tissue. In this sense, the well-mixed condition of liquid suspension culture becomes detrimental toward further development. In addition, the size of the tissues becomes so large, that issues arise for transport of oxygen as a result of the greatly increased flux requirements at the surface of a large growing tissue[8,15]. Limitations such as these that are encountered while working with large organized tissues are in part, a goal to overcome in the proposed temporary immersion bioreactor.

CHAPTER III

BIOREACTOR APPLICATION TO PLANT TISSUE CULTURE

Bioreactor Overview

As the name implies, bioreactors are vessels in which a biological reaction is carried out. Where the term 'bioreactor' usually conjures up images of complex vessels with instrumentation, the generalized concept of a bioreactor extends to a simple petri dish. In the case of plant tissue culture, the need for such a contained culture system is the result of growing plant tissues on nutrient media that would support the prolific growth of contaminants; therefore, a major goal is to achieve asepsis and exclude other organisms. In addition to providing the ability to study an isolated monoculture (as with microbial cultures for example), the plant tissue culture environment allows the study of isolated tissues, such as shoots, roots or even de-differentiated suspensions of plant cells. Different types of tissues have different environmental requirements which can be accommodated by altering the bioreactor configuration. In this thesis, the goal is to develop bioreactors for the propagation of plants, and therefore the requirements of the bioreactor can be different for different stages of plant development.

A bioreactor system provides the opportunity to control the environment experienced by the plant cells including responses to temperature, light, salinity, cold and heat. In addition, the in-vitro environment allows the study of complex interactions under controlled conditions. As an example, the first honors thesis in Curtis laboratory examined the interaction of phosphate availability on the defense response of plant roots exposed to plant fungal pathogen extracts[16]. The degree of complexity of the 'bioreactor,' therefore, depends upon the specific experimental goals. Ironically, the need to provide the aseptic environment often limits the amount of

complexity that is introduced for fear of contamination. As a result, the largely ‘closed’ environment of plant tissue culture bioreactor vessels often leads to a lack of control of the tissue culture environment that is not appreciated. First, it should be understood that plants grown in tissue culture are almost exclusively heterotrophic – meaning they eat sugar and require oxygen to consume that sugar. This is often a point of confusion, since plant growth is typically associated with photosynthesis. While a goal of this thesis is to enable photoautotrophic growth of plant tissues in bioreactors[17], this represents an extremely small subset of how these plant tissue culture bioreactors are used. A typical plant tissue culture medium contains 20-30 grams per liter of sucrose[18–20]. Noting that biomass yield is roughly 50%, this means that approximately 15g sucrose / liter will be respired to CO_2. Since a typical culture vessel has a volume of 200 mL, with 50 mL of liquid volume, 0.75 g sucrose must be consumed in the vessel. Based on the simple stoichiometry of sucrose combustion, this means that approximately 0.21 grams of oxygen must be consumed in the reactor vessel. An application of the ideal gas law indicates that 22 mmol of gas are present in the culture vessel. The atmosphere is composed of only 21% oxygen, there are only 0.03 g of oxygen present in the vessel for consumption. As a result, at least six additional volumes of air must diffuse into the reactor vessel simply to account for the respiration of the plants. Based on empirical observation, plant tissues are capable of growing to this maximum yield in such a ‘closed’ reactor vessel, thus the reactor must have significant exchange with the air. Clearly this must take place very slowly, and is accomplished without compromising the sterility of the contents within the vessel. Noting that the respiration quotient for oxygen use is roughly 1:1 with the production of CO_2, this gas exchange must also accommodate the removal of this waste product as well.

By applying this type of simple engineering analysis to plant tissue culture ‘bioreactors’ the Curtis lab has provided a much needed perspective of the true environmental conditions that are experienced in plant tissue culture systems. Understanding the evolution of CO_2 within a

shake flask, and the exposure of this gas to reduce stress by inhibiting the hormone action of ethylene, is the basis of the recommended operational strategy to provide supplemental CO_2 during the initial phases of bioreactor operation where sparging of air through the system would otherwise remove the CO_2[21]. More issues associated with scale-up are discussed below with a focus on relevance to propagation bioreactors after a review of much simpler plant tissue culture bioreactor systems.

Simple Plant Tissue Culture Vessels

The Gyratory Shake Flask: While many may not think of the simple shake flask as a bioreactor it is clearly the most prevalently used system for a wide variety of microbial and tissue culture applications. It provides the essence of a sterile batch culture containing dissolved nutrient media with mixing for suspension and to facilitate gas exchange. For the application of plant tissue culture, the gyratory motion can be attenuated to accommodate 'shear sensitivity'. The complexity of a shake flask is often overlooked, as the depth affects both mixing and mass transfer, as does the distance of the stroke of the shaker. As indicated above, the consumption of oxygen and production of carbon dioxide in a gyratory shake flask require that a significant degree of gas exchange be possible. Factors as simple as how firmly one seals the aluminum covering of such a culture can cause observable changes in the culture's growth.

Figure 3 – BMA roots grown in a gyratory shake flask.

Figure 4 – Watermelon organogenic tissue growing in a Magenta GA7 vessel.

GA7: The clear polycarbonate (autoclavable) plastic box (sometimes called a 'Magenta' after the company that manufactures it) and associated double-rimmed lid are a mainstay of many tissue culture laboratories for maintaining plant tissues on an agar-solidified media. Unlike the shaker flask, the application is largely non-submerged culture, so that a considerable amount of the gas exchange can take place between the plant and the gas phase within the vessel. None-the-less, there still must be some gas exchange to accommodate the overall consumption of sugar from the media. Often, however, the amount of tissue that grows in differentiated form, and the rate at which it grows, is much lower than would be experienced for a plant cell suspension; therefore, the requirements for aseptic gas exchange are considerably lower. It is worth noting, that various 'gas permeable' tops are also available for the GA7 to improve gas exchange if needed. As an illustration of the modification of these simple batch vessels to be utilized as different bioreactor modes, an undergraduate research project fabricated air-lift, trickle-bed and inclined plane 'bioreactors' out of the basic GA7 polycarbonate box to study different strategies for the scale up of plant root cultures[22].

Disposable culture containers: There has been a transition to disposability in tissue culture environments to reduce need for cleaning and autoclaving. This has led to the proliferation of a wide variety of gamma-irradiated disposable plant tissue culturing vessels that are single use, non-autoclavable plastic. This concept of avoiding cleaning and low cost use of plastic materials is also incorporated into our temporary immersion bioreactor design for plant propagation.

Bioreactor Scaleup of Plant Tissue Culture.

From an engineering perspective, a goal of particular interest for plant tissue culture bioreactors is the scaleup of these systems. For plant cell suspensions, there was historically significant discussion of problems of 'shear sensitivity'; however, the reality is that these problems are readily overcome by utilizing relatively standard stirred tank or air-lift bioreactor systems[23,24]. In fact, plant cell suspensions were grown at pilot scale as early as 1960[25], and full scale production of taxol has been taking place in 70,000 liter stirred tank systems for more than a decade. The most recent research in the Curtis laboratory involved growing up cells expressing pathogenic *E. coli* antigen in a 50-L stirred tank for use as a vaccine in cattle. This work emphasizes that while there are special considerations for dealing with the relatively large size of plant cell aggregates (sedimentation, port-plugging etc.), the scale up of undifferentiated plant suspensions in bioreactors is relatively straight-forward[26].

The scale up of differentiated plant tissues such as root cultures is much more difficult but this is also amenable to engineering principles[27,28]. For the scale up of somatic embryos, the approach is dependent on the degree of differentiation. For embryonoic suspensor masses, the Curtis lab was successful at implementing both a wave bioreactor system, as well as a 'roller carboy' to allow for initiating the culture from extremely small volumes with fed batch accumulation of biomass[14]. This work with somatic embryos was limited to very early stage amplification, with subsequent development on agar plates. It is hoped that the temporary immersion bioreactor system of this work will be able to be implemented for extended somatic embryo development, and possibly maturation and 'hardening' to allow for more successful transfer to greenhouse conditions.

CHAPTER IV

TEMPORARY IMMERSION GENERAL DESIGN PRINCIPLES

Temporary Immersion Bioreactor Basics

While simple cell suspensions can be grown in conditions similar to bacteria, differentiated tissues make this much more difficult. By definition, the formation of embryos or meristems is defined by a clear 'polarity' of the plant, where there is an 'up' and a 'down'. This is relevant at the macro-scale in terms of gravity, but also at the biochemical scale in terms of the maintenance of chemical gradients. As a result, the free-floating and tumbling of a suspension system is not advantageous. Similarly, a well-mixed nutrient environment may also not be beneficial towards the maintenance of gradients. In a temporary immersion bioreactor, the tissue is only briefly subjected to suspension, and in the case of relatively high density propagation, the tissue can become relatively fixed in place in a typical plant orientation. This was particularly evident in work with propagation of hosta plantlets carried out in the Curtis Lab in the 'rocker' bioreactor noted below.

While it is relatively easy to avoid 'shear damage' for plant cell suspensions using low-shear impellers and air-lift bioreactors, this becomes increasingly difficult as the size of the tissue gets larger. The theoretical basis for cell damage in a bioreactor is often discussed in terms of the turbulent eddy length, which is a function of the power input per unit volume[23]. Stated simply, the more power that goes into a bioreactor by agitation or sparging of air, the smaller the flow patterns become and the greater the likelihood of damaging larger tissues. In the case of propagated tissues, the tissue size is so large, that such a turbulent flow analysis is not as meaningful, though the concept of keeping the energy within the system to a minimum is still

valid. Even the bubbling (sparging) of air can cause damage; therefore, by operating the TIB very infrequently and allowing only a gentile ebb-and-flow of nutrient media, tissue damage is minimized.

Hyperhydricity is a phenomenon of 'water-logging' in which the plant tissue can take on a glassy appearance and is usually associated with abnormal phenotypes[29]. Temporary immersion bioreactors have been particularly useful in preventing the development of hyperhydricity in liquid culture[30]. It should not be overly surprising that areal plant tissues prefer to not be submerged, and a temporary immersion provides the needed exposure to media nutrients on an intermittent basis.

An important engineering observation contributed to the analysis of plant tissues in liquid culture was the somewhat surprising limitations to oxygen mass transfer that result from the large size of the plant tissue[7]. This mass transfer limitation is quite different from the typical gas-liquid oxygen mass transfer limitation observed in microbial culture, and takes place at the solid-liquid boundary layer. As a result of the need to transport the oxygen needed throughout the tissue mass through the surface, the flux requirements for oxygen can be quite high, even for what is a relatively slow growing tissue. This has been analyzed in detail for somatic embryos, and experimentally assessed in germinating seeds that experienced oxygen deprivation and associated activation of an alcohol-inducible promoter[8]. With the assistance of the present author, this supply and demand analysis was extended for root tissue culture and watermelon shoot cultures by in a recent honors thesis[31]. Rapid growing meristems can readily become oxygen limited. By minimizing the liquid boundary through which gasses must move, a TIB can help to alleviate this mass transfer limitation.

Commercial TIB Systems:

There are a wide variety of micropropagation bioreactors that have been developed around the concept of temporary immersion[32], and coffee plant propagation in particular is now highly developed[33]. While the concept of intermittent exposure to media is common to all of them, the implementation approaches are creatively diverse. A recent analysis has examined the economic advantages of utilizing mechanized systems associated with plant propagation[34].

One of the simplest methods to achieve temporary immersion is by placing tissue in a long vessel that sits on a platform that rocks the media from one end of the vessel to the other. This system was commercialized as the Liquid Lab Vessel ® otherwise known as "The Rocker" in which the vessels were blow-molded polycarbonate and utilized canning ring threads for the opening[35]. The liquid lab system has been replaced by simple plastic bags as well. There are variations on the concept of using a rocking motion to provide for intermittent media contacting. A recent rendition (the BioMINT ®) is based on a cylindrical design with a partition between the two sections to keep the plant tissues on only one side of the reactor[36].

An obvious way to provide temporary immersion is to pump the media in and out of the plant culturing vessel with a mechanical pump. This is alternatively referred to as an ebb-and-flow bioreactor (EBFR), and is more useful in situations where the flow resistance is quite high. Experimental work in the Curtis lab with root cultures utilized such an ebb-and-flow system[37], though trickle bed systems were superior. EFBR has been reported for microprogation of pineapple[38]. The disadvantage of utilizing mechanical pumping to move liquid between reactors lies in both the cost of the pumping and the reliability of peristaltic pump tubing (required for asepsis) over extended periods of time.

Alternatively, the media can be pushed between culture vessel and reservoir using pneumatic gas pressure. This can be accomplished either using a three-way valve, or placing the

culture vessel higher than the reservoir, and letting gravity drain the media back down into the vessel. A tremendous variety of configurations have been tested, with volumes of 10-L or greater[39]. One of the earliest commercialized versions for lab scale work included the reservoir integrated within the same vessel called the RITA®[40], and the introduction of air would push the media into the growth chamber followed by gravity drainage back into the reservoir compartment.

Proposed Temporary Immersion Bioreactor Design

The primary goal of this work is to develop a scalable, economic plant propagation bioreactor, primarily by reducing the costs associated with its construction. As a temporary immersion bioreactor, one of the most critical components of the design is the capability handle liquids within the system. Where many typical TIB designs utilize pneumatic gas flows and/or switching solenoids to force liquid from a standing reservoir into the reactor vessel and back, our design seeks to utilize gravity as a driving force for the flow in both directions utilizing a liquid siphon. When the reservoir is raised above the reactor vessel, the tissue is flooded with media. This is reversed by lowering the reservoir below the reactor vessel, causing pressure equilibration to suck the media from the reactor vessel to achieve a full cycle.

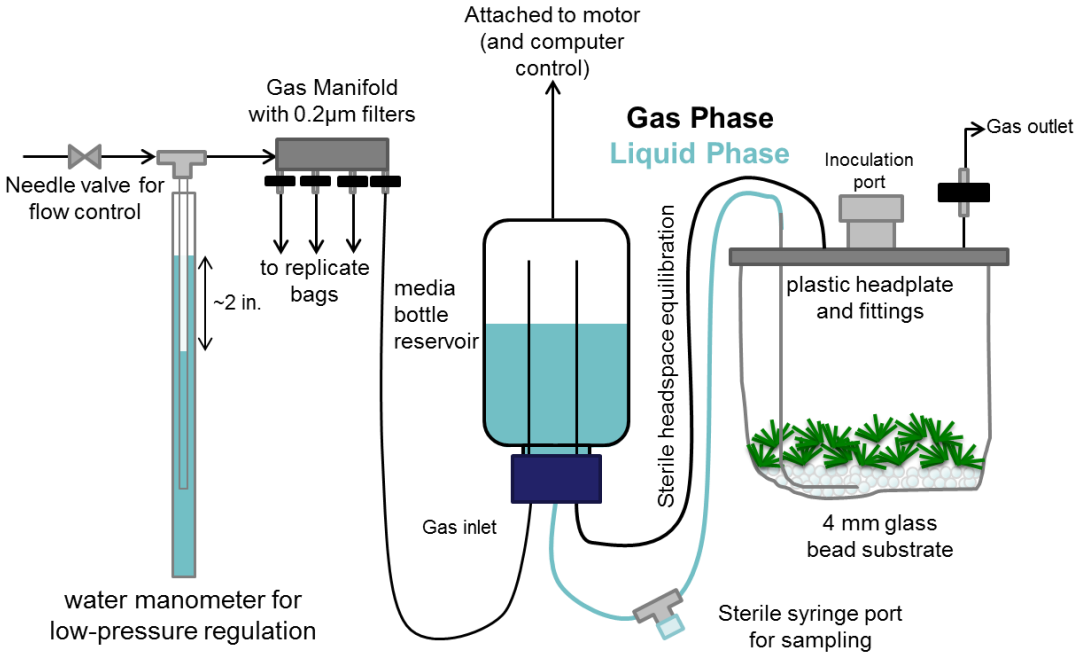

Figure 5 – Design schematic for proposed temporary immersion bioreactor.

This leads to several advantages over contemporary systems. Primarily, the volume requirement for compressed gas is reduced. This is very relevant when gas mix is used because even at a very low flow of 50mL/min this corresponds to 1800 L per month. The Curtis Laboratory has significant experience in the development of low cost bioreactors by foregoing pressure rated vessels to make use of disposable polypropylene bags[41]. These bags are affixed to a simple polycarbonate headplate to allow inoculation, gas and liquid feeds and outlets, and monitoring equipment. All of the materials used in the construction of these reactors are autoclavable, allowing sterile operation within a closed system without using pressure-rated vessels. In addition, because the reactors designed operate under ambient conditions, they can be easily moved from a growth chamber to a sterile laminar flow hood environment to allow manipulations of the tissue grown, as well as the serial replacement of liquid media to facilitate the plant regeneration and hardening processes.

An additional advantage of this TIB design decoupling the liquid and gas flows through the system is the fact that it becomes economically feasible to supplement the gas phase headspace of reactors. In a standard TIB reactor, a significant portion of this costly supplementation would be vented during the cycling process (see calculation on previous page); however, our design allows the constant introduction of a very low gas flow rate to the reactor. Despite the fact that a major advantage of a TIB system is the mitigation of oxygen limitation in growing tissues, it is still expected that the primary growth limitation in the reactor will be oxygen transport. By supplementing the reactor with higher concentrations of oxygen, the mass transfer into the liquid boundary on the plant tissue is enhanced, allowing a further advantage to fast-growing tissues. It has also been observed that CO_2 has an important role in the inhibition of ethylene action. Supplementing CO_2 to reactor runs has already been shown to reduce the stress of growing plants, and could significantly enhance the productivity of a temporary immersion bioreactor. In addition, CO_2 supplementation could allow the production of a fully photoautotrophic bioreactor system[42]. This is described more fully in the Future Work section.

CHAPTER V

DESIGN OF LIQUID HANDLING

Mechanical Support Systems

One of the primary innovations of the TIB system designed in this project over current commercial systems is the attempt to decouple gas and liquid flows by utilizing gravity as a driving force for the cycling of liquid media. By placing a drain at the bottom the liquid reservoir, the media will naturally drain to reach equilibrium of liquid level between the reservoir and reactor vessel. So long as a siphon is maintained, media can be transferred from the reservoir to the reactor vessel and back simply by adjusting the relative height between the two.

It was determined that the liquid reservoirs should be raised and lowered for a variety of reasons. Primarily, this minimizes the motion of the growing plant material itself. Unexpected errors ranging from power failure to computer glitches could result in the potential impact of the moving vessels with the ground, walls, or other vessels. By maintaining the plant tissue in a stable and stationary environment, it is protected from physical damage. As will be shown later, avoiding the perturbation of reactor bags is also an important consideration for minimizing contamination risk at low gas flow rates. Another major consideration is the power requirement to hold a vessel at a high, rather than low position. The vast majority of the time, the media reservoirs will be in the drained position below the reactor vessels. By selecting to move the reservoirs, the system can be designed such that this base position requires no power, and motors can be powered off until they are needed. It should also be noted that we envision manual cycling of reservoirs as large groups in a greenhouse (photoautotrophic) setting, where moving the reservoirs makes most sense.

Because the reactor vessels remain stationary, a rigid PVC support was built to hold the headplates in place as the plastic bags hanged beneath them. This stand was tall enough that the upside down media bottles could be lowered sufficiently to allow a siphon to drain the full contents of the reactor vessel. Small sections of the PVC pipes were cut out to allow the headplates could rest stably on the support.

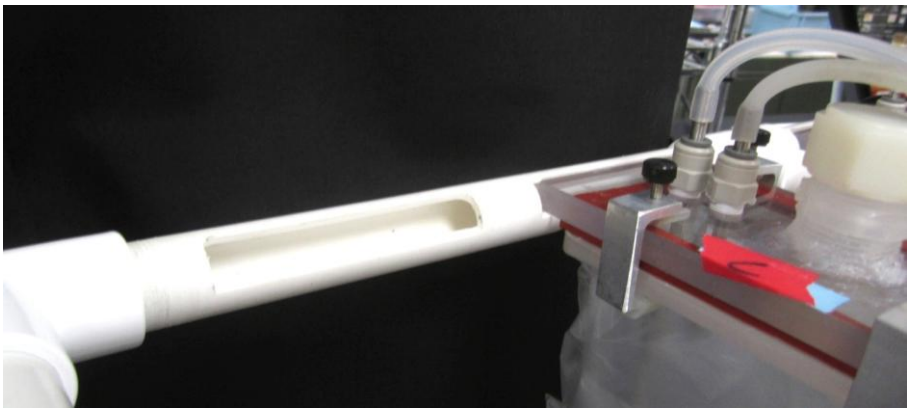

Figure 6 – PVC reactor support showing a fully assembled reactor headplate seated in slots.

To facilitate the raising and lowering of media bottle reservoirs a support system was designed to simultaneously cycle four media bottles at a time. These were held in place upside down by ¼ inch polypropylene sheeting with 2.25 inch holes cut, allowing the bottle to rest on only its neck. Additional ¼ inch slits were cut to allow the bottles to be slid in and out of the rig with sterile tubing connections, while an aluminum corner bar was used to prevent the support from flexing under the weight of the reservoirs. This assembly stabilized by hanging from an aluminum T-bar.

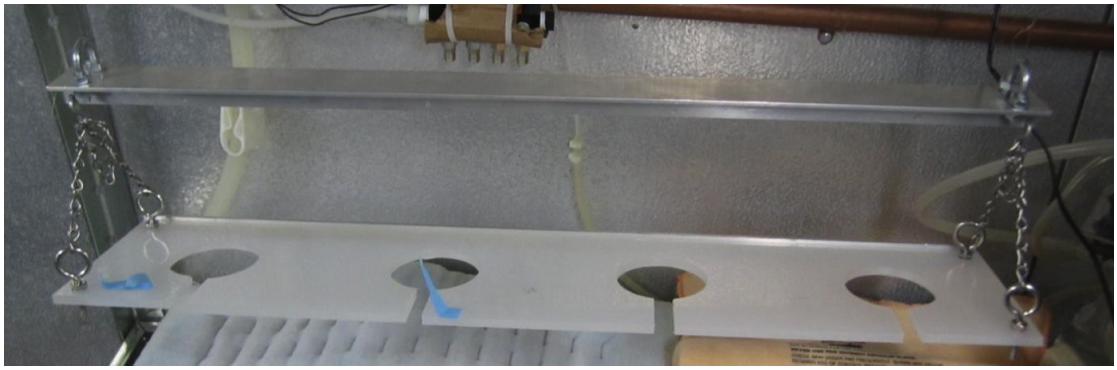

Figure 7 – Reservoir support facilitates the hanging of four standard media bottles.

The stepper motor selected for this application is described in more detail in Chapter VII Instrumentation. This motor was attached to a delrin pulley, allowing it to act as a simple winch. To decrease the torque load on the system, a four pulley block and tackle system (double tackle) was devised to give a fourfold mechanical advantage to allow the motor to raise the reservoirs. While this design was effective, its complexity and space requirements made it somewhat unwieldy in a growth chamber where space is limited. As a result, the design was modified to make use of a 2:1 gearing ratio, allowing a significant reduction in the required vertical space for the reactor to run.

Figure 8 – Methods used to provide additional mechanical advantage to the stepper motor. (Left) A double tackle. (Right) A 2:1 gearing ratio.

In both cases, the mechanically advantaged line from the motor is spooled around a stacked pulley staged below the motor. The additional pulleys act as anchors for the spooling of two secondary lines, as detailed in Figure 9. When tension is applied to this center pulley by the motor, it causes its rotation by unspooling the innermost pulley. This causes the spooling of the secondary lines that in turn attach to the reservoir assembly, causing it to rise from its resting position.

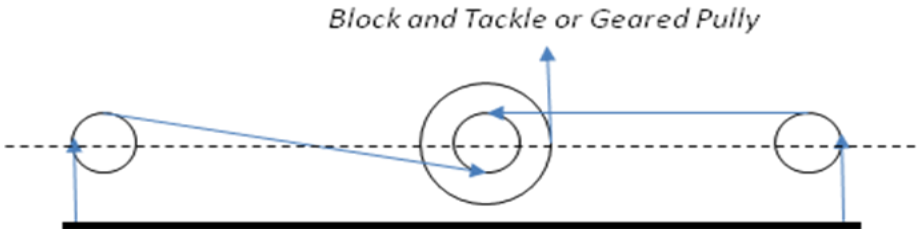

Figure 9 – Diagram of center pull spooling mechanism raising both sides of the reservoir support evenly.

Once the reservoirs are lifted such that their liquid level is higher than the reactor headplate, they begin to siphon until liquid levels are equal. Care must be taken to ensure that the reservoirs are not raised so high that the reservoirs drain completely, as the siphon cannot be reinitiated from the reactor vessel side without applying significant additional pressure to the system, causing potential damage to the plastic bag in the process.

A final mechanical support that must be considered in a TIB is a matrix upon which the tissue grows. This substrate must be small enough to prevent tissue from washing through and becoming trapped in the substrate or media reservoir, while being large enough to prevent tissues from growing around and incorporating the substrate in their weight. A support matrix of 4 mm glass beads was added to each reactor vessel to this end.

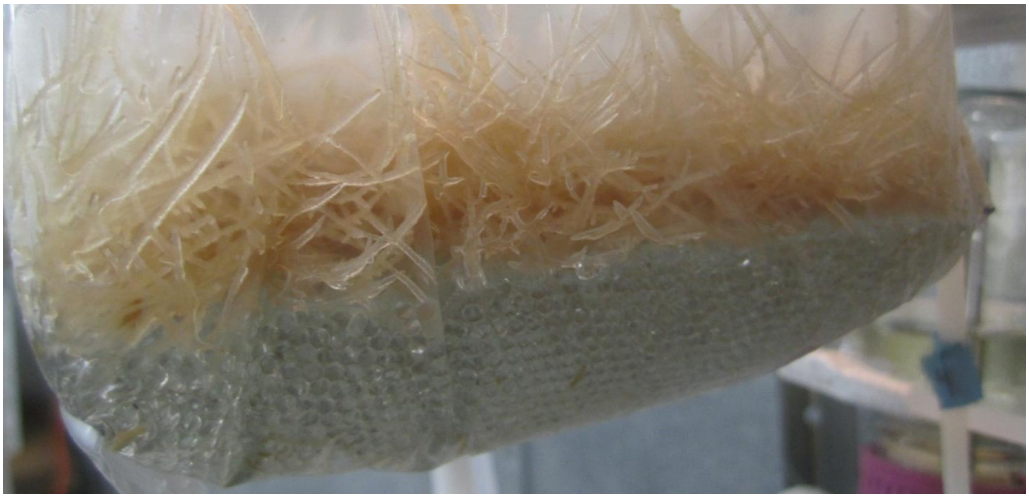

Figure 10 – *Nicotiana benthamiana* hairy roots growing over 4mm glass bead substrate.

Liquid Tubulation

All tubing connections in the sterilizable reactor itself were made with 3/8 inch silicon tubing. A 1.5 inch long piece of stainless steel tubing was pushed flush with the base of the silicon stopper in the media bottle reservoirs, such that the reservoir could drain completely when hung upside down. This was attached directly to the reactor vessel headplate to allow liquid transfer between the two vessels.

The media enters the reactor through a stainless steel liquid distributor. To facilitate the distribution of media into the reactor vessel and prevent the glass bead substrate from preventing liquid flow, a simple screen was fabricated at the end of the liquid distributor by cutting 2 mm slits from the end of the stainless steel tube and bending the remaining material inward into a rounded tip. This replacedan initial 'L' shaped design with slits that was found to accumulate gas bubbles which could substantially alter flow rates, or even occasionally break the

Figure 11 – Liquid distributor rounded to prevent clogging by glass beads and puncture of plastic bag.

siphon[43]. This piece of tubing was fitted through a ¼ inch NPT polypropylene bulkhead in the reactor headplate.

It was noted throughout testing that a significant advantage of liquid shake flask cultures was the capability to easily perform sterile manipulations during growth. It has been shown that media sampling can be used to monitor the growth of plant tissue culture, thus it desirable to have that capability as the prototype reactors are evaluated. To that end, a polypropylene leur-lock 'T' was added to the liquid transfer line to incorporate an in-line leur-lock injection port, allowing the sterile media sampling for purposes ranging from contamination testing to obtaining growth curves. There is some concern that this experimental convenience introduces contamination risk, which should be tested offline for reliability.

Figure 12 – In-line sterile inoculation port for liquid sampling.

CHAPTER VI

DESIGN OF GAS HANDLING

Gas Supply

To achieve the control of the gas-phase headspace inside the reactor, the gas sources needed first to be stepped down from their high pressure storage sources. Compressed air was generated by an in-house compression system, maintaining a 60 psi source. This source for air controls of the system was stepped down through an in-line pressure regulator to 15 psi. For use in gas headspace experimentation, however, gas mixture tanks at 2000 psi were used, allowing the utilization of both high CO_2 (5%), and high oxygen (40%) mixtures. Once again, pressure regulators were set such that a 15 psi source could be achieved.

From this 15 psi gas source, the pressure was stepped down once more utilizing inexpensive needle valves to achieve the desired flow rate through the reactor. The very small flow rates in the system were monitored utilizing brooks mass flow meters for our specific implementation, utilizing both metal and glass bead floats capable of registering between 5 and 150 mL gas flow per second. It was later determined that these gas flow meters were relatively imprecise at low flow rates, and that the flow rate through the system could be readily correlated to the pressure drop directly preceding the gas manifold. A very simple water manometer was used as a secondary indicator of the gas flow through the system. This proved to be not only more accurate a measure to maintain positive pressure in the reactors, but also represents a significantly lower capital cost in the production of a bioreactor.

To prevent the reservoirs from drying out during operation leading to variations in the liquid concentrations of media components, each gas input was passed through a humidification

train prior to introduction to the system. The train consisted of four side-arm flasks in which gas was bubbled through small ceramic aquarium spargers to maximize mass contacting. The sparger was left out of the last flask in the series to facilitate the counting of bubbles as an alternative means for verifying the flow rate through the system. In the past, the Curtis Lab has utilized industrial steel fittings designed for use in high-pressure systems to achieve this goal, however, both the low required flow rate and low pressure of the delivered gas allowed a much less complex system to be designed. A series of 4 tapered holes were cut in both a 3/16" polypropylene sheet, while smaller holes fitting only the stainless steel outlet of the stopper were cut into a 1/16" polypropylene sheet, with holes tapped in both for screws. The necks of these flasks could then be placed through the headplate fitted with a tapered rubber shim (Grainger, 1DPK5) so that the neck cannot fit back through the headplate, and the second sheet placed over the stoppers and tightened down to greatly stabilize the daisy-chained side arm flasks.

Figure 13 – Humidification train composed of four sidearm flasks, used to saturate incoming air.

Before entering each reactor, the humidified gas had to be split such that each reactor would receive close to the same gas flowrate. This was achieved by using an aluminum gas manifold (McMaster-Carr, 5469K113) fitted with ¼ inch leur-lock fittings (McMaster-Carr, 51465K153). For gas flow rates to be equal through a manifold, it is necessary that the resistances between the manifold and gas purge from the reactor system be the same. Minor variations between each reactor setup, however, will cause differences in these resistances. In the attempt to mitigate the effect of the reactor system on gas flow, very high pressure drop teflon filters (Restek, 0.22 microns, 4 mm diameter, PTFE) were attached at the leur-lock fitting, reducing the impart of smaller resistances to flow beyond this filter on the flow rate through each system. An additional advantage of utilizing these filters is that they could be used to separate the sterile and nonsterile environments of the gas supply setup.

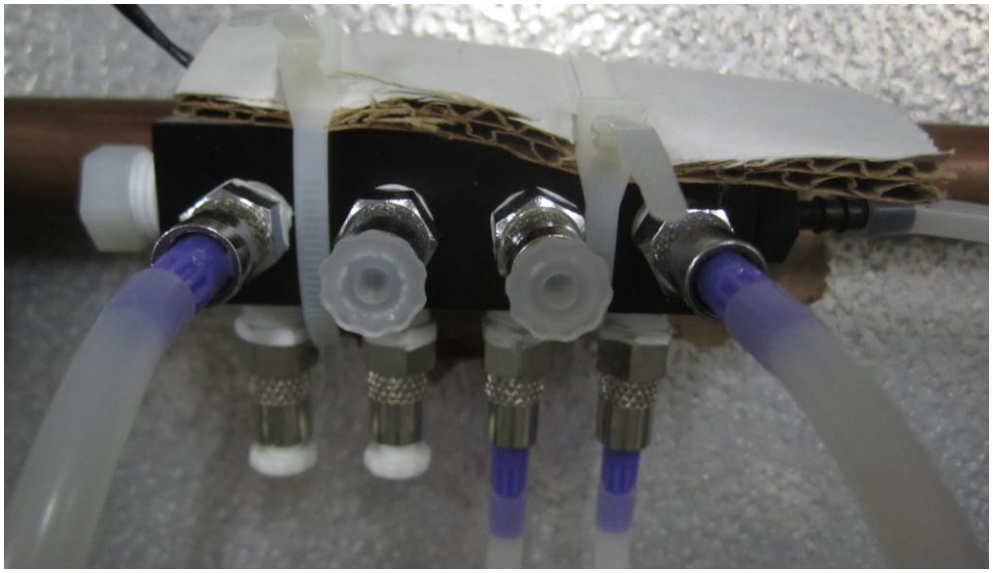

Figure 14 – Gas manifold supplying air to four separate bioreactors.

It was rapidly noted that these very small area filters had a tendency to become plugged with condensation when attached directly to the humidified gas feed. This issue was rectified by attaching low wattage resistive heating elements directly to the gas manifolds (15V, 0.4A, McMaster-Carr, 35765K265). The slight increase in temperature at the manifold decreases the relative humidity of feed gas, preventing condensation in the filters from interfering with gas flow.

Vessel Gas Configuration

As with the liquid tubing, all gas connections within the reactor system were made with 3/16 inch silicon tubing. The 0.22 micron filters were attached to a gas inlet line entering directly into the media bottle reservoir with a sufficiently long (14 cm for our application) stainless steel tubing spacer to prevent liquid from entering this line. To allow the proper headspace equilibration required during siphoning, and to allow the constant flow through of supplemented

gases, a headspace equilibration line was added running from a second stainless steel tubing spacer in the media bottle reservoir into the headspace of the plastic bag. A gas vent was also included in the headplate with a somewhat larger filter to keep the reactor system sterile while maintaining back-pressure in the reactor. By using a 0.22 micron filter with a 13 mm diameter, a low positive pressure could be maintained within the plastic bags at gas flow rates in the range of mL/min, preventing the contamination of the run due to imperfections in the seal achieved between the hanging plastic bag and headplate.

Initial attempts to run these reactors were made with much larger filters in place. At the gas manifold, the 13 mm diameter filters were used, while a 43 mm filter was used to maintain sterility of the vent. It was found that using this configuration, however, all attempts to run the reactor for extended periods of time became contaminated. Simple tests performed by simply running air through the media reservoirs showed that contamination was not a result of the gas feed to the system; however, it was found that the running TIB became contaminated without inoculation with plant tissue. To evaluate the pressure drops experienced within the reactor vessel during cycling, a series of tests were performed using integrated circuitry pressure sensors that allowed the precise pressure to be monitored.

A time course of the pressure in the reactor at a gas flow rate of 10 mL/min can be seen Figure 15. When the gas handling lines are attached to two rigid media bottles in the same configuration as describe above, liquid cycling has very little effect on the pressure in the the system, and the low gas flow rate used is sufficient to maintain a positive pressure at all times. When utilizing the plastic bag, however, it was seen that there is a significant decrease in the pressure as the media flows into the reactor vessel. This drop in pressure was very surprising since the interconnected headspace of the culture bag and reservoir should allow equilibration. Any time the pressure in the reactor is lower than atmospheric, the possibility arises for contaminating organisms to be introduced to the reactor through imperfect seals in the system.

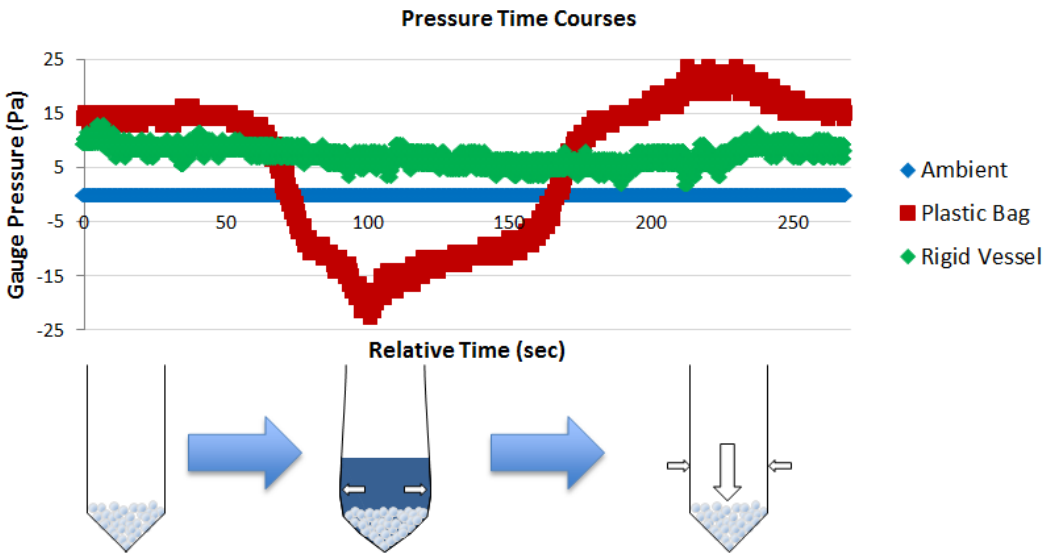

Figure 15 – (Top) Time courses of pressure inside a TIB during liquid cycling. (Bottom) Physical explanation of pressure variations seen in Plastic Bag reactor cycling relative to time above.

The decrease in pressure was found to be a result of the non-rigid plastic reactor vessel. As media fills the plastic bag, it was observed to expand slightly, increasing the total volume of the system thus decreasing the pressure as illustrated Figure 15. The simplest way to rectify this problem is to increase the gas flow rate through the system such that the overall pressure is greater. Not only does the increased pressure prevent the expansion effect from dropping the overall pressure in the system below ambient, but it also has a tendency to inflate the plastic bag such that this effect is not as great.

Pressure time courses were performed at a variety of gas flow rates to evaluate how these factors affect each other. It was noted early in testing that the pressure as measured in the liquid manometer prior to the gas manifold was a better indicator of the behavior of individual bags than the total flow rate of gas delivered, and could be measured more accurately than the gas flow rate through the system. After each change in flow rate, the system was allowed to reach pressure equilibrium before running through three liquid cycles. The top left pane of Figure 16 shows the

equilibrium pressure of the reactor vessel as a function of manifold pressure, showing the expected linear profile with higher bag pressures at higher flow rates. By measuring the difference between the high and low pressures experienced in the system, the effect of bag pressurization could also be evaluated, also shown in top right pane. The change in pressure during a cycle tended to be smaller at higher flow rates. Combining these effects, the minimum gauge bag pressure observed over the course of liquid cycling was correlated with the manifold supply pressure using the original larger filter setup. As expected the relationship is linear, and shows that setting a pre-manifold pressure of approximately 4 cm of water should be sufficient to prevent contamination of the system. Unfortunately, this manifold pressure drop was achieved by using a flow rate of approximately 35 mL/min, nearly five times higher than desired based on the consumption of oxygen in the reactor. Because the intention of this reactor is to supplement the gas phase, excess gas exiting the system represents an economic disadvantage, limiting to feasibility for controlling gas composition in the TIB reactor.

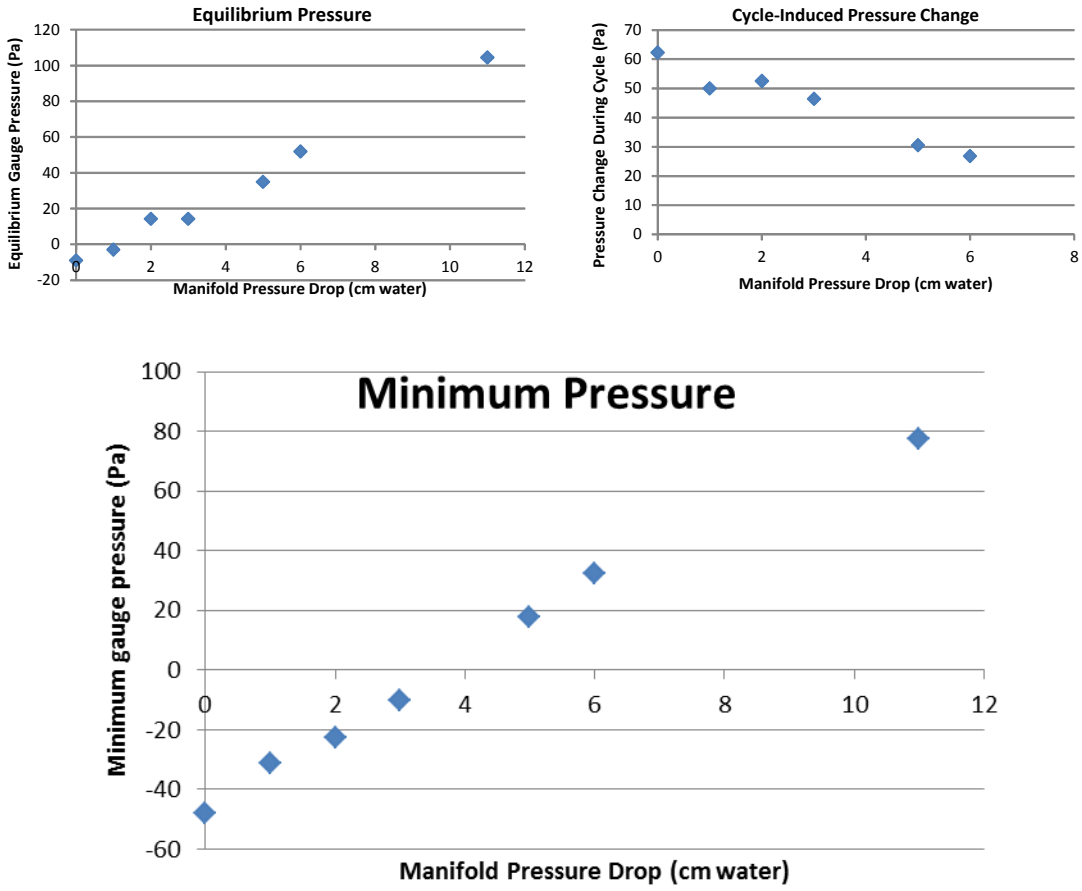

Figure 16 – Trends in plastic bag reactor vessel pressures as a function of pre-manifold pressure drop. (Top Left) Equilibrium Pressure. (Top Right) Pressure change experienced during liquid cycling. (Bottom) Overall minimum pressure experienced in the reactor vessel.

To try to reduce the required flow rate through the system, a series of filter configurations were tested. As expected, smaller filters with a greater pressure drop allowed the pressure within the reactor to build up to a greater degree. Due to the fact that the manifold's function is based on equal pressure drop between reactors, however, it was necessary to maintain a much smaller filter on the manifold inlet than on the reactor vent to prevent flow inconsistencies. The final optimal configuration of a 4 mm filter at the manifold and a 13 mm filter on the vent was arrived at to allow the proper pressurization of the reactor vessels at a flowrate of approximately 10 mL/min/reactor, corresponding to 2 cm on the manometer.

CHAPTER VII

INSTRUMENTATION

Hardware

In this testing environment, a very precise motor was desirable to reproducibly raise and lower the media reservoirs to precisely the same height, hold the reservoirs at this position without slipping, then reversing gently to lower the reactors back to their resting height. To this end, a stepper motor from Anaheim Automation was selected. Stepper motors are driven electromagnetically, rotating shaft through a single toothed "step" by the switching of signals to the motor head. The stepper motor selected completes 200 steps per full rotation. Based on the weight load of four media bottle reservoirs containing up to 200 mL of media each, a motor with a relatively high bipolar torque of 262 oz-in was selected (Part number 23Y204S-LW8). Because of the nature of the stepper motor's electromagnetic driving force, the actual torque that can be exerted is based on the current run through the motor. Simple load testing on a variable power supply showed that the rig was capable of lifting 2 liters of water without slipping when provided with approximately 3.3 A.

In order to make use of the stepper motor, electrical signals must be modulated to each of the electromagnetic coils in the motor. To achieve this, a low cost DIY stepper motor controller circuit was purchased and assembled from Hobby Engineering (Catalog # 12591). The board allows for both standalone operation allowing the onboard signal generation for the speed and direction of motor rotation. Alternatively, the motor controller can be interfaced to a computer to allow more precise control of the rotation. While it would be possible to develop a series of simple hardware timers to achieve the desired reactor cycling, the LabVIEW interface described

below provides a series of advantages ranging from precise tuning of the system to automated mid-run documentation.

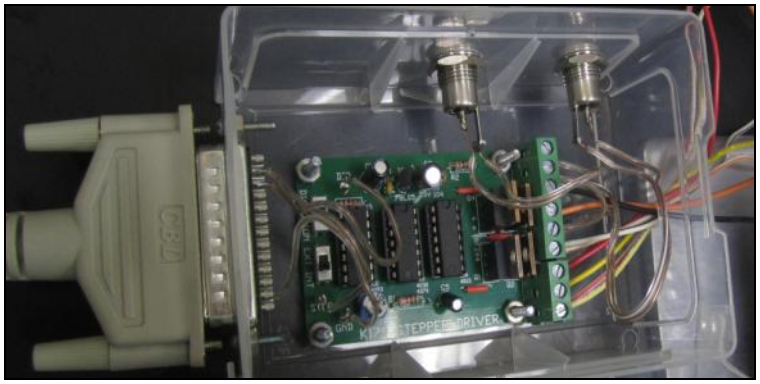

Figure 17 – DIY stepper motor controller with parallel port interface.

Software

The overall organization of the LabVIEW program is relatively straightforward; however, the symbolic language of LabVIEW is very different from traditional programming. Upon initialization, the program resets any booleans or buttons that may have been pressed that could interfere with the proper running of the program. It then enters into four primary loops: the System Time Display Loop, the Push-Button Event Loop, the Motor Control Loop, and the Camera Control Loop. The System Time Display Loop simply queries the system clock and outputs the current time on the front panel. The Push-Button Event Loop allows the program to operate in one of two modes: Automate Control, and Manual Control. In the "Automate Control" mode, the program takes full control of the reactor cycling. Upon the start of each cycle, it raises the reactor fully to the set height, holds the reactor there for the specified wait time, and then lowers it back to the resting position. Variations of the program have been written to perform this every six hours for watermelon organogenic tissue, and hourly for hairy root culture. The Manual

Control mode was designed primarily for troubleshooting and testing purposes. While in this mode, the set height can be modified by user input, and the motor will adjust the height of the reservoirs accordingly. As a result, it is possible to cycle the reactors off-schedule in the case that a reactor failed to siphon correctly.

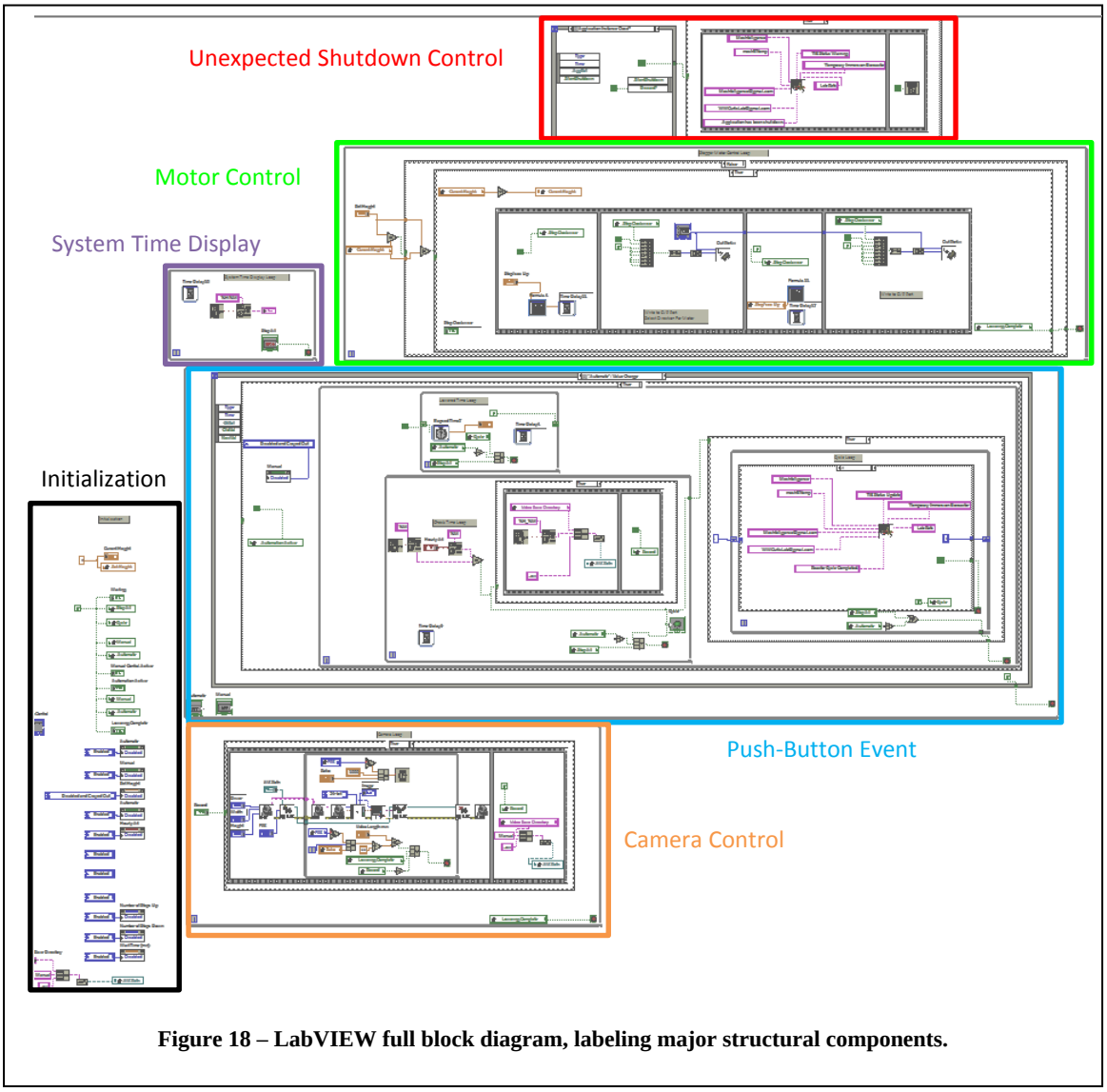

Figure 18 – LabVIEW full block diagram, labeling major structural components.

The front panel of the running virtual interface (VI) has several specifications that must be set, and that vary depending on the precise reactor setup. The “Number of Steps” control defines very precisely how far the stepper motor will rotate before reaching its highest position.

This number is determined by utilizing the manual mode to slowly raise the reservoirs until they are at an appropriate height to properly drain without breaking the siphon completely when empty. Generally, the "Number of Steps Down is set the same value as number of steps up to lower the reactor to the same point. This option was left within the program to allow intervention should the reservoirs fail to drain completely. The "Wait Time (min)" control defines how long, in minutes, the reactor will remain at its highest position before lowering. This is defined by timing approximately how long it takes for the reservoirs to drain completely after being raised to the maximum position.

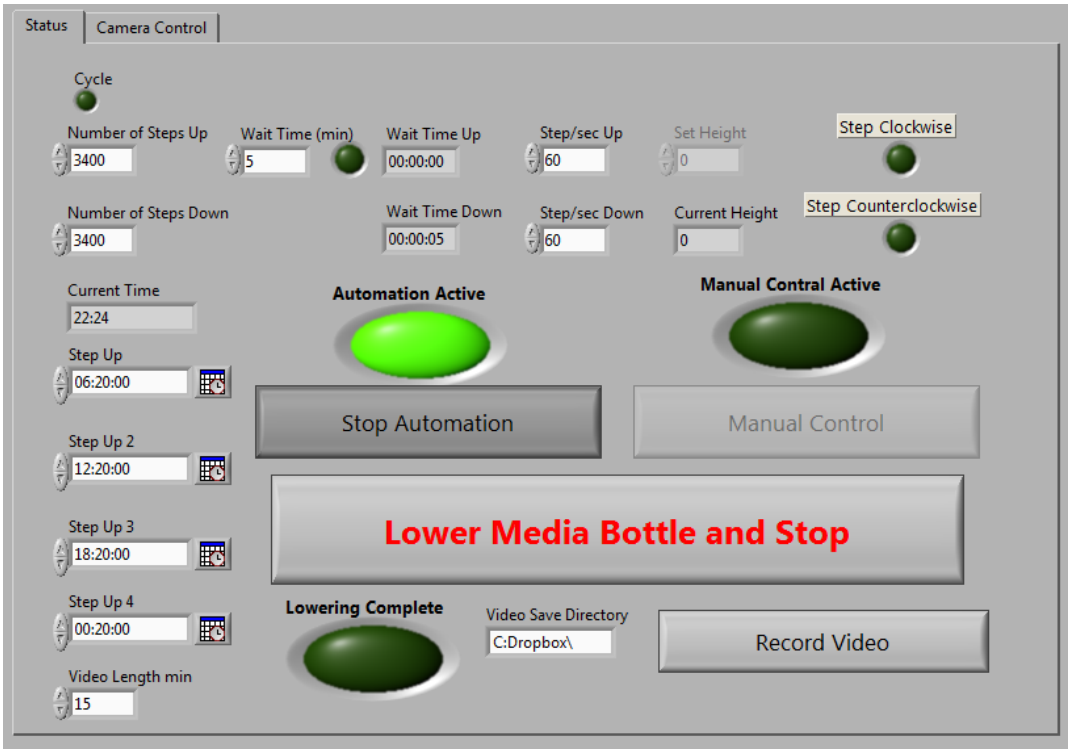

Figure 19 – LabVIEW application front panel during watermelon tissue organogenesis run.

The stepper motor output from the computer to the DIY controller board was transmitted through a parallel (printer) port. The stepper motor controller hardware requires only 2 inputs: a direction, and a signal to step. Using built-in port controllers, the directional input was set to pin 2 of the parallel cable where a high signal corresponds to counterclockwise rotation, and a low

signal corresponds to clockwise (Outputs are all nominal logic, 0 or 5 volts). The step signal was set to pin 3 of the port output. To achieve one step, the program must set this pin from low to high, then back to low again. This was performed using the sequential pane labview data structures. In addition to these two signal lines, and additional ground signal was connected to the grounding pin 26 to ensure the proper communication between these two devices.

The control of the reservoir height was designed to allow both manual and automated control of the system. As such, the control loop shown below checked once per second whether the set height had been changed from the current height. If so, the program enters a secondary loop that sends a pulsing signal at the rate of specified on the front panel to move the stepper motor. Tests showed that the maximum step rate was limited primarily by the processing speed of the computer in use; however, to prevent excessive strain on the fishing line used in the pulley strain and jostling of the reactor, the step rate was set to achieve a velocity of approximately 1 inch every 5 seconds.

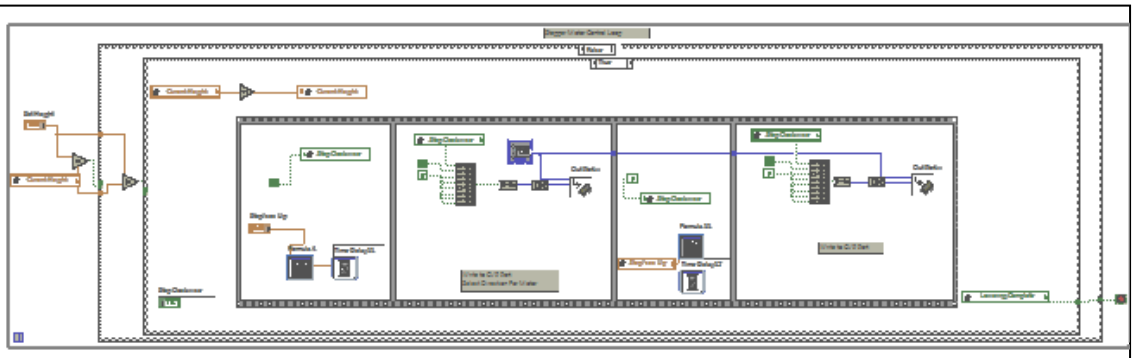

Figure 20 – Motor Control Loop block diagram sample.

One of the major advantages of utilizing the LabVIEW programming environment for the control of the stepper motor in this testing environment is its connectivity with the internet. Taking advantage of the internet connection, a VI was developed to allow the programmatic sending of emails. By utilizing SMS gateways, these emails could be converted into text messages to allow the instant dissemination of critical information regarding the program to as

many individuals as necessary. Initially, this system was utilized primarily to send status reports. At the end of each reactor cycle, a message would be sent to all participants indicating that the program had executed successfully. While performing reactor runs with BMA root cultures cycling every hour, however, it rapidly became clear that receiving hourly text messages 24 hours per day was an unrealistic method of monitoring the program's activity, and the program could stop for hours before the error was noticed. It was noted that the vast majority of failures were caused by unscheduled Windows Updates, thus a script was written to detect the automatic shutdown signal "If Application Session Close?", sending a text message warning before computer fully shut down, as shown in Figure 21. While this system could not account for critical failures of the windows machine, no such failures have occurred since its implementation, and the response time to reset the program after windows updates was significantly improved.

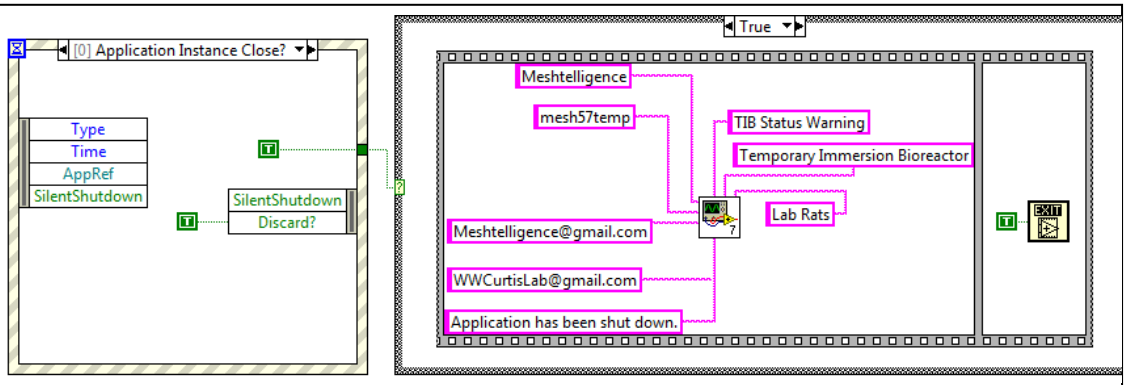

Figure 21 – Unexpected shutdown event handling block diagram sample.

In addition to the ability to send alert messages, internet connectivity also allowed the online monitoring of the reactor. Utilizing a the IMAQ toolset from National Instruments, images from a standard webcam could be captured and manipulated within the LabVIEW program. To allow the verification of proper liquid cycling in the reactor, images are taken every two seconds during a cycle run. These images are sequentially stitched together into a simple AVI video format, creating a rudimentary time lapse video of the reactor run that significantly compresses

the length of a full cycle video. By placing a webcam behind the reactor focused such that it could view both the reactor vessel bags, and the raised reservoirs, it is possible to capture the full extent of liquid cycling in this time lapse video. The program Dropbox was configured to automatically upload and synchronize these video files with an online server, allowing lab members to check the proper operation of the reactors remotely.

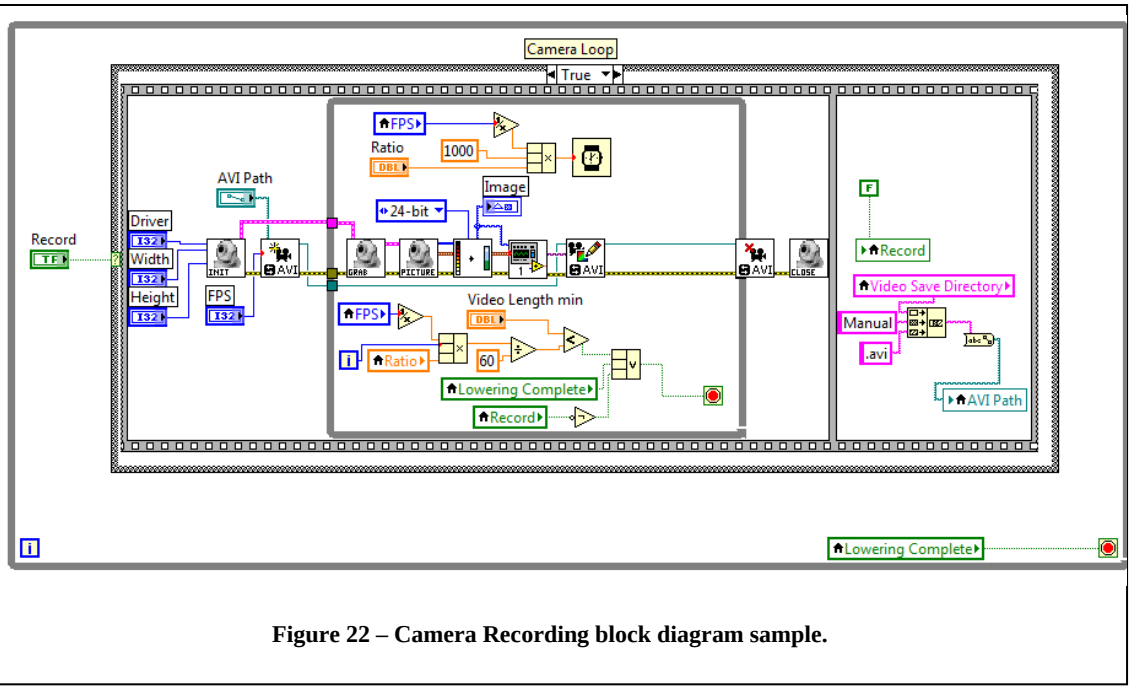

Figure 22 – Camera Recording block diagram sample.

Camera controls can be found on the second tab of the application's user interface. This window serves primarily to give a preview of the image field that will be captured by the camera; however, there are several important controls that affect the quality, and thus size of the captured video. The Width and Height controllers on this tab define the resolution of the image captured by the webcam. The maximum possible resolution settings are defined by the camera itself, however, it was found that utilizing a 640X480 resolution, it was possible to fit all four reactors within the frame of view and reliably observe changes in the liquid levels in both the reactors and reservoirs. The FPS setting defines the playback rate of the video created. Monitors generally operate at a maximum frame rate of approximately 60 Hz, which would correspond to 60 frames

per second, however, there is little need for that seamless a transition between frames. The Ratio control defines how much faster playback will occur than movie capture. It was determined that a video played at 20 times original speed allowed images to be captured quickly enough to produce a relatively seamless video, yet significantly reduced the file size of a recording that could span as long as ten minutes over the course of a cycle. This allows a video generated over 15 minutes to be viewed and evaluated for proper reactor operation in only 45 seconds.

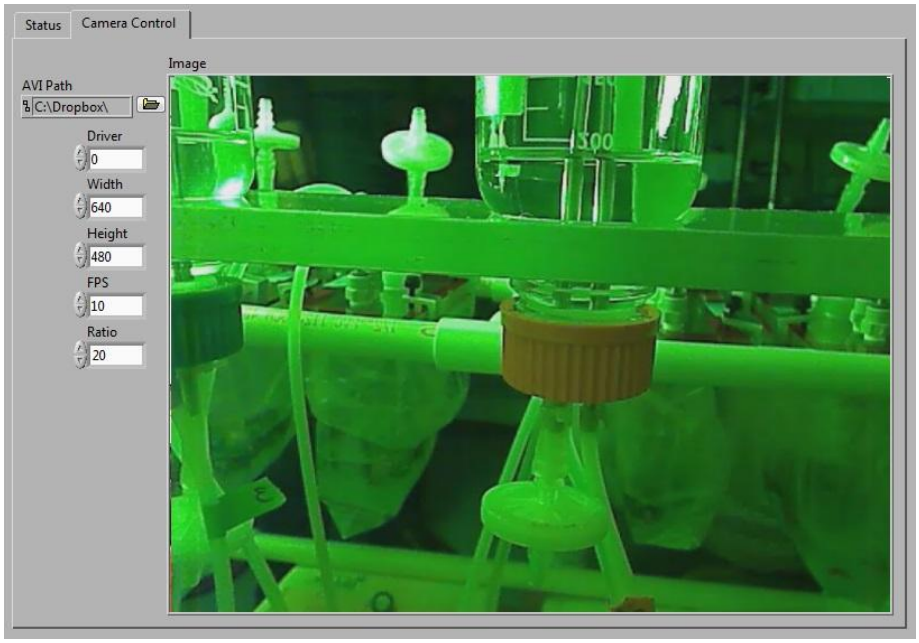

Figure 23 – Camera Control tab showing mid-cycle video capture of watermelon tissue organogenesis run.

Due to the limited photoperiod of typical plant growth, video monitoring would not normally be possible during the night. In addition, it has been shown that bursts of light at different wavelengths can have significant effects on plant growth and development. To attempt to document reactor cycles overnight with as little an effect as possible on the plant species grown, a lamp was configured to turn on and off hourly with the reactor cycle. Considering that the green coloration of plants is a result of their inability to absorb wavelengths in that spectrum, a double layer of green cellophane was added to the light to prevent possible developmental interference.

CHAPTER VIII

CURRENT BEST PRACTICES

Current Bioreactor Configuration

For each particular application of the bioreactor, small modifications are necessary to optimize the reactor environments for a specified plant tissue. The bioreactor is currently undergoing testing for the growth of hairy root cultures. A Metro rack was chosen as the ideal setup environment for both the wireframe shelves that allow tensioned lines to run from one level to the next, and the ease with which shelf levels can be adjusted to optimize the usage of space. The motor is placed on the top of three levels, with the stacked pulley spreader on a shelf six inches below it. A gap of 3 feet is left between the second and third shelf to allow ample room for media bottles to be raised and lowered.

The reactor headplates themselves are are made of ¼" polycarbonate sheeting. Holes are drilled and tapped for two ¼" NPT bulkhead with a ⅜" quick connect fitting (US Plastics, 58155), a ¾" diameter threaded 'close nipple' to serve as an inoculation port, and a final ⅛" NTP to ¼" hose barb for the reactor vent. 8" by 10" 2-mil polypropylene bags were pulled over a 13" by 8" polypropylene rectangular polypropylene ring to hold them open at a constant size, and these were compressed against a 3/16" thick silicon gasket (McMaster-Carr, 9010K841) of the same size on the headplate by small C-clamps cut from an aluminum bar (McMaster-Carr, 1 ¼" base, 1/8" thickness, 9001K36) with 5/8" holes threaded for thumb screws (McMaster-Carr, 91185A332). To maintain a rectangular shape, the bags were sealed 1" from their corners. As a result, the base of the bag hangs 11 cm from the headplate. The liquid distributors used were

made of ⅜” stainless steel tubing, and were placed through the quick connect fitting (carefully drilled out from the NPT side to prevent damage to the O-ring) such that they extended 10 cm into the reactor vessels to be covered in the beads. The connector for the gas headspace was made of a 4 cm long stainless steel tube through a second drilled out quick connect fitting such that it extends just into the reactor headspace.

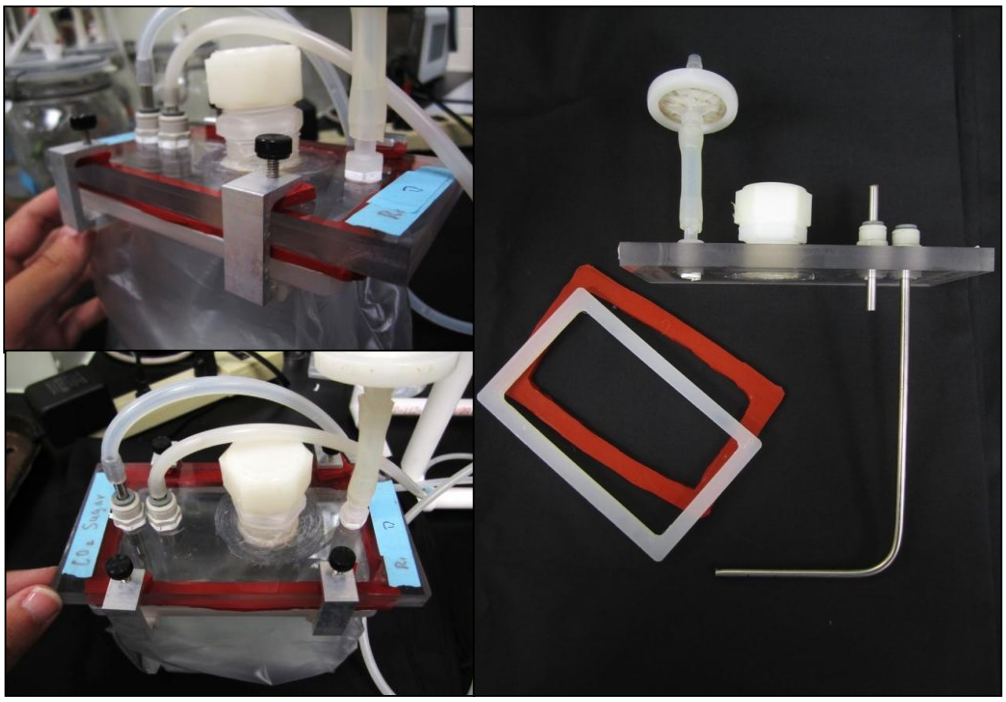

Figure 24 – (Left) Fully assembled headplate side and top views. (Right) Individual components of the headplate. Liquid distributer shown is an obsolete version.

The media bottle reservoir was fitted with a silicon stopper (stopper #6) with three holes cut using a borer. Three ⅜” stainless steel tubes were placed through these holes, two of which were 14 cm long, extending into the headspace of the reservoir for gas connections, while the last was 4.5 cm long, pushed flush with the silicon stopper to allow complete liquid draining. The total length of liquid tubing was found to be important for proper siphon operation. The liquid connection from the media reservoir to the inoculation port was made with a 13 cm long piece of ⅜” silicon tubing. This was then fitted into the leur-lock ‘T’ connector (Cole Parmer, EW-45500-

56), followed by a 37 cm piece of tubing to connect the liquid distributor. The gas connection between the reservoir and reactor headspaces was made with a 49 cm long piece of ⅜" silicon tubing. Each reactor was filled with 50 mL of 4 mm diameter glass bead substrate (VWR 4mm soda lime, 26396-563).

In the preparation of the reactor, all tubing connections were made save for the placement of a sterile injection port (Cole Parmer, EW-45503-04) in the leur-lock 'T', as these cannot withstand autoclave temperatures. The 'T' was wrapped in a double layer of aluminum foil to maintain its sterility, and the entire assembly was autoclaved. Immediately after removing the reactors from the autoclave, sterile injection ports were attached in a laminar flow hood, and covered with the same aluminum foil to prevent contamination of their exterior. The media reservoirs were autoclaved containing DI to improve sterilization, and the silicon stopper/tubing assembly was switched to freshly autoclaved media bottles containing appropriate media 3 days prior to running the reactor. Care must be taken not to insert the silicon stoppers too deeply into the media bottle, or they become very difficult to remove, which can compromise the sterility when switching the assembly to a new media bottle. The silicon stopper is held in place by a media cap with a 1 in diameter hole to allow for tubing penetrations. This should not be screwed down too tightly, as it will press the stopper more tightly into the bottle and make its removal extremely difficult. This setup was run under typical cycling conditions for 2-3 days to insure that no contamination was present prior to inoculation.

Root Culture Operation

For *Nicotiana benthamiana* hairy root culture, reactor vessels were inoculated with 2 grams of tissue. This tissue was prepared beforehand in a sterile laminar flow hood. Prior to inoculation, 125 mL Erlenmeyer shake flasks were used to grow the appropriate tissue. To plan

for inoculation requirements, each flask produces approximately 4 grams of tissue approximately 2 weeks after subculturing. The tissue was cut in a criss-cross pattern to facilitate removal from the flask, then blotted on filter paper to mass the inoculum. Both reactor inoculum and controls were placed in 50 mL of Gamborg's B5 media[18] in 125 mL wide mouth shake flasks for two days to prevent excess stress associated with the cutting of tissue, as well as to check for contamination. The tissue was then introduced into the bioreactor system through the ¾" inoculation port.

The LabVIEW application was set up for root operation such that the maximum height reached and maintained allows the siphon to begin at 1500 steps. Because of the rate at which the reservoirs drain, a wait time of 0.5 minutes was used during which the roots are submerged. Due to the high rate of growth of roots, the reactor is set to cycle hourly. Video recordings are placed in the directory C://Dropbox, which is set up to automatically upload and share all files placed in it into a shared folder for individual users to monitor online using the WWCurtisLab@gmail.com dropbox account.

Watermelon Operation

The seedless watermelon line from Nunhems seed company (#0904) has been maintained by subculture every 2-4 weeks onto fresh WSIM media[31]. It was observed that the cultures were relatively permissive to extended periods without subculture, in large part because the subculturing procedure involved the careful excision of the proliferating shoot meristems (and removal of older leaves, callus and stem tissue). A healthy clump of watermelon shoots would generate 4-6 excised meristems; since these were being subcultured 5 explants per GA7 container (Figure 4), this provided for a 5-fold amplification during subculture if needed. Since a previous subculture effort ended with contamination of all the cultures with a slow growing bacteria which

we were not able to 'cure', an additional precaution for contamination testing was implemented where use of the polystyrene petri-dishes for dissection was limited to providing tissue for 1 or 2 GA7 culture boxes. In addition, 3-5 small fragments left over from dissection where added to 4 mL of LB media in a 25mm ID culture tube to incubate at 25°C. During this procedure, each corresponding contamination test and GA7 were numbered so that any cultures failing the contamination test could be removed from the culturing (and greatly reduce risk of cross-contamination). In the future, it is recommended that the broad growth testing Difco™ R2A agar be used for this contamination test because it provides for growth of fungi and bacteria (this media is often used for broad testing of environmental contamination).

Figure 25 – Organogenic watermelon explants used for statistical analysis on Dec 6, 2012.

Looking toward the inoculation and growth of watermelon cultures in the TIB system, an examination of the size of typical explants being subcultured by Dr. Curtis was undertaken (December 6, 2012; 0904_{17}). Twelve additional explants were dissected and blotted and fresh weights taken. The resulting explants varied by about 25% giving an average of 78 ± 23 mg FW per explant. While this is a relatively large variation, if the plan is to inoculate comparable to the root culture biomass, this would require 2 gFW or roughly 25 explants. If this large number of explants is used, then the variation will even out between different bioreactor inoculations.

Due to the slow rate of watermelon organogenisis, it is significantly less likely that the explants will become nutrient limited while in the dry phase of the reactor cycle. Typical organogenic cultures are observed to grow fully over a period upwards of 6 weeks where hairy root cultures can exhibit the comparable or greater extent of growth in 2 weeks. As a result, it may be advantageous to reduce the frequency of reactor cycling to allow the development normal

physiological gradients within the tissue. Simple calculations using observed growth rates indicate that cycling the reactors once every six hours should be sufficient to provide the required nutrients to the explants in a liquid film.

Future Work

While the root culture work described above focused primarily on tissue that becomes mass transfer limited with oxygen, utilizing hairy root cultures bypasses one of the major advantages of working with plants: they are capable of growing photoautotrophically. By supplementing the gas phase with additional CO_2 and growing plant tissue under lighted conditions, it is possible to generate fully autotrophic tissue culture[42]. Besides the obvious advantage of reducing substrate costs by not feeding plants sugar, the reduction of available sugar in the media prevents the overgrowth of heterotrophic bacteria and fungi. As a result, the risk of contamination in such a system is significantly mitigated, increasing the feasibility of industrial scale applications of the bioreactor. Preliminary results have shown organogenic watermelon tissue growing on a 5% CO_2 mixture where those fed with air displayed minimal growth; however, the sample size of these tests was not large enough to draw significant conclusions, and there were problems with contamination is the tissue used for the experiment.

Another major goal of the Curtis Lab is to enable the propagation of recalcitrant species through the transient introduction of transcription factors to induce somatic embryogenesis. Collaborating with the Guiltianan Lab, a project is underway to propagate *Theobroma cacao*, the chocolate tree. Due to the recent spread of diseases such as ‘witches broom’ there are significant crop losses that are adversely effecting supply and the subsistence farmers that produce cacao[44]. This project utilizes the recent development of an auxotrophic strain of *Agrobacterium* that can be used to deliver genetic information to plants without risk of overgrowth[45]. While *Agrobacterium*

is commonly used to genetically engineer plants with stable insertion of DNA, the goal of this project is to use a transient expression system to begin the process of embryogenesis without genetically engineering the plant itself. Using this method, the genetic environment of plant tissues can be controlled along with the gas and liquid environments to further enhance the rate of production of propagated plants.

Based on a presentation of this work at the 2012 In Vitro Biology Congress held in Bellevue, WA, an interaction with a Nigerian scientist was established that resulted in the funding of a collaborative project through the NSF-PEER program with Dr. Morufat O. Balogun (Geneticist, University of Ibadan, Nigeria). This project seeks to further develop this TIB system toward the application of growing the food crop *Dioscorea spp* in Africa. Known more commonly as the yam, this crop is a significant caloric intake of roughly 300 million people in the tropics[46]. Production of this crop is constrained by the availability of planting materials and low natural multiplication rates[47]. More specifically, the plan is to increase propagation rates and manipulate the dormancy of this plant through application of hormones[48] and manipulation of the gas phase[49] within the TIB system. The potential for replacing computer-controlled elements with routine (inexpensive) manual labor is particularly appropriate for technology implementation Nigeria. The success of this project could result in the full-scale development of this bioreactor system to increase availability of ‘seed’ stock of a food crop that is critical to millions.

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