Aeration and Mode of Nutrient Delivery Affects Growth Of Peas in a Controlled Environment

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AERATION AND MODE OF NUTRIENT DELIVERY AFFECTS GROWTH OF PEAS IN A CONTROLLED ENVIRONMENT

by

Joseph Francis Romagnano

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Abstract

The development of a plant growth chamber capable of sustaining plant growth over multiple generations is a necessary step towards the attainment of a Controlled Ecological Life Support System (CELSS). The studies herein examine the effects of aeration abilities and rates on plants grown in three model nutrient delivery systems during germination and over the life-cycle of the plant. These studies are the first time a porous tube nutrient delivery system was compared to another active nutrient mist delivery system. During germination an indicator of hypoxic stress, alcohol dehydrogenase (ADH) activity, was measured and was more affected by aeration rate than mode of nutrient delivery. Over the life-cycle of the plant, however, plants grown in the porous tube system had the least ADH activity and the highest levels of shoot (leaf + stem), root and leaf biomass. None of the plants in any system, however, produced viable seed. This study highlights the need to optimize aeration capabilities in the root zone of enclosed chambers.
Acknowledgements

Contrary to popular image, even the scientist toiling alone late into the night is not so isolated that he owes no thanks to anyone. As such, I owe a debt of gratitude to many people who, directly or indirectly, supported me and helped further my work in this undertaking. First and foremost, thanks must go to the members of my family. There has been one person who has been with me from the beginning, mom, thank you from the bottom of my heart. From growing corn plants in Tupperware, and FedExed books on Biosphere II, to home-built jerry-rigged hydroponics systems in the basement, you’ve shown support and tolerance for the often-strange activities of your son. You’ve been the best mom a son could ask for, even if the road has, at times, been rocky.

Special thanks must also go to my Grandmother and Grandfather. Thank you for allowing me to spread gas chromatography data across your living room. Thank you for the cookies. Thank you for your kind words of support and encouragement. Thank you for the summer adventures that piqued the curiosity that led to my interest in science.

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Special thanks must also go to those outside researchers who have provided valuable support and insight. Mary Musgrave, University of Connecticut, provided valuable comments, information, and reviews of some portions of this thesis. Tom Dreschel, Gary Stutte, and Oscar Monje of Dynamac Corporation at Kennedy Space Center provided valuable information, porous tubes, and sample substrate material. Marshall Porterfield of the University of Missouri also provided valuable insight and guidance.

Finally, to those whom I’ve forgotten and did not record here, you have my apologies and also my thanks. It has been a good long slog but I’ve enjoyed every minute of it and I wouldn’t change one thing, thank you all.
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Introduction

The quest for knowledge is one of the primary motivators of human action. Concomitant with that quest is humanity’s drive to explore, understand, and expand into new regions. The benefits of current space exploration efforts to humanity have been numerous and far-reaching. Examples include satellite global positioning and communications, development of new materials, physiological studies, and many other things great and small. However, with the exception of robotic probes, limited trips to the moon, and studies in low earth orbit, mankind’s exploration of our solar system and beyond has been restricted by our inability to safely journey in the void for extended periods of time.

Many systems, such as propulsion and guidance need to be developed before a long duration mission can occur. One of the chief systems yet to be fully developed is a system capable of providing for and sustaining human life over the duration of a long-term mission. Such a life support system must provide for regeneration of air and water, waste removal, food supply, and psychological diversion (Olson, et al., 1988). To this end, one of the proposed means of achieving some of the aforementioned goals is a system that incorporates plant species and their abilities into the system. Such a plant-based system has been termed a Controlled Ecological Life Support System (CELSS) or a Bioregenerative Life Support System (BLSS).

For use in a life support system, plants offer numerous advantages; plants have the ability to fulfill part or all of the requirements of a self-sustaining CELSS system. Through the process of photosynthesis a plant can remove carbon dioxide from the air and replenish oxygen. Transpiration can be harnessed to purify grey water into potable
water. Generational growth of crop plants ensures a continuous source of fresh food essential for the maintenance of proper nutrition. In order to minimize the need to carry extra seed mass or the need for a re-supply mission, it is exceptionally important that the plant growth chamber of a CELSS system be able to support production of viable seed in order to provide the next crop generation As such, the ability to grow and maintain crops in a microgravity environment is a necessary and key step towards obtaining a complete CELSS system (Dreschel, et al., 1994).

Technologies developed for use in a CELSS system, in addition to use in space, have the potential to also benefit those on Earth. Modification of organic waste for use as nutrient stocks, the ability to grow plants in a highly dense manner, conversion of wastewater into potable water, clearance of excess carbon dioxide, and subsequent replenishment of oxygen are end products that can be adapted for use on the ground. Furthermore, once stable, controlled growth of plants in microgravity is achieved, the door is opened to new studies about the fundamental physiology of plants.

Information gained from studies used to develop those technologies may lead to additional spin-off technologies. The data herein, can be used to further our understanding of responses of plants to hypoxic stress. In addition to comparing two active modes of nutrient delivery over the life cycle of a plant, variations in the aeration abilities and rates fed into the root zone were also tested. Similar aeration applied to a farmer’s flooded field, for instance, may temporarily alleviate hypoxic stress, lessening the likelihood of crop loss. Understanding media aeration and its impact in the root zone is also applicable to dense root beds commonly found in hydroponic systems. Root zone
optimization of nutrient flow and gas exchange can lead to more productive plants thus lowering operating costs.

**Growing Plants in Microgravity**

Conventional hydroponic systems, while effective on the ground, are not readily adapted for use in a microgravity environment. Growing plants in an enclosed and controlled environment dictates that many plant needs must be serviced artificially. Specifically, light, air, temperature, humidity, nutrients and the removal of wastes must all be taken into account (Heathcote, *et al.*, 1996).

Lack of a gravity vector greatly affects the movement and availability of metabolic gases ($O_2$, $CO_2$, $C_2H_4$) and liquid nutrient media to plant tissues in both root and shoot zones (for reviews, Monje, *et al.*, 2003; Porterfield, *et al.*, 2003). In the shoot and root zones of a nutrient delivery system’s culture chamber, buoyancy-driven convection currents are not present (Porterfield, 2002; Bingham *et al.*, 1996; Bingham *et al.*, 1997; Jones and Or 1998; Salisbury, F.B., 1997; Yendler *et al.*, 1995). Under normal gravity conditions such currents are responsible for the replenishment of metabolic gasses around plant tissues. When these currents are absent poor gas exchange occurs which results in the formation of stagnant air pockets around plant tissues subsequently resulting in poor plant growth and development (Monje, *et al.*, 2003; Porterfield, *et al.*, 2003; Porterfield, 2002; Porterfield, *et al.*, 1997; Musgrave, *et al.*, 1998).

As was first demonstrated in the Chromex series of experiments, the addition of a fan to circulate air in the shoot zone greatly reduced the stress induced by poor gas exchange in plant shoots (Musgrave, *et al.*, 1998). The roots of plants from the same experiment, however, had significantly greater amounts of hypoxic stress compared to
normal gravity controls (Porterfield, et al., 1997). Nutrient delivery systems for use in space must account for water’s surface tension and lack of gravity-induced flow. Therefore, conventional hydroponic and aeroponic nutrient delivery systems are not feasible (Levine, et al., 2001).

**Plant Growth Systems Previously used in Microgravity**

Porterfield, et al., (2003) and Monje, et al., (2003) provide excellent reviews of hardware previously used in microgravity and the concerns and limitations of growing plants in microgravity. To date, there have been few nutrient delivery systems that have sustained plants over the course of their entire life cycle (Table 1). Key experiments and systems have included: Phyton on Salyut-7, the Chromex series of experiments conducted on the space shuttle, and the Greenhouse 3 experiment using the Svet greenhouse onboard Mir. These are summarized in Table 1. The Phyton system on Salyut-7 marked the first time that a complete seed-to-seed life cycle was completed under microgravity conditions (Merkys and Laurinavicius, 1983). The Chromex series of experiments were the first ones to compare the same nutrient delivery system under varying conditions. It was found that the addition of a fan to the shoot zone permitted pollination to occur thereby showing how critical it was to carefully consider provision of metabolic gases to plants (Musgrave, et al., 1997). Finally, the Svet greenhouse experiment allowed for a multi-cycle study, using seed material produced from the first cycle as material for the second, to be conducted.

One of the key issues highlighted by these past studies has been the need to better understand gas and liquid movement and distribution in the root zone. Until recently,
Table I. Abbreviated history of plant nutrient delivery systems used in microgravity. * Data and observations compiled from Musgrave, et al. (1997).

<table>
<thead>
<tr>
<th>Plant Type</th>
<th>Flight</th>
<th>Chamber</th>
<th>Nutrient Supply/Delivery</th>
<th>Substrate</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Epidendrum radicans</em></td>
<td>Salyut 6</td>
<td>Malachite</td>
<td>Water supplied to bed</td>
<td>Ion exchange resin</td>
<td>Flowers present at launch senesced. No new flowers formed. (Nechitailo and Mashinsky, 1993)</td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td>Salyut 6</td>
<td>Oasis</td>
<td>Water through hydrophilic membrane</td>
<td>Ion exchange resin</td>
<td>Plants died at flowering, due to open design of system (Nechitailo and Mashinsky, 1993).</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>Salyut 6</td>
<td>Svetoblok-1</td>
<td>None</td>
<td>1.5% agar-based medium</td>
<td>Plants flowered. No viable seed produced. (Kordyum, et al., 1983)</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>Salyut 7</td>
<td>Phyton</td>
<td>None</td>
<td>1.5% agar-based medium</td>
<td>First complete life-cycle achieved in space. 33% of siliques contained aborted ovules. 50% of seeds were viable upon germination (Merkys and Laurinavicius, 1983).</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>Mir</td>
<td>Svetoblok-M</td>
<td>None</td>
<td>Agar-based</td>
<td>Chlorophyll a + b levels reduced (ratio unchanged). Poor growth, delayed growth, sterile and viable seed produced (viability determined on ground). (Mashinsky, et al., 1994).</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>CHROMEX-03 (STS-54), CHROMEX-04 (STS-51), CHROMEX-05 (STS-68)</td>
<td>Plant Growth Unit</td>
<td>Agar-based</td>
<td>Phenolic foam</td>
<td>Rosettes initiated flowering shoots on-orbit. Equal flowering in microgravity and ground controls. 65% of pollen found non-viable. Poor formation of reproductive structures. Increased ADH activity relative to ground controls. (Kuang, et al., 1996; Crispi, et al., 1996; Musgrave, et al., 1997; Porterfield, et al., 1997).</td>
</tr>
<tr>
<td><em>Brassica rapa</em></td>
<td>“Greenhouse 3” (STS-84 to Mir, STS-86 return)</td>
<td>Svet</td>
<td>Water delivered via porous tube</td>
<td>Turface / Osmocote mix</td>
<td>Over 122 days, two full life cycles and part of a third were achieved. Plants grown from seeds produced on-orbit had inhibited growth and development. Silique ripening was found to be basipetal, rather than simultaneously along the length (Musgrave, et al., 2000).</td>
</tr>
</tbody>
</table>
nutrients have been delivered into the root zone in a passive manner and work has primarily been directed towards optimizing conditions in the shoot zone. The Phyton system and the Plant Growth Unit both used agar-based nutrients and, in the case of the Chromex experiments, a phenolic foam substrate (Merkys and Laurinavicius, 1983; Kuang, et al., 1996). More recent systems, such as the Astroculture™ and the Svet greenhouse have used porous tubes buried in a granular substrate like Turface (Morrow, et al., 1995; Porterfield, et al. 2000b; Musgrave, et al., 2000).

Movement of liquids through a granular substrate has been the subject of many studies (Bingham et al., 1996; Bingham et al., 1997; Jones and Or 1998; Salisbury, F.B., 1997; Yendler et al., 1995). Currently, it is believed that as liquid media moves radially outward from a porous tube into the surrounding substrate, pockets of air become trapped against the wall of the culture vessel enclosing the plant root zone (Monje, et al., 2003). When plant roots penetrate these pockets and deplete them of gasses necessary for the maintenance of metabolism, particularly oxygen, roots become stressed and contribute to an overall reduction in plant health (Porterfield, et al., 1997, Monje, et al., 2003; Porterfield, et al., 2003). Due to these observed problems there is a need for the development of ground-based systems that can mimic conditions encountered in microgravity (Levine, et al., 2001).

**Oxygen and ADH Activity**

Oxygen is necessary for normal maintenance of the Krebs cycle and electron transport chain (Moore, et al., 1998). When plentiful, oxygen serves as the ultimate electron acceptor in the electron transport chain, allowing for the regeneration of NAD$^+$ from NADH. Under normoxic conditions, NAD$^+$ is used by triose phosphate
dehydrogenase to oxidize glyceraldehyde-3-phosphate to 1,3-bisphosphoglyceric acid. Under hypoxic or anoxic conditions, however, regeneration of NAD\(^+\) is not readily completed, leading to a breakdown of the glycolytic pathway (Moore, et al., 1998).

Other electron acceptors must, therefore, be used to regenerate NADH to NAD\(^+\) and fermentative metabolism is initiated. Rather than undergoing conversion to acetyl-CoA and entering into the Krebs cycle, pyruvic acid is converted via pyruvate decarboxylase to acetaldehyde. As a final step, acetaldehyde conversion into ethanol is catalyzed by alcohol dehydrogenase (ADH). During the catalysis, the hydrogen of NADH is removed and NAD\(^+\) is thus regenerated (Moore, et al., 1998). Due to the fact that ADH activity correlates with relative amounts of root hypoxia, and its activity can be readily calculated by measuring the decrease of NADH in a spectrophotometric reaction, ADH serves as an ideal indicator of hypoxic stress (McKee and Mendelssohn, 1987; Appendix C).

**Ethylene and its Indicators**

Under conditions of normal aeration and gas exchange, ethylene is a plant hormone that is responsible for the development and maturation of flowers and fruit as well as controlling the abscission of fruit and leaves (Moore, et al., 1998). In an enclosed environment with poor gas exchange, such as that experienced by plants in microgravity, ethylene concentrations can accumulate and inhibit flowering. As ethylene concentrations increase, fruit and flowers that would normally develop are negatively affected, resulting in the onset of premature senescence, particularly of flowers (Beyer, 1976). This response to ethylene is similar to reproductive problems observed during the Chromex series of experiments (Kuang, et al., 1996; Musgrave, et al., 1997). It was reported that the
reproductive organs, particularly flowers and siliques, were incapable of producing either fruit or viable seed in *Arabidopsis* (Table 1).

Other adverse effects of increased ethylene concentrations have been documented. In *Lactuca sativa* increased ethylene concentrations significantly reduced overall dry weight and enhanced the number of leaves while reducing overall leaf size and dry weight (Mortensen, 1988). Also, waterlogging of the root zone, coupled with the onset of hypoxic conditions, is known to lead to the build-up of small amounts of ethylene in root tissues. The trapped ethylene stimulates cellulase and pectinase resulting in the breakdown of cell walls and the formation of aerenchyma which help to facilitate the diffusion of oxygen into surrounding root tissue (Moore, *et al.*, 1998).

It has been suggested that ethylene acts as part of a signal pathway indicating hypoxia in roots (Drew, *et al.*, 1997). Besides formation of aerenchyma, waterlogging in roots also stimulates the conversion of ethylene precursors in the shoot, resulting in the onset of epinasty (Moore, *et al.*, 1998). Under anoxic conditions, however, ethylene production is blocked due to the absence of at least a small amount of free oxygen for the conversion of ACC (ethylene precursor) to ethylene (Drew, *et al.*, 1997). Furthermore, germinating shoots have been shown to produce increased ethylene levels when encountering mechanical barriers (Schwarzbach, *et al.*, 1991). It is, therefore, not unreasonable to expect that ethylene levels may also increase when roots encounter mechanical barriers.

Due to the above effects of ethylene, levels of this gas have been specified at less than 5 ppb for the Plant Research Unit (PRU) to be developed for use in the international space station (Heathcote, *et al.*, 1996). In order to sustain such low amounts of ethylene,
care must be taken to design growth culture systems that can maintain a well oxygenated root zone that is minimally waterlogged, and large enough in size to contain the roots of mature plants of any of the CELSS candidate crops.

**CO₂ in the enclosed environment**

Carbon dioxide, in addition to its use in photosynthesis, also influences stomatal conductance and transpiration rates (Moore, *et al.*, 1998; Wheeler, *et al.*, 1999). Although decreased CO₂ levels, particularly in the interior of leaves, are responsible for stomatal opening and transpiration increases (Moore, *et al.*, 1998), a greater amount of research has been devoted to CO₂ enriched atmospheres similar to those in enclosed chambers. One of the primary goals of CELSS research is to use the photosynthetic pathway in plants to fix CO₂ from stale incoming cabin air and replenish it with O₂ released from the plant. Another chief goal is to use transpiration as a way to filter and purify water from waste sources. Thus, CO₂ availability, particularly with a widely fluctuating source such as cabin air, must be accounted for in the design of a plant growth chamber. For example, from May to September of 1997 CO₂ concentrations in the cabin of Mir varied from 2,000-10,000 ppm (Monje, *et al.*, 2003). In the Chromex series of experiments supplemental CO₂ was provided to the plants (up to 302 µl l⁻¹), contributing to premature reproductive organ development (Musgrave, *et al.*, 1997). Also, studying plant responses to varying CO₂ levels, particularly increased concentrations, can provide insight into plant responses under global warming conditions.

Besides photosynthesis, carbon dioxide also affects root growth. The effects of increased CO₂ availability on roots have been studied in cultures of beet and safflower hairy roots grown in an enclosed culture system (DiIorio, *et al.*, 1992), and in whole
plants (Weathers and Zobel, 1992). In the cultures of beet and safflower hairy roots, it was found that an increase in dry weight was effected by an increase in CO₂ availability up to 1.3, 1.5% CO₂, respectively. Past that point (1.6 or 2.0% for beets and safflower, respectively) the mass increase was significantly less and ultimately negligible (DiIorio, *et al.*, 1992). This suggests that, for roots, an optimal CO₂ concentration exists and, once levels go beyond that point detrimental effects are seen. For example, in whole plants, an increase of CO₂ concentrations in the root zone to 45% was found both to be beneficial when applied for a short period of time and, when applied for longer than a few hours, growth was inhibited (Weathers and Zobel, 1992). Long term treatments in the root zone with CO₂ concentrations from 0.1-0.5%, however, showed a 20-25% stimulation in growth (Weathers and Zobel, 1992).

Dwarf wheat plants grown in the superoptimal CO₂ concentrations (350-10,000 μmol mol⁻¹ range) used by Reuveni and Bugbee (1997) showed a decrease in seed yield, harvest index, mass per seed and number of seeds per head as CO₂ levels increased. Also, it was found that photosynthetic and dark respiration rates decreased by 8% and 25%, respectively, when CO₂ levels were raised to 2600 μmol mol⁻¹ CO₂. Furthermore, high CO₂ concentrations did not correlate with ethylene production by flag leaves or wheat heads (Reuveni and Bugbee, 1997).

The studies herein mark the first time plants grown using a porous tube system have been compared to another system also capable of actively delivering nutrients. These, comparisons should provide valuable insight into potential benefits and drawbacks not readily apparent when compared to passive delivery systems. Finally, since aeration
is provided strictly through the root zone, the impacts of that provision on shoot and overall plant health can also be examined.

Objectives

In order to better understand the impacts of mode of nutrient delivery and media aeration in the root zone, two studies were undertaken. The first sought to understand how changing aeration rates delivered to the root zone would impact root health during germination irrespective of mode of nutrient delivery. The second sought to understand if the mode of nutrient delivery into the root zone would affect not only root health but shoot health as well.

Organization of Thesis

Due to the fact that both studies were self-contained this thesis is organized into chapters dedicated to each specific study and consisting of each manuscript as it is to be submitted for publication.
**Chapter 1: Germination Studies**

Media aeration affects hypoxic stress in germinating pea roots more than mode of media delivery.

**SUMMARY**

To grow plants in microgravity, porous tubes have been used to deliver liquid media into root zones of nutrient delivery systems. Growth has, however, been less than desirable. The lack of a gravity vector likely leads to the formation of stagnant air pockets and inadequate gas availability to the roots. Effects of media aeration have been overlooked.

We have compared substrate-based nutrient delivery systems using a mist or porous tube to feed germinating peas. Roots from peas germinated in mist and porous tube systems using a 15.2 cm long culture trough were compared against normoxic and hypoxic controls. Of the two experimental and two control systems, the mist system yielded the greatest fresh root mass per germinated seed. To measure hypoxic stress alcohol dehydrogenase (ADH) activity of the roots was assayed. ADH activity of roots grown in the mist, porous tube, and normoxic controls were all approximately 10% of the activity measured in the hypoxic control. When the culture trough was scaled in length threefold, mist and porous tube systems supported about the same amount of root biomass. ADH activity, however, varied greatly between modes of nutrient delivery and level of aeration. Roots grown in porous tube and drip systems showed slightly less hypoxic stress than roots from an equivalently aerated system. These results suggest that both the type of nutrient delivery system and media aeration are important in alleviating hypoxic stress in the root zone.

* This section submitted for publication.
INTRODUCTION

Understanding the various stresses whole plants encounter when subjected to culture in an artificial environment is crucial to the design and implementation of a bioregenerative life support system that will use plants to provide food, recycle water and replenish oxygen. Successful development of a plant culture system that is capable of supporting generational crop growth in a spaceflight environment is a necessary step if a Controlled Ecological Life Support System (CELSS) is to be realized (Dreschel et. al, 1994). The microgravity environment presents several challenges for the design of a plant growth system. Light, air, temperature, humidity, nutrient delivery and the removal of wastes must all be taken into account (Heathcote et. al, 1996). Due to water’s high surface tension and tendency to stick to itself under microgravity conditions, conventional hydroponic and aeroponic nutrient delivery systems are not feasible (Levine et. al, 2001). The successful nutrient delivery system must be able to deliver a reliable nutrient flow without adding stress to the plants in the system.

Active nutrient delivery systems used to date have employed porous tubes that exploit capillary action and system pressure (positive or negative) to control the delivery of liquid media into surrounding substrate (Dreschel et. al, 1994; Porterfield 1996; Tibbitts, 1995; Duffie et. al 1995; Turner et. al, 1999). To date, porous tube nutrient delivery systems (with or without a surrounding substrate) have been the only active nutrient delivery systems used in space (Porterfield et. al, 2003). While progress has been made in optimizing conditions in the shoot zone, stress in the root zone, particularly due to hypoxia, has been difficult to eliminate (Monje et. al, 2003).
Hypoxia has been shown to occur in the roots of plants grown in several different microgravity experiments (Porterfield et al., 1994). It is hypothesized that as liquid media is drawn from the porous tube into the surrounding substrate, air/liquid boundary layers form causing the formation of air pockets (Monje, et. al, 2003). Thus, roots penetrating these trapped air pockets deplete them of the oxygen necessary to maintain aerobic metabolism thereby leading to the depletion of bioavailable oxygen in the media (Monje, et. al, 2003). Therefore a system that is capable of providing not only aerated media but also preventing the formation of trapped air pockets should be able to effectively reduce or eliminate hypoxic stress in roots of plants grown in microgravity conditions. A mist-based nutrient delivery system in which liquid media and oxygen can be concurrently delivered has the potential to fulfill these requirements.

Mist-based systems have the potential to optimize conditions in the root-zone as well as to increase gas exchange between the root and shoot zones. Mist bioreactors have been shown to have several advantages over conventional bioreactors (Weathers et. al, 1999; Kim, et. al, 2002). One of the major advantages of mist systems is the ability to control the gas mixture entering into the culture vessel (Weathers et. al, 1997). In cultures of transformed roots, advantages of mist systems over conventional systems have been demonstrated for both O₂ (Weathers et. al, 1999) and CO₂ controlled gas levels (DiIorio et. al, 1992). We considered, therefore, that a nutrient delivery system using an ultrasonic nozzle has the potential to offer these same advantages to plant roots suffering from hypoxia in microgravity.

In this study, we compare two substrate-based systems employing different modes of nutrient delivery: a mist and a porous tube. We measured root growth and
alcohol dehydrogenase (ADH) activity of germinating pea roots in response to changes in aeration rate.

MATERIALS AND METHODS

Culture Systems

Culture Trough Fabrication: For all experimental systems, culture troughs were fabricated from plastic rain gutters (WormsWay, Worcester, MA; Figure 1). Gutters were cut to just over 15.2cm (short systems) or 45.7cm (long systems) in length to allow for end-cap overlap. End pieces modified for respective delivery systems were fitted to each gutter. Fabricated end-caps were formed from 6.35 mm thick clear polycarbonate plastic (Plastics Unlimited, Worcester, MA).

Figure 1. Empty experimental and control nutrient delivery systems used in short culture trough experiments. A. Mist based system. Arrow indicates hard-plastic air line at bottom of culture trough. Mist is injected along the center axis. B. Close up of ultrasonic nozzle. C. Porous tube system. Outside of system at end, tube is clamped off ensuring a positive liquid flow. Arrow indicates direction of liquid flow. D. Normoxic rag-doll control. Air feed line enters at top. E. Hypoxic rag-doll control. During germination all units were covered with foil to prevent light from entering.
**Substrate and Media:** Sieved (1mm obtained diameter) TurfaceMVP™ (PROFILE Products, LLC, Buffalo Grove, IL) was used as the substrate in the experimental units for all replicate runs. Deionized water, kept in 1L Gibco™ bottles, was the medium used for the duration of each experiment.

**Mist Delivery:** An ultrasonic nozzle operating at 60 Hz with 115 V (model# 2-60TS, Sono-Tek, Milton, NY) was used to generate and propel mist into the root zone of the mist system. This nozzle (Figure 1, B) generates a mist with a nominal particle size of 30 µm. Maximum liquid flow capacity for the nozzle was found to be approximately 15 mL/min. For all experiments, the nozzle was run at 10 mL min⁻¹ for one minute every two hours. Supplemental aeration of the root zone was accomplished by either co-injection through the sonic nozzle (45.7 cm culture trough), or by addition of air into a perforated hard plastic airline located along the length of the bottom of the culture trough (15.2 cm culture trough).

**Porous Tubes:** Experiments using short culture troughs incorporated a 15 cm ceramic porous tube with a nominal pore size of 10 µm (courtesy of Tom Dreschel, Dynamac Corp., Kennedy Space Center, FL) with an o.d. of 1.5 cm and an i.d. of 1.0 cm. The tube was mounted using cored rubber septa attached to Tygon tubing. By sealing the outflow side of the tube it was ensured that media fed under a positive pressure would exit only through the tube walls into the root zone. The top of the porous tube was set 1 cm below the Turface surface.

Experiments using the longer culture troughs (45.7 cm) used a 45.2 cm long alumina porous tube with a nominal pore size of 12 µm (Refractron Technologies Corp., Newark, NY 14513-9796) with an o.d. of 1.3 cm and a 0.79 cm i.d. With the exception of
the addition of a center support fabricated from a shaped 1 mL plastic pipette tip, the tube was mounted as described for the shorter system. For all porous tube experiments, a media delivery cycle of 10 mL min\(^{-1}\) for 1 min every 2 hours was used.

**Plants and Growth Conditions**

For all experiments “Little Marvel” peas, *Pisum sativum*, (Chas. C. Hart Seed Co. Wethersfield, CT 06109 or Harris Seeds Rochester, NY 14624) were used due to availability and short time till germination (less than two days). Seeds were imbibed in diH\(_2\)O overnight prior to planting and then grown for seven days in the four experimental culture systems. In mist and porous tube systems (Figure 1 A, C), the imbibed peas, placed at a depth of 1 cm, were spaced 2.5 cm apart along the length of the culture trough with three rows across the width (Figure 2). Eighteen and fifty-four total peas, respectively, were planted in the 15.2 cm and 45.7 cm culture vessels.

For normoxic and hypoxic controls, seeds were germinated in dampened, rolled, paper towels systems that were then placed in sealed or air-flushed Rubbermaid sport-bottle (1L capacity) containers (Figure 1, D, E). From planting until harvest the germinating seeds in all systems were kept in the dark by placement of a loose-fitting foil cover over the culture trough. Thus, some gas exchange still occurred over the substrate surface.

**Aeration rates:** Germinating peas in normoxic and mist systems using the shorter culture troughs were flushed with humidified ambient air (accomplished by passing through a bubbler) at a rate of 500 mL min\(^{-1}\) for 32.25 min every two hours. In the normoxic control an air line was dropped down to just above the water level. A separate hole at the top of the system was created to allow for excess gas to vent. Aeration into
the root zone of the mist system was accomplished through the use of a perforated hard-plastic tube placed along the bottom of the trough.

Peas germinating in systems using the longer culture troughs had substantially different aeration cycles. In the mist system, ambient air was co-injected through the ultrasonic nozzle at 20 mL min\(^{-1}\) for 1 min every two hours. In the porous tube system, aeration, when used, was fed at a rate of 20 mL min\(^{-1}\) for 1 min every two hours into the media reservoir from an air line. The drip control system, when aerated, followed the same protocol as the porous tube system. The drip system was also run at a tenfold higher aeration rate of 200 mL min\(^{-1}\) for 1 min every two hours.

**Figure 2.** Inlet to outlet section grouping of peas germinated in long culture trough experiments. Each section represents a total grouping of roots from nine peas pooled together.

*Harvest and Extraction:* At harvest, plant roots were extracted following a procedure modified from the works of Porterfield (1996) and McKee and Mendelssohn (1987). Experiments using the shorter culture troughs had roots from the entire system pooled and extracted together. Roots from the longer culture troughs were pooled into six sections, each consisting of nine peas. Sections were divided from the inlet to the outlet with the first three rows from the inlet composing section one (Figure 2). Freshly harvested roots were rinsed, blotted dry, and the fresh weight was then measured. The roots were then frozen in liquid nitrogen. Time from system shut down until all of the
roots were frozen was kept under an hour. If roots were not immediately extracted, they were stored at –80°C until further processing was possible.

To facilitate disruption, frozen roots were re-dipped in liquid nitrogen just prior to grinding in a mortar and pestle using 2-4 mL of Tris breaking buffer per gram of root tissue. The breaking buffer, brought to pH 7.5, consisted of: 20 mM Tris, 10 mM MgCl₂, 40 mM dithiothreitol and 4% (w/v) polyvinylpyrrolidone (MW 40,000). After grinding, the resulting homogenate was then centrifuged at 26,000 g for 15 minutes. The supernatant was decanted into a clean test tube and stored at –80°C until assayed for ADH activity.

Assays: The ADH assay was modified from McKee and Mendelssohn (1987), Porterfield (1996) and Sigma Aldrich (quality-control document, product A3263) with stock reagents as follows: 50 mM sodium diphosphate adjusted to pH 8.8 using phosphoric acid (SPP), 19.8 mM acetaldehyde (acetaldehyde) and 7.5 mM β-NADH (NADH). SPP can be prepared and stored at 4°C for long periods of time. NADH stock solution has a useful life of 1-2 weeks if stored at 4°C. Acetaldehyde should be prepared the day of the assay. During the course of the assay NADH and SPP can be kept at room temperature, but acetaldehyde and crude extracts are kept on ice.

One unit of ADH activity is defined as 1 µmol NADH oxidized min⁻¹ mg⁻¹ soluble protein at 25°C (McKee and Mendelssohn, 1987). NADH oxidation is measured as a decrease in absorbance at 340 nm over a course of six minutes. An extinction coefficient of 6.22 (Sigma-Aldrich) was used in calculating the enzyme’s specific activity. Total protein was measured using the Bradford (1976) assay.
All assays were run using a Hitachi U-2001 dual-beam spectrophotometer and a final reaction volume of 1 mL. Sample measurement consisted of the following mixtures: in reference cell, 934 µL SPP, 33 µL acetaldehyde, 33 µL NADH; in the sample cell, acetaldehyde and NADH volumes were the same and 868 µL of SPP and 66 µL of crude enzyme extract were used. Reagents were mixed before enzyme extract was added. Only data from the linear portion (defined as an $r^2$ value > 0.95) of the assay was used to calculate initial velocity and specific activity. All reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Data Analysis

Except where noted, the data presented are the result of two independent replicate runs for each experimental system. Section number indicates a group’s position, not distance, from inlet to outlet. Data from each experiment were gathered and entered into Microsoft Excel (Microsoft, Redmond, WA) and analyzed using the data analysis expansion pack.

RESULTS

Given the problem of providing adequate oxygenation to the roots of plants growing in microgravity and the capability of the mist bioreactor to reduce this problem we considered that a mist-based system using an ultrasonic nozzle may reduce hypoxic stress experienced by plants growing in a ground-based nutrient delivery system. To this end, roots of germinated peas experiencing less hypoxic stress should have higher biomass yields and lower specific ADH activity compared to roots experiencing greater amounts of hypoxia.
**Root Growth**

Roots harvested from the mist delivery system had the highest overall fresh weight per germinated pea (Figure 3A). Roots harvested from the mist and porous tube systems both had greater root mass than hypoxic and normoxic controls. As expected, roots in the normoxic control had a higher fresh weight per germinated pea than the hypoxic control (Figure 3A).

To determine if use of a mist for nutrient delivery was practical in a scaled-up culture system, we tripled the length of the initial culture trough. We also altered aeration rates in the porous tube and drip control systems to ascertain if an increase in aeration, although fed into the media reservoir and not directly into the root zone, would alter root growth and hypoxic stress. There were no statistically significant differences in growth of roots taken from either the porous tube or the mist-based systems. Indeed, root mass per germinated pea was comparable for all nutrient delivery systems and sections therein (Figure 4A). Furthermore, there was no apparent gradient, which might be seen as an upward or a downward trend in root growth as one compared peas from the inlet to the outlet of each of the scaled-up systems (Figure 4A).

**Hypoxic Stress**

Roots were assayed for ADH activity to determine their relative response to hypoxic stress. We first measured responses of roots grown in the short culture systems. Although not statistically significant, roots harvested from the mist system showed slightly lower ADH activity than roots grown in the porous tube system (Figure 3B) suggesting better aeration occurred via misting. Roots in hypoxic and normoxic controls had the expected high and low levels of ADH activity, respectively (Figure 3B). Roots
Figure 3. Growth and ADH responses of germinated pea roots grown 7 days in the short culture vessels. Error bars represent one standard deviation. Results are averages of three replicate experiments. A. Average root mass per germinated pea. All roots from each system were pooled and the weight divided by the number of germinated peas. B. Specific ADH activity.
grown in the mist and porous tube systems had ADH activities more similar to the normoxic control than the hypoxic control (Figure 3B).

When the culture system length was scaled up threefold, there was no discernable gradient of ADH activity in roots harvested from plants in any of the six sections, from inlet to outlet of all the systems tested (Figure 4B). Overall, roots harvested from the most-highly aerated drip system had the lowest levels of specific ADH activity (Figure 4B) whereas roots harvested from the porous tube system run without supplemental aeration had the highest levels.

When the nutrient medium in the reservoir was aerated, roots grown in the porous tube and the drip systems had lower specific ADH activities compared to parallel unaerated runs (Figure 4B). Although roots grown in the aerated porous tube and in the drip systems had ADH levels somewhat lower than roots grown in the mist system, some sectors were equivalent (Figure 4B). Interestingly, when the aeration level was increased tenfold, from 20 to 200 mL air min⁻¹ in the drip system there was not as large a corresponding decrease in activity as the initial 0 mL/min to 20 mL/min drop. This suggests that the ADH activity in roots grown using 200 mL/min aeration rate is the minimum level achievable for peas under these conditions (Figure 4B).
Figure 4. Growth and ADH responses of germinated pea roots after 7 days in the long culture vessels. Data represent the average of two replicate experiments. Vertical bars indicate the high and the low points from the average. Each point represents a pooling of roots from nine peas in each section. A. Root fresh weight per germinated pea. B. Specific ADH activity.
DISCUSSION

To our knowledge this is the first report of an active nutrient delivery system that is suitable for use in microgravity and does not use a porous tube (Porterfield, et. al, 2003). A decrease in root hypoxia, as measured by ADH activity, relative to roots in the porous tube system was seen in pea roots grown in the mist-based system. There are three principle differences between the porous tube and mist-based systems. First, the ultrasonic nozzle of the mist-based system is capable of co-injecting gas with the liquid media. This allows for direct manipulation of gas flow and composition into the root zone. Second, the generation and propulsion of aerated mist into the substrate should inhibit the formation of stagnant air pockets. Finally, increased surface-to-volume ratios of the fine mist droplets should also allow for enhanced air transport capabilities when compared to bulk liquid transport through a porous tube.

It has been hypothesized that as capillary action radially draws liquid into the root zone, stagnant air pockets form as the liquid media seals off pockets of gas (Monje, et. al, 2003). Aeration of the media fed into the root zone, however, should not be overlooked. Recent surveys of nutrient delivery systems demonstrate that, to date, studies have chiefly relied on passively using temperature to control the content of dissolved oxygen. No effort has been directed into actively enriching the oxygen content of media (Porterfield, et. al, 2003; Monje, et. al, 2003). Our data, particularly from roots in the long culture vessels (Figure 4B), suggest that mode of media delivery into the root zone may not wholly be responsible for hypoxic stress. This is seen particularly in the great decrease in specific ADH activity in roots from the porous tube and drip systems when provided with some supplemental aeration.
Despite a tenfold increase in aeration rate, roots in the drip system did not show proportionate decreases in ADH activity (Figure 4B). Thus, although media aeration can play a role in alleviating hypoxic stress, it is limited in its ability to solve all media-transport issues. Also, in spite of being aerated, it is possible that trapped air pockets will still form if a delivery system cannot provide for adequate media exchange through the root zone. In addition to studying movement of liquids through the substrate, future work should also measure dissolved oxygen content of media entering and leaving the root zone. Furthermore, as suggested by Poerterfield et al. (2000b) it is also important to measure not only delivered oxygen but also biologically available oxygen to determine what gas is actually accessible to the roots.

Although this report shows a reduction of hypoxic stress in roots from plants grown in aerated systems, these promising results should be viewed as valid only for the first week of germination. It remains to be seen if reduction in hypoxic stress indicators in the roots can be maintained over the full lifespan of the plants. Also, it remains unclear if overall plant health and development will be affected by altering the mode and level of gas delivered to the roots. Issues relating to mode of nutrient delivery, not supplemental aeration, may also arise when growth extends beyond a week.
ACKNOWLEDGEMENTS

The authors would like to thank Mary Musgrave (University of Connecticut) and Marshall Porterfield (University of Missouri) for insight and guidance, and Tom Dreschel and Gary Stutte (Dynamac Corporation) for the donation of porous tubes and technical specifications. Finally, thanks to Melissa Towler, Timothy Iskra and other members of WPI’s plant research group for comments, techniques and support. Financial support from WPI is greatly appreciated.
Chapter 2: Life Cycle Study*

Evaluation of various modes of nutrient delivery over the lifespan of peas grown in a controlled environment

SUMMARY

In a previous study it was shown during the first week of germination that media aeration affected shoot health more than mode of media delivery. The purpose of this study was to evaluate pea plant responses to various modes of nutrient delivery over the life cycle of the plant. Earligreen peas, a dwarf variety, were grown in sealed culture vessels that were well aerated but only via three different modes of nutrient delivery into the surrounding substrate of the root zone: mist, porous tube and a drip control. Measurements recorded for each system included fresh weight (roots, shoots, leaves), hypoxic stress of the roots and chlorophyll content. Of the three systems, plants grown in the porous tube system consistently had the highest levels of biomass and the lowest levels of hypoxic stress. Plants grown in the porous tube system also had the lowest total chlorophyll and a chlorophyll a/b ratio similar to the mist system. No plants produced fully mature seed. This study marks the first time the porous tube system has been compared with another active nutrient delivery system over the life cycle of a chosen species.

* This section submitted for publication.
INTRODUCTION

To sustain a long-term mission in an enclosed environment, it is necessary to provide food, recycle air and water, and maintain the psychological wellbeing of the humans residing in the environment. This is the primary goal in the development of a controlled ecological life support system (CELSS) (Monje et al., 2003; Porterfield, et. al, 2003). The development of such a system necessitates growing plants in an enclosed and highly regulated environment. Factors that must be considered in the design of such an environment include light, air, temperature, humidity, nutrient delivery, and waste removal (Heathcote et. al, 1996). In the root zone, water’s high surface tension and cohesiveness under microgravity conditions rules out conventional hydroponic and aeroponic nutrient delivery systems (Levine et. al, 2001). In both the shoot and root zones understanding gas flow, particularly the movements of oxygen, carbon dioxide and ethylene, is especially important.

In microgravity, the lack of a significant gravity vector is responsible for the absence of buoyancy-driven convection currents that under normal gravity are responsible for the delivery of oxygen and carbon dioxide to plant tissues (Porterfield et. al, 1997, Monje et al. 2003). Under microgravity conditions it is thought that boundary layers depleted of CO₂ and O₂ form around plant tissues and limit the access of those gases to the plant tissue (Monje et al., 2003, Porterfield, 2002). Data from spaceflight studies using Arabidopsis have shown various problems in the shoot zone that affect normal reproductive organ development (Kuang et. al, 1996; Musgrave et. al, 1997) when compared to ground controls (Table 1). These problems include: flower/reproductive organ senescence, decreased biomass, deformed reproductive organs,
Table 1. Abbreviated history of plant nutrient delivery systems used in microgravity. * Data and observations compiled from Musgrave, et al. (1997).

<table>
<thead>
<tr>
<th>Plant Type</th>
<th>Flight</th>
<th>Chamber</th>
<th>Nutrient Supply/Delivery</th>
<th>Substrate</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Epidendrum radicans</em></td>
<td>Salyut 6</td>
<td>Malachite</td>
<td>Water supplied to bed</td>
<td>Ion exchange resin</td>
<td>Flowers present at launch senesced. No new flowers formed. (Nechitailo and Mashinsky, 1993)</td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td>Salyut 6</td>
<td>Oasis</td>
<td>Water through hydrophilic membrane</td>
<td>Ion exchange resin</td>
<td>Plants died at flowering, due to open design of system (Nechitailo and Mashinsky, 1993).</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>Salyut 6</td>
<td>Sveto-1</td>
<td>None</td>
<td>1.5% agar-based medium</td>
<td>Plants flowered. No viable seed produced. (Kordyum, et al., 1983)</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>Salyut 7</td>
<td>Phyton</td>
<td>None</td>
<td>1.5% agar-based medium</td>
<td>First complete life-cycle achieved in space. 33% of siliques contained aborted ovules. 50% of seeds were viable upon germination (Merkys and Laurinavicius, 1983).</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>Mir</td>
<td>Sveto-M</td>
<td>None</td>
<td>Agar-based</td>
<td>Chlorophyll a + b levels reduced (ratio unchanged). Poor growth, delayed growth, sterile and viable seed produced (viability determined on ground). (Mashinsky, et al., 1994).</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>CHROMEX-03 (STS-54), CHROMEX-04 (STS-51), CHROMEX-05 (STS-68)</td>
<td>Plant Growth Unit</td>
<td>Agar-based</td>
<td>Phenolic foam</td>
<td>Rosettes initiated flowering shoots on-orbit. Equal flowering in microgravity and ground controls. 65% of pollen found non-viable. Poor formation of reproductive structures. Increased ADH activity relative to ground controls. (Kuang, et al., 1996; Crispi, et al., 1996; Musgrave, et al., 1997; Porterfield, et al., 1997).</td>
</tr>
<tr>
<td>Brassica rapa</td>
<td>“Greenhouse 3” (STS-84 to Mir, STS-86 return)</td>
<td>Svet</td>
<td>Water delivered via porous tube</td>
<td>Turface / Osmocote mix</td>
<td>Over 122 days, two full life cycles and part of a third were achieved. Plants grown from seeds produced on-orbit had inhibited growth and development. Silique ripening was found to be basipetal, rather than simultaneously along the length (Musgrave, et al., 2000).</td>
</tr>
</tbody>
</table>
unviable pollen and seed, decreased chlorophyll a and b levels (but no change in ratio), and increased ADH activity and transcription (Table 1). A number of these problems are related to poor gas exchange both in the root and shoot zones.

The poor gas exchange around shoots in microgravity is readily obviated by facilitating air exchange using a fan as shown in the CHROMEX series of experiments (Table 1). It is still necessary, however, to monitor and control levels of CO$_2$ and ethylene as shown in the Svet series of experiments where wide fluctuations in CO$_2$ levels were observed despite use of a fan in the shoot zone (Musgrave, et al., 2000).

Although progress has been made in eliminating gas flow problems in the shoot zone, plant stress caused by poor aeration in the root zone has been difficult to avoid (Monje, et al., 2003). Prior to the introduction of porous tube based systems most nutrient delivery systems relied upon nutrient-charged agar gel or fibrous ion exchange resins to provide nutrient delivery and root support (Table 1). Problematic to the use of agar gel and resin exchange systems was the low redox potential of the agar media coupled with the lack of media exchange capabilities (Porterfield, et al., 1997). The porous tube systems now in use provide a means for uniformly distributing media across the root zones of nutrient delivery systems. However, as the liquid moves radially outward from the tube into the surrounding substrate, it is likely that trapped air pockets form that subsequently become depleted of oxygen when penetrated by roots (Monje, et al., 2003). It is thought that the hypoxic stress experienced by roots grown in porous tube systems contributed to the overall decrease in health of plants grown in microgravity (Monje, et al., 2003; Porterfield, et al., 2000b). Indeed, hypoxia in roots, as measured by alcohol dehydrogenase (ADH) specific activity was elevated in roots from plants grown in
microgravity compared to ground controls (Porterfield et al., 1997; Porterfield et al., 2000 a, b; Monje et al., 2003).

A nutrient delivery system that uses a mist to facilitate penetration of nutrient media across the entire cross-section of the root zone may inhibit the formation of stagnant air pockets thus leading to a decrease in hypoxic stress in roots. Indeed, hypoxic stress, as measured by ADH expression and activity, was eliminated when nutrient mists were fed to dense beds of roots (Weathers et al., 1999). Besides elimination of hypoxia, another benefit of a mist-based system is control of gas composition fed into the root zone (DiIorio, et al., 1992; Weathers, et al., 1997, Wyslouzil, B.E., et al., 2000). For example, roots of many plant species grow best in air enriched with low levels of CO₂ (Weathers and Zobel, 1992; DiIorio, et al., 1992).

In a previous study (Romagnano and Weathers, 2004) we examined growth and hypoxic stress of peas germinated in mist and porous tube nutrient delivery systems. Although, roots from germinated peas grown in the mist and porous tube systems produced similar amounts of biomass, roots from the porous tube system showed more hypoxic stress than those from the mist system. Supplemental aeration of the medium reservoir of the porous tube system, which is difficult at best under microgravity conditions, eliminated the disparity in hypoxic stress between the modes of nutrient delivery. Here we use the conditions established from that work to study the impact that the mode of delivery of both gas and liquid nutrients to the root zone has on peas over the course of their life cycle. This is the first study that compares full life cycle plant growth in a porous tube system to growth in another active nutrient delivery system.
MATERIALS AND METHODS

Culture Systems

Culture Trough Fabrication: For all experimental systems, culture troughs were fabricated from plastic rain gutters (WormsWay, Worcester, MA; Figure 1 A). Gutters were cut to just over 45.7 cm in length to allow for end-cap overlap. End pieces modified for respective delivery systems were fitted to each gutter. Fabricated end-caps were formed from 6.35 mm thick clear polycarbonate plastic (Plastics Unlimited, Worcester, MA).

Substrate and Media: Sieved (1 mm obtained diameter) TurfaceMVP™ (PROFILE Products, LLC, Buffalo Grove, IL) was used as the substrate in the experimental units for all replicate runs. One quarter strength Hoagland’s solution, kept in 1L Gibco™ bottles, was the medium used for the duration of each experiment.

Drip Control: Liquid media propelled using a peristaltic pump was dripped from a tube located at one end of the culture trough and at 1 cm depth into the system and allowed to freely flow to the drain. Media was fed at a rate of 10 ml min⁻¹ for 1 min every two hours.

Mist Delivery: An ultrasonic nozzle operating at 60 Hz with 115 V (model# 2-60TS, Sono-Tek, Milton, NY) was used to generate and propel mist into the root zone of the mist system. This nozzle (Figure 5, A at inlet) generates a mist with a nominal particle size of 30 µm. Maximum liquid flow capacity for the nozzle was found to be approximately 15 mL min⁻¹. For all experiments, the nozzle was run at 10 mL min⁻¹ for one minute every two hours. Aeration of the root zone was accomplished by co-injection of ambient air (see below) through the sonic nozzle.
Figure 5. Experimental system and plant morphology. A. Example of a mist experimental unit. Gas samples were removed from the root and shoot zones via rubber septa (example indicated by arrow). B. Example of a yellow, curling, pod. Such pods were seen on plants from all systems. C. Example of a primary root bent to 90° from the original axis due to shallow depth of culture trough.
**Porous Tube**: Culture troughs with a length of 45.7 cm used a 45.2 cm alumina porous tube with a nominal pore size of 12 µm, o.d. of 1.3 cm, and a 0.79 cm i.d. (Refractron Technologies Corp., Newark, NY 14513-9796). By sealing the outflow end of the tube it was ensured that media fed under a positive pressure would exit only through the tube walls into the root zone. The top of the porous tube was set 1 cm below the Turface™ surface. A center support fabricated from a shaped 1 mL plastic pipette tip was used to stabilize the tube in the trough. A media delivery cycle of 10 mL min⁻¹ for 1 min every 2 hours was used.

**Plants and Growth Conditions**

For all experiments “Earligreen” peas (Western Regional Plant Introduction Station, Acession # 365417, Pullman, WA) were used due to their dwarf size (~25 cm) and defined morphology. Seeds were imbibed in diH₂O overnight prior to planting and then grown for forty days in the three experimental culture systems. In mist and porous tube systems (Figure 5 A, C), the imbibed peas, placed at a depth of 1 cm, were spaced 2.5 cm apart along the length of the culture trough as a single row down the center axis (Figure 2). In total, eleven peas were planted in each system and grown in a temperature controlled room at 20°C and 50% relative humidity.

Light was continuously provided by six 1500 watt fluorescent bulbs suspended directly above the experimental systems. Light intensity at the surface of the Turface in the culture trough was 500 µmol m⁻² s⁻¹.

**Developmental Milestones**: The day a particular life cycle milestone was achieved was recorded during the course of the experiments. The milestones were: germination, first leaves, mature tendrils, flowering, pod formation, and mature pods.
Germination was defined as that time when the cotyledon was first visible above the substrate. First leaves were recorded after they had unfolded from the shoot. Mature tendrils were defined only as those tendrils that had a tripartite differentiation. Flowering was recorded as the first opening of the first flower bud. Pod formation was noted as soon as the pod emerged from the fertilized flower. Finally, pods were considered mature only if they had swollen and contained mature seed. Mature seeds were subsequently harvested and germinated to determine viability.

Aeration rates: In the mist system, ambient air was co-injected through the ultrasonic nozzle at 200 mL min$^{-1}$ for 1 min every two hours. In the porous tube and drip control systems ambient air was fed at a rate of 200 mL min$^{-1}$ for 1 min every two hours into the media reservoir from an air line. The aeration rate was chosen based on previous germination studies (Romagnano and Weathers, 2004). To simulate the poor gas exchange in microgravity the shoot zone was sealed using clear boxes fabricated from 3.17 mm thick polycarbonate (Plastics Unlimited, Worcester, MA). Chamber height was 30 cm. The chamber was sealed to the culture trough using aquarium safe 100% silicone glue (DAP, Inc, Baltimore, MD). Although convection currents would still be present, the only gas entering into the experimental troughs would be that gas transported by the nutrient delivery system into the root zone. Thus, the data generated would mainly be the result of differences in modes of media delivery rather than aeration effects. Septa for the gas samples were placed as shown in Figure 5A: half way up the shoot zone enclosure and along one side of the culture trough at 1 cm below the lip.
**Harvest and Extraction**

**Roots:** Plant roots were harvested and extracted following a procedure modified from the works of Porterfield (1996) and McKee and Mendelssohn (1987). The roots from individual plants were harvested from each culture system, assigned a plant number from inlet to outlet, rinsed, blotted, and the fresh weight recorded. The roots were then frozen in liquid nitrogen. Time from system shut down until all of the roots were frozen was kept under an hour. If roots were not immediately extracted, they were stored at –80°C until further processing was possible.

To facilitate disruption, frozen roots were re-dipped in liquid nitrogen just prior to grinding in a mortar and pestle using 2-4 mL of Tris breaking buffer per gram of root tissue. The breaking buffer, pH 7.5, consisted of: 20 mM Tris, 10 mM MgCl₂, 40 mM dithiothreitol and 4% (w/v) polyvinylpyrrolidone (MW 40,000). After grinding, the resulting homogenate was centrifuged at 26,000 g for 15 minutes. The supernatant was decanted into a clean test tube and stored at –80°C until assayed for ADH activity.

**Shoots:** Prior to root harvesting, shoots were removed from their root systems at the transition region. Shoot height and weight were recorded along with any measurements for pods. Shoots were then individually wrapped in aluminum foil, immersed in liquid nitrogen and then transferred to a –80°C freezer for storage until chlorophyll assays were performed.
Assays

Root Hypoxia: The ADH assay was modified from McKee and Mendelssohn (1987), Porterfield (1996), and Sigma Aldrich (quality-control document, product A3263), and described in detail in Romagnano and Weathers (2004).

Chlorophyll: Prior to assay, shoots were removed from storage and thawed. Number of leaves and leaf weight were recorded before the leaves were ground in a mortar and pestle containing fine glass beads (< 1 mm diameter, enough to cover the bottom) and 100% acetone (≈ 5 mL to start). Extraction was done in a darkened room to prevent photobleaching of the chlorophyll. The acetone extract was then vacuum filtered through Whatman #1 filter paper into a clean flask. Tissue was exhaustively extracted until the ground material either turned completely white or freshly added acetone no longer tinted green when ground with the leaves. Crude extract was then transferred from the filtrate flask into a graduated cylinder. After the flask was rinsed three times with acetone (using a squirt bottle to “spray” the sides), the final volume of the acetone extract was measured. A 1:20 or 1:40 dilution of the extract with acetone was performed and the O.D. of the resulting solution was measured at 664 nm, 647 nm, 630 nm and chlorophyll a and b content calculated with an additional turbidity correction at 730 nm according to (Jensen, 1978).

Gas Analyses

Ethylene: A Perkin-Elmer Sigma 3 gas chromatograph using a flame ionization detector coupled with a poropak-N column (Supelco, Inc.) was used to separate ethylene from 200 µL air samples drawn from either the shoot or root zone (Figure 5A). Carrier gas flow-rate was 15 ml/min. The limit of detection was 7 ppm. Retention time for the
ethylene standard was at 0.83 min ± 0.005 min. Air samples were drawn using a 1 mL syringe with 26 ga needle (Becton-Dickenson) from rubber septa located in the root and shoot zone walls (Figure 5A). A Hewlett-Packard numerical integrator was used to record peak data from the detector. Triplicate samples were taken just after media/air cycling occurred in individual systems.

**CO₂ and O₂:** A Perkin-Elmer 8500 gas chromatograph using a hot wire detector coupled to a Carboxen 1000 (Supelco, Inc) molecular sieve column was used to separate and detect CO₂ and O₂. Aliquots of 200 µL samples were taken as described for ethylene. Retention times for O₂ and CO₂ were 2.14 and 8.45 min ±0.05 min, respectively. Limits of detection were 0.4% and 0.2% for O₂ and CO₂, respectively.

**Data Analysis**

The data presented are the result of two independent replicate runs for each experimental system. Plant number indicates a plant’s position, not distance, from inlet to outlet. Data from each experiment were gathered and entered into Microsoft Excel (Microsoft, Redmond, WA) and analyzed using the data analysis expansion pack.

**RESULTS**

In evaluating pea plant responses to various modes of nutrient delivery over the life cycle of the plant, we observed that, overall, plants grown in the porous tube system were healthier and more productive than those grown in the other two systems. In no case, however, did plants in any of the three systems produce mature seed.

**Biomass/Morphology:** Plants grown in the porous tube system had the highest amount of shoot, root and leaf biomass (Figure 6 A, B, C). Whereas plants grown in the
drip and porous tube systems showed a steady decrease in shoot, root and leaf biomass from inlet to outlet, plants grown in the porous tube system maintained a fairly uniform biomass across the total length of the system (Figure 6 A, B, C) indicating that a more uniform distribution of nutrients occurred via the porous tube. A similar trend was also observed for shoot height and number of leaves (Figure 7 A, B). When data from all three experimental systems are pooled, a general positive correlative trend was seen for shoot height and leaf mass and also between root mass and leaf mass (Figure 8 A, B).

**Achievement of Life Cycle Milestones:** Through the first week of growth, plants grown in the various delivery systems were consistent in time to germination and emergence of first leaves (Figure 9). Beginning with tendril differentiation, however, plants growing in the porous tube system began to show a delay in development when compared to their counterparts in the mist and drip systems, 15 d on average compared to 11 d and 12 d for mist and drip systems, respectively (Figure 9). This delay carries through to flowering and pod development. No plant in any system, however, produced fully mature pods with set seed (Figure 9). This is interesting considering that plants previously grown using a similar system but with an open shoot zone achieved mature seed in 40 days (data not shown).

**Hypoxic stress:** Consistent with the higher biomass produced, plants grown in the porous tube system had the lowest amount of specific ADH activity in their roots (Figure 6 D) indicating that they had the least hypoxic stress overall. Roots from plants grown in the mist system and the drip controls showed increasing ADH activity, respectively (Figure 6 D). In contrast to biomass, which decreased from inlet to outlet in plants from the mist and drip systems, no apparent gradient in ADH activity was present. Low ADH
levels also appeared to correlate with higher biomass (leaf, shoot+leaf, and root) levels. The decreasing pattern in biomass, porous tube > mist > drip systems, mirrored the increasing ADH activity, porous tube < mist < drip. No correlative trends were seen between ADH and chlorophyll, root mass, shoot mass, leaf mass, number of leaves or shoot height (data not shown).

Figure 6. Biomass and hypoxic stress of peas harvested after 40 days growth from drip, mist and porous tube systems. A, Average shoot (stem + leaves) fresh weight (g). B. Average root fresh weight (g). C. Average leaf mass (g). D. Average specific ADH activity. Bars mark high and low difference from the average of two independent replicate experiments. Plant # is indicative of position from inlet, not distance.
**Chlorophyll:** Specific chlorophyll levels increased in plants from all systems from inlet to outlet (Figure 7 C). This increase is mirrored in both chlorophyll a and b levels (data not shown) and is in contrast to what was observed for biomass (Figure 6 A, B, C). Although the chlorophyll a/b ratio remained constant for leaves from all systems across the length of the culture trough, the ratio in shoots from the drip system was higher than from either the mist or porous tube systems (Figure 7 D). When total chlorophyll levels from all the systems were pooled and compared with root and leaf weights, there were generally negative correlation trends (Figure 8 C, D).

**Gas Analyses**

No ethylene was detectable in any system at any time before harvest. Although analysis at harvest time showed possible trace levels of ethylene (~7ppm), the data were not conclusive. Physiological indicators of ethylene, however, were observed and included shortening of stressed shoots, leaf and flower senescence, and shortened, yellow, curled pods (Figure 5 B). No changes from ambient (i.e., ~21% O₂, ~0.1% CO₂) in either oxygen or carbon dioxide levels were observed in any of the systems in either the root or shoot zones at any time. However, since samples were taken just after the addition of nutrients and air into the root zone it is possible that this stability in gas levels is the result of flushing rather than any physiological responses.
Figure 7. Shoot morphology and chlorophyll content of peas harvested after 40 days growth from drip, mist and porous tube systems. A. Average shoot height (cm). B. Average number of leaves per plant. C. Total chlorophyll content (mg gF.W.\(^{-1}\)). D. chl a/ chl b ratio. Bars mark high and low difference from the average of two independent replicate experiments.
Figure 8. Correlations between different measured parameters for data pooled from all three systems. A. Shoot height vs. leaf mass. B. Root mass vs. leaf mass. C. Total chlorophyll vs. root mass. D. Total chlorophyll vs. leaf mass.
DISCUSSION

In order to simulate poor gas exchange conditions found in microgravity, the environment of the plant growth chamber was sealed to prevent free gas exchange. This allowed for an examination of the capabilities of the nutrient delivery systems to support plant life by feeding gases only through the root zone irrespective of poor conditions in the shoot zone. Furthermore, this work supplements previous studies, such as the Chromex series of experiments and their ground-based follow-ups, in which the mode of nutrient delivery was constant but aeration in the shoot zone varied (Musgrave, et al., 1998; Crispi, et al., 1996; Porterfield, et al., 2000 b).

Taken together, data from the root and shoot biomass coupled with ADH activity indicated that, in general, plants growing in the porous tube system were more productive and suffered less hypoxic stress than those growing in the other two systems (Figure 2). Furthermore, it appears that the porous tube system is better able to maintain uniform

Figure 9. Timeline of Developmental Milestones. G, germination; L, appearance of first leaves; T, mature tendrils; F, flowering; P, pod formation; MP, mature pods. Superscripts for P and MP symbols in the greenhouse row indicate average day observed. Open chamber indicates plants grown in culture vessel in climate-control chamber with no shoot zone enclosure and passive water-on-demand nutrient delivery.
plant growth than the other systems suggesting that there is a more homogenous
distribution of nutrients from inlet to outlet (Figures 2, 3 A, B). These results agree with
the original characterization of the system by Porterfield using dwarf wheat (Porterfield,
1996).

Although ethylene was barely detected analytically (7 ppm), physiological
evidence of its presence was seen. Plants that were more hypoxically stressed were
shorter, had senesced leaves and flowers, and produced pods that remained short,
yellowed and curled. Indeed the short, yellow, curled pea pod is strongly reminiscent of
the classic “triple response” assay originally described by Neljubow (1901) and used as a
bioassay for ethylene until the advent of gas chromatography. The premature senescence
of flowers and leaves seen in plants from all our experimental systems are also classic
ethylene symptoms documented by Beyer (1976). Also, the fact that developmental
milestones were attained later in plants from the porous tube system suggests that
premature aging in plants from the mist and drip systems could have been brought on by
hypoxic stress coupled with higher ethylene production.

Hypoxic stress alone may not be the only source of premature ethylene evolution.
The physical shape and depth of the root zone, particularly mechanical barriers, may play
a role in exacerbating excess ethylene production. Indeed, many of the primary roots
from plants in all systems were bent 90° from the vertical axis to subsequently travel
along the bottom of the culture trough (Figure 1 C). This is consistent with changes in
morphology seen by Schwarzbach et al. (1991) who observed increased ethylene
production by germinating pea seedlings when their shoots encountered a plexiglass
barrier. It is, thus, conceivable, that the bent roots (Figure 1C) harvested in these
experiments could be producing ethylene as a stress response. Although good oxygenation would be expected in a shallow root zone it may be obviated by root bending resulting in production of ethylene that disrupts reproductive development.

In addition to physical size requirements, attention must also be paid to obstacles in the root zone. The porous tube, while ensuring a uniform distribution of medium into the surrounding substrate, also presents a significant barrier for germinating radicles to grow around; it also complicates root harvest. The latter point is relevant when one considers an astronaut with limited time who must efficiently remove an old crop and plant a new one. Although the mechanical barrier is not contributing to hypoxic stress, it may be contributing to the overall concentration of ethylene in the system as evinced by observed physiological indicators of ethylene.

There have been a number of studies to determine how liquid media moves out of the porous tube into the surrounding substrate under microgravity conditions (Bingham et al., 1996; Bingham et al., 1997; Jones and Or 1998; Salisbury, F.B., 1997; Yendler et al., 1995). Liquid media flow experienced by plants growing under the normal gravity conditions in which these experiments were conducted should be different than flows under microgravity conditions. As such, benefits that the mist-based system could potentially deliver, particularly the ability to directly inject and control gas composition in the root zone (DiIorio, et al., 1992; Weathers, et al., 1997; Weathers et al., 1999; Wyslouzil, et al., 2000) may not have become evident. For example, beet roots grown under a controlled atmosphere with slightly elevated levels of CO\textsubscript{2} attained a higher biomass than those grown with a decreased level (DiIorio, et al., 1992; Weathers and Zobel, 1992). Also considering that unused media could freely drain from the culture
vessels the formation of stagnant air or media pockets probably did not occur which is in contrast to what likely occurs in microgravity (Porterfield, 2002). Furthermore, mist penetration into the root-zone is likely to be affected by variations in the gravity vector. Experiments under microgravity conditions would be more likely to truly ascertain the performance capabilities of each system.

By using culture vessels with sealed shoot zones, this study shows that we designed a good ground-based system for testing and developing more effective nutrient delivery systems for growing plants in microgravity. This design allowed analysis of plant growth where only root zone aeration was directly controlled. To our knowledge, this is also the first time the porous tube system has been directly compared to another active nutrient delivery system over the complete life cycle of a chosen plant species. Although, in terms of ADH activity and biomass yields, the porous tube out-performed the mist and drip control systems, the method used here to aerate the media fed into porous tube system is not practical for use in microgravity and remains to be improved.

This study should be used as a starting point for refining methods of nutrient delivery aimed at eliminating the formation of stagnant air and media pockets in the root zones of plant systems used in microgravity. Microgravity studies in which multiple modes of nutrient delivery are employed should provide insight not only into factors affecting plant stress but also data for use in models of fluid movement through substrate in microgravity.
ACKNOWLEDGEMENTS

The authors would like to thank Mary Musgrave (University of Connecticut) and Marshall Porterfield (University of Missouri) for insight and guidance and Tom Dreschel and Gary Stutte (Dynamac Corporation) for technical specifications and support. Finally, thanks to Melissa Towler, Timothy Iskra, and other members of WPI’s plant research group for comments, techniques and support. Financial support from WPI throughout the course of this study is greatly appreciated.
CONCLUSIONS AND FUTURE WORK

The goal of this work was to understand how hypoxic stress experienced by plants is impacted not only by mode of nutrient delivery but also by varying aeration rates into the root zone. To this end, two studies were conducted. Both studies were the first time that growth of plants in a porous tube system was compared to other active nutrient delivery systems. The first study, presented in chapter one, sought to examine how altering mode of nutrient delivery and aeration rates affected biomass and ADH activities of germinating pea roots.

Effects of altering the length of the culture trough were also examined and compared against hypoxic and normoxic controls. Roots grown using different modes of nutrient delivery in short culture vessels all had comparable levels of biomass. ADH levels for roots grown in the mist and porous tube systems were comparable to each other and the normoxic control. When the length of the culture vessel was scaled up three-fold, root biomass levels remained consistent not only between roots grown using the different nutrient delivery systems, but also, with the exception of roots from the hypoxic control, with roots from short culture trough systems. ADH levels from roots grown in all modes of nutrient delivery were markedly higher in plants from the culture troughs than those grown in short troughs. Roots grown using the porous tube system in its native configuration (i.e., without aeration) had the highest levels of ADH activity. With the addition of a moderate amount of aeration, however, ADH activities dropped to levels comparable to the mist system. Addition of aeration (20 mL/min) to the drip control system also resulted in greatly reduced ADH activity levels. A further increase in aeration to the drip control system (200 ml/min), resulted in a decrease of ADH activity, although
it was not as great as the drop seen with the lower (20 mL/min) aeration rate. This also
suggests that other factors in the root zone, such as waterlogging or vessel size, may
contribute to hypoxic stress. These results demonstrated that the addition of aeration into
the root zone could effectively reduce amounts of hypoxic stress. The short length of time
that the peas were grown, however, did not allow for comparisons of plant health in the
shoot zone.

The second study sought to compare both shoot and root development among the
three modes of nutrient delivery. Unique to this study was the fact that the shoot zone in
the plant growth chamber was sealed and that aeration was provided strictly through the
root zone at the maximum rate (200 mL/min) used in the previous study. At harvest,
plants from the porous tube system had the highest shoot, root and leaf fresh weights, and
the lowest levels of ADH activities. Shoot height, number of leaves and total chlorophyll
levels were all roughly similar to each other. There was also a notable delay in reaching
the life cycle milestones of plants from the porous tube; this parallels a similar delay
observed for plants grown in the greenhouse or with an open shoot zone. Measured levels
of oxygen, and carbon dioxide did not deviate from ambient during the course of the
study. Ethylene was barely detected at analytical limits (~7ppm), and then only at
harvest.

Although plants from the porous tube system showed increased growth and lower
ADH levels, the mechanism used to aerate the medium reservoir is not feasible for use in
microgravity. During the course of the second study, no mature pods or seed were
produced. This problem may be eliminated when aeration is applied to both shoot and
root zones. More research is needed to determine optimal plant-growth conditions not only for this species, but also for other CELSS candidate crop plants.

Once we have gained more practical knowledge and experience with optimized nutrient delivery systems a key element of a bioregenerative life support system will be attained. A complete system, however, still requires the integration of more elements. For instance, the conversion of waste liquids into usable nutrient media still needs to be integrated. Also, the disposal of solid organic matter (such as non-edible plant tissue), and its subsequent conversion into available nutrients also needs to be pursued. Long duration missions need to be designed so that even packaging materials can be converted into a usable supply of nutrients. Spin-off technologies developed for CELSS projects can also be used to handle waste processes on a large scale. Only when these goals are achieved will we truly be able to break the bonds that tie us to mother Earth and set sail (solar or otherwise) to the stars.
REFERENCES


Appendix A
Schematic of System Configurations
Figure 10. Schematic of equipment and connections used for various modes of nutrient delivery. – Denote equipment and pathways used for the mist-based nutrient delivery system. ▶ Denote equipment and pathways used for the porous tube or drip control nutrient delivery systems.
Appendix B
Gas Analysis Protocols
Ethylene
Perkin Elmer Sigma 3 FID Gas Chromatograph

1. Close oven lid.

2. Turn Power “ON”.

3. Turn Recorder Power “ON”.

4. Hold “Zone Temp” button down, while pressing in sequence:
   “CE MOD”, “1”, “0”, “0” and “ENTER”.

5. Hold “Init Temp” button down, while pressing in sequence:
   “CE MOD”, “5”, “0” and “ENTER”.

6. Hold “Final Temp” button down, while pressing in sequence:
   “CE MOD”, “5”, “0” and “ENTER”.

7. Hold “Init Time” button down while pressing in sequence:
   “CE MOD”, “0”, “0”, “0” and “ENTER”.

8. Turn on all three gas regulators:
   Ultrazero air, 50psi ; Hi Pure N₂, 80psi ; H₂, 30psi   all at regulator.

9. For C₂H₄: set carrier flow 15 ml/min for column A. Column: Poropak N.

10. Wait until all three “Not Ready” lights go off. (about 5 minutes)

11. Pull “Hydrogen A” toggle switch towards you (open) and leave it open for about
    15s. Close it again by pushing it back. Press “Ignite A” button while opening
    toggle switch again. You should hear a definite POP and the pen should jump.

12. Confirm ignition: Open panel to right of injector ports and hold smooth metal
    surface in front of each vent. Condensation on metal indicates ignition. If it is not
    lit, close toggle switch and repeat ignition process (# 11 above).

13. Wait 30 minutes, or until all “Not Ready” lights go off, then press “ready” button.

14. Turn on numerical integrator. Assay time is 1 minute. Set the integrator to the
    following parameters: Stop, 1 min; Threshold 1. All other settings are integrator
    default.

15. Rinse out 100µL Gas-Tight syringe with dH₂O to check for free flow.
16. Inject 200µL into upper injection port. Ethylene comes off at approx. 0.8 min, (see example chromatogram).

Figure 11. Example chromatogram of an ethylene standard.

To Turn off GC (Approx. 10 minutes after last peak)

1. Put syringe away in drawer.

2. Turn off integrator.


4. Turn power “OFF”.

5. Close all gas regulators tightly. Hydrogen won’t go all the way off immediately (the needle will eventually fall), but it doesn’t matter. Compressed air must go all the way off, as judged by needle on 0-4000 regulator gauge (i.e. needle must go to 0).

Notes

C₂H₂: polarity – atten. x 1. range x 1 detector A.
Oxygen and Carbon Dioxide

Perkin Elmer 8500 HWD Gas Chromatograph

Assumes instruction manual read and some basic software navigation skills. Adapted from “Application Note 10” by Supelco, Inc.

1. Turn on GP-100 printer.
2. Check septa for holes.
3. Turn on Helium tank (92 psi line pressure, no less than 500 psi in tank)
4. In “Carrier Gas” control panel:
   a. Supply off → on
   b. Column A mode flow → pressure
   c. Column B mode flow → pressure
5. Turn machine on, wait till initialization is over.
7. Check that method matches sample (press “accept section” to move to next section)

![Printout of HWD GC methods section for CO₂ detection.](image)
8. When satisfied, press F1 “Accept Method” when in Section 1 of Methods section. This brings you to the run-screen.

9. Press “InstTerm” button to call up “Terminal Functions” screen.

10. Select F6 “Plot/Replot” to call up “Plot Control” screen.
   a. Set “Attn” (attenuation) to 8
   b. Set “Plot Run” to NO
   c. Press “InstTerm” button to accept changes and bring back to run-screen.

11. Run protocol without injection twice to clean & purge the column. Start run by pressing “run” button.

12. Once baseline has settled, run air sample spiked with CO₂.
   a. Inject 200 µL sample into column “A” and press “Run” button.


14. Report screen is automatically displayed at end of run. Select F3 “PrintReports” to print a copy of the report.

15. Press F8 “Return to Index” to go back to the “Terminal Functions” screen.
   a. Select F6 “Plot/Replot” to go to the “Plot Control” screen.
   b. Press the “Page” key to go to the replot screen.
   c. Select F6 “Replot on P/P” to print a chromatogram of the sample.
   Example chromatogram is from a 500 µL air sample from an aerial port. Oxygen and Nitrogen do not completely resolve. At an attenuation of 8 O₂ and N₂ peaks are off-scale.

![Figure 13. Printout of HWD GC chromatogram for CO₂ standard.](image-url)
d. You MUST be in front of printer while chromatogram is printing!!! If printing stops, rapidly turn printer off and on to continue. If you miss and the printer stops too long you will have to shut the entire machine off and start over!

16. Press “InstTerm” to go back to run screen. Repeat 12a-15 for each sample.

17. To shut down machine from run screen:

   a. Press “InstTerm” to call up “Terminal Functions” screen.
   b. Select F1 “Method” to go to the “Current Method” screen.
   c. Press F3 “Modify Section 1”
   d. Press number 0 on numeral keypad and then F8 “HWD Range” to stop current from running through the detector. THIS IS IMPORTANT, as it will keep the wires in the HWD detector from breaking.

18. Turn power off, reverse “carrier gas” switches, turn tank off, turn printer off.
Appendix C
Buffer, Extraction, and ADH Assay
Tris Buffer Solution
For crude enzyme extraction.

20 mM (0.020mol) Tris (Sigma # T-1503), pH 7.5 \( \rightarrow \) F.W. 121.1 g/mol
- Adjust using 0.4 M HCl and 0.5 M NaOH

10 mM (0.010mol) MgCl\(_2\)·6H\(_2\)O \( \rightarrow \) F.W. 203.3 g/mol

40 mM (0.040mol) dithiothreitol (DTT) \( \rightarrow \) F.W. 154.24 g/mol

4% (w/v) PVP (Sigma # P-2307)

For 100 mL Total:

Tris: \( \frac{2.4220g}{1000mL} = \frac{x}{100mL} \) \( \therefore \) \( x = 0.2422g \)

MgCl\(_2\)·6H\(_2\)O: \( \frac{2.0330g}{1000mL} = \frac{x}{100mL} \) \( \therefore \) \( x = 0.2033g \)

DTT: \( \frac{6.1696g}{1000mL} = \frac{x}{100mL} \) \( \therefore \) \( x = 0.6170g \)

PVP: 4g
Crude Enzyme Extraction Process

Excised Plant Roots

Rinse in deionized water and blot dry.
Take and record fresh weight of roots.
Freeze roots in liquid nitrogen (N). This is accomplished by pouring the N into a suitable deep container. The roots should be placed into a smaller container, dipped into the N and removed.

Frozen Roots

Grind roots in pre-chilled (-20°C) mortar and pestle using 2-3 ml of pre-chilled Tris buffer (see separate protocol) as the breaking buffer.

Homogenate

Add homogenate to appropriate centrifuge tube for centrifuge being used and add and equal volume of pre-chilled Tris buffer. Vortex thoroughly and centrifuge at 26,000g for 15mins in a chilled centrifuge. Take care to keep temperature between 2-4°C.

Pellet

Supernatant

Pipette supernatent solution from centrifuge tube into sealable test tube. Place on ice immediately.

Assay for ADH
Assay for total soluble protein.

Note: Entire process must be carried out at a temperature between 2-4°C. Keep all materials on ice while working with them.
Assay for Alcohol Dehydrogenase

The ADH assay was modified from McKee and Mendelssohn (1987), Porterfield (1996), and Sigma Aldrich (quality-control document, product A3263). Due to the fact that this assay is partly a reverse of the Sigma process volumes and mole concentrations have been kept the same, where applicable, while the reagents listed in the Sigma process have been changed to match the reversal of the reaction. This is made possible due to the stoichiometric balance of this reaction.

Reaction Equation:

$$\text{Acetaldehyde} + \beta\text{-NADH} \xrightarrow{\text{Alcohol Dehydrogenase}} \text{Ethanol} + \beta\text{-NAD}^+$$

Abbreviations Used:

ADH: Alcohol Dehydrogenase

Acet.: Acetaldehyde (F.W. 44.05; d= 0.79 g/ml = 0.018 mol/ml± )

NADH: β-Nicotinamide Dinucleotide, Reduced Form (M.W. 663)

NAD*: β-Nicotinamide Dinucleotide, Oxidized Form

SPP: Sodium Pyrosulfate Buffer at pH 8.8, 50mM

Reagent Preparation:

Crude enzyme supernatent: see attached protocol

Acetaldehyde: Into 20 ml of water, add 22.00 µL (.198 mmol) of acetaldehyde. This will result in 10 ml of aqueous acetaldehyde solution with the number of moles comparable to the 10ml ethanol solution described in the Sigma process. Resulting solution is 19.8 mM.

NADH: Required, 20 ml of 7.5 mM solution. Into 10 ml of water add 0.09945 g of NADH. Stir until dissolved into solution. Bring volume of solution to 20 ml by slowly adding more water.

To make 100 mL of 50 mM Sodium Pyrophosphate Buffer, pH 8.8 at 25 °C.

1. Add 2.2305 g of Sodium Pyrophosphate (F.W. 446.06), Decahydrate to 60 ml of deionized water.

2. Allow SPP to dissolve completely while on a stir plate.

3. Adjust pH (dropwise) using 8% (v/v) Phosphoric Acid to 8.8 at 25°C.
Assay Procedure: This reaction is monitored by a spectrophotometer that must be pre-set to 25°C. Light path is one centimeter. Absorbance (A) is measured at 340nm (A_340nm). Reactions are performed in a 1ml UV cuvette.

Zeroing the Instrument:

<table>
<thead>
<tr>
<th>Reagent Solution</th>
<th>Reference Cell (uL)</th>
<th>Experimental Cell (uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPP Buffer</td>
<td>967</td>
<td>967</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>1000</td>
</tr>
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</table>

Auto Zero the instrument after OD has stabilized (1-2 min).

Blank NADH: OD should not exceed 2.0 OD. NADH volume can be adjusted if too high or too low.

<table>
<thead>
<tr>
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<th>Reference Cell (uL)</th>
<th>Experimental Cell (uL)</th>
</tr>
</thead>
<tbody>
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<td>934</td>
</tr>
<tr>
<td>Acetaldehyde</td>
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<td>33</td>
</tr>
<tr>
<td>NADH</td>
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<td>33</td>
</tr>
<tr>
<td>Crude Enzyme Extract</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

Use buffer solution that has previously been brought to 25 °C. Let cells stabilize to 25°C for approximately 10s. Have spec. then record reaction for 6 minutes. This provides a baseline for background NADH oxidation. Do not re-zero the instrument!!

Experimental Measurement: Prepare new reference and experimental cells. Acetaldehyde is added last, this starts the reaction in the experimental cell.

<table>
<thead>
<tr>
<th>Reagent Solution</th>
<th>Reference Cell (uL)</th>
<th>Experimental Cell (uL)</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Crude Enzyme Extract</td>
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<td>66</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

Mix cells by inversion and place in spectrophotometer. Allow cells to stabilize for 30s to 1min then record the reaction for 6 minutes. Perform protocol for all systems’ fresh extract first followed by heat-killed extract for all systems. If reaction runs too quickly, add less enzyme and increase the volume of the buffer. If reaction runs too slowly, add more enzyme and decrease the volume of the buffer.
Appendix D
Bradford Protein Assay Protocol
**General Bradford Procedure**

**Dye Reagent Prep**

5x concentrate of Bradford Reagent

Dilute 1 volume of dye reagent to 4 volumes of DI water
*(you’ll want at least 40 ml of solution in order to assay 6 samples as well as construct 3 standard curves)*

Crude Reagent

Thoroughly mix crude reagent using a spin bar for 20 minutes.
Filter solution through Whatman #1 paper or equivalent.

**Prepare Standard Protein Concentrations**

Use purified Bovine Serum Albumin (BSA)
Prepare 10 ml of 2 mg/ml Protein solution in DI H₂O.

Add 20mg of BSA to 10 ml of DI H₂O.

**Concentrated Protein Stock Solution**

Create known Concentration Solutions of Protein

Prepare 200µl of each known solution in separate glass tubes. The following dilutions (in mg/ml) are required: 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0

The following is to obtain 200µl of the specified concentrations:

1.0 is obtained by measuring out 200 µl of stock and adding 0 µl of DI H₂O.

0.8 is obtained by measuring out 160 µl of stock and adding 40 µl of DI H₂O.

0.6 is obtained by measuring out 120 µl of stock and adding 80 µl of DI H₂O.

0.4 is obtained by measuring out 80 µl of stock and adding 120 µl of DI H₂O.

0.2 is obtained by measuring out 40 µl of stock and adding 160 µl of DI H₂O.

0.1 is obtained by measuring out 20 µl of stock and adding 180 µl of DI H₂O.

0 is obtained by measuring 200µl of the buffer in which the protein, normally, is dissolved.

**Known Concentrations Protein Solutions**
Create Standard Curve

This is how to create the standard curve against which you will measure your unknown concentrations.

Prepare a blank by placing 50 µl of DI H₂O in a cuvette and adding 2.5 ml of Bradford reagent. Vortex thoroughly. When placed in spectrophotometer this becomes the blank the other reactions will be measured against.

Measure 50 µl of each known solution into separate cuvettes.

Add 2.5 ml of Bradford reagent to each cuvette containing a protein solution.

Vortex most thoroughly.

Allow each cuvette to wait 5 minutes to 1 hr before putting in Spec.

After chosen time place each cuvette, in turn, in spectrophotometer and measure OD₅₉₅ versus the blank.

Plot OD₅₉₅ versus known protein concentration values. To create Standard Concentration Curve.

Repeat two more times to create Three Standard curves that should look remarkably the same. If the curves are different repeat procedure until three matching curves are created.

Measure OD₅₉₅ of unknown, read value from standard curves to determine concentration of protein in unknown solutions.

Protocol Adapted From:
