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Parameters Necessary for *In Vitro* Hydroponic Pea Plantlet Flowering and Fruiting

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1. Introduction

Flowering *in vitro* has been reported infrequently in tissue culture and the subsequent occurrence of fruiting structures from these flowers is rare (Al-Juboory et al., 1991; Bodhipadma & Leung, 2003; Dickens & Van Staden 1985; 1988; Franklin et al., 2000; Ishioka et al., 1991; Lee et al., 1991; Pasqua et al., 1991; Rastogi & Sawhney 1986; Tisserat & Galletta 1993; 1995). Fruits are complex organs composed of unique tissues that are a source of many important food products, nutrients and phytochemicals. The biosynthesis of phytochemicals common in fruits by cultured vegetative cells and tissues is difficult and usually not achievable; when achieved they usually occur at lesser yields than in vivo derived fruits (Tisserat et al., 1989a; 1989b). Unfortunately, fruit tissues and organs are difficult to establish, maintain and proliferate *in vitro* as such, mainly because they fail to retain their unique tissue and organ integrity within a sterile environment and often generate into a undifferentiated mass (*i.e.* callus) with an altered biochemical metabolism compared to that obtained from the original fruit tissues (Hong et al., 1989; Tisserat et al., 1989a; 1989b). Nevertheless, development of sterile fruit production systems would be useful in order to study the reproductive processes, provide a source of important secondary natural products *in vitro,* provide sterile produce for at-risk populations with weakened immune systems, and aid in breeding projects (Bodhipadma & Leung, 2003; Butterweck, 1995; Kamps, 2004; Ochatt et al., 2002; Pryke & Taylor, 1995).

According to the Centers for Disease Control and Prevention (CDC) each year 76 million people in the USA get food sickness, of these 325,000 are hospitalized and 5,000 die (Anon., 2004). One in every 6 Americans becomes a victim to a serious food poisoning per year (CDC, 2011) The majority of these victims have a weakened immune system and can not effectively fight infections normally (U.S. Department of Agriculture-Food Safety and Inspection Service, 2006; Hayes et al., 2006). Peoples with high risk to food borne infections include: young children, pregnant women, older adults, and persons with weakened immune systems, including those with HIV/AIDS infection, cancer, diabetes, kidney disease, and transplant patients, or those individuals undergoing chemotherapy (U.S. Department of Agriculture-Food Safety and Inspection Service, 2006; CDC, 2011; Hayes et al., 2006; Kamps, 2004). An analysis of food-poisoning outbreaks from 1990-2003 revealed

that contaminated produce is responsible for the greatest number of individual food-borne illnesses (Table 1) (Anon., 2004).

Food Source	Outbreaks No. (%)	Illnesses No. (%)	Common Food Pathogens
Seafood	720 (26%)	8,044 (10%)	*Vibrio parahaemolyticus*
Produce	428 (16%)	23,857 (29%)	*Salmonella*
Poultry	355 (13%)	11,898 (14%)	*Clostridium perfringens*
Beef	338 (12%)	10795 (13%)	*C. perfringens, Escherichia coli*
Eggs	306 (11%)	10449 (13%)	*Salmonella*
Multi-ingredient foods	591 (22%)	17,728 (21%)	Multiple, unknown
Totals:	2,738	82,771	

Table 1. Analysis of monitored food-poisoning outbreaks and number of illnesses from 1990-2003 (Anon. 2004).

Other studies suggest that of the 200,000 to 800,000 cases of food poisoning occurring daily in the USA over one-third to as much as one-half are due to produce contaminates, with ≈90% of that being bacterial (U.S. Department of Agriculture-Food Safety and Inspection Service, 2006; Kamps, 2004; Wagner, 2011). In addition, food poisoning is also caused by heavy metals, fungus, viruses, chemicals (e.g., pesticides), and parasites. All of these contaminates are commonly found on produce, and still produce food poisoning is usually overlooked as the source of the food borne illness (CDC, 2010; Kamps, 2004). Obviously, growing sterile produce would have a niche market for at-risk populations susceptible to food poisoning. One can speculate that the high cost of sterile produce would be acceptable to at-risk populations in certain situations.

Elucidation of the wide range of parameters responsible for flowering and fruiting from cultured plantlets on demand in a single study has not been conducted. A sterile hydroponics culture system, termed the automated plant culture system (APCS) has been employed to obtain reproductive activity (i.e. flowering and fruiting) from cucumber and pepper plantlets (Tisserat & Galletta 1993; 1995). The APCS mimics the hydroponic system by employing a large plant growth culture vessel and immersing and draining the plant periodically with nutrient medium kept in a larger medium reservoir (e.g. 1000-ml aliquot) (Fig.1). The in vitro hydroponic system differs from traditional agar tissue culture vessel (e.g. 25-ml aliquot) in a number of characteristics: 1) plant cultures in the APCS remain intact and stationary for the duration of their growth once established whereas plantlets grown in agar-based cultures must be repeatedly and frequently removed and replanted to obtain fresh medium, 2) the APCS provides considerably more nutrient medium (≈ 40 x) for culture growth (stored in a separate reservoir) than the agar media provided for plantlets grown in culture tubes, 3) plantlets grown in the APCS can achieve considerably higher growth rates and physical sizes than plantlets grown in agar grown cultures, and 4) flowering and fruiting is more readily achievable in plantlets grown in the APCS than in agar cultures tubes.

The very fact that plantlets grown in the APCS can obtain larger sizes than plantlets grown in smaller culture vessels may in it self be the prerequisite to obtain subsequent reproductive activities *in vitro* compared to plantlets grown in the tissue culture vessel (Tisserat & Galletta, 1993; 1995). Further, intermittent forced ventilation of the culture vessel

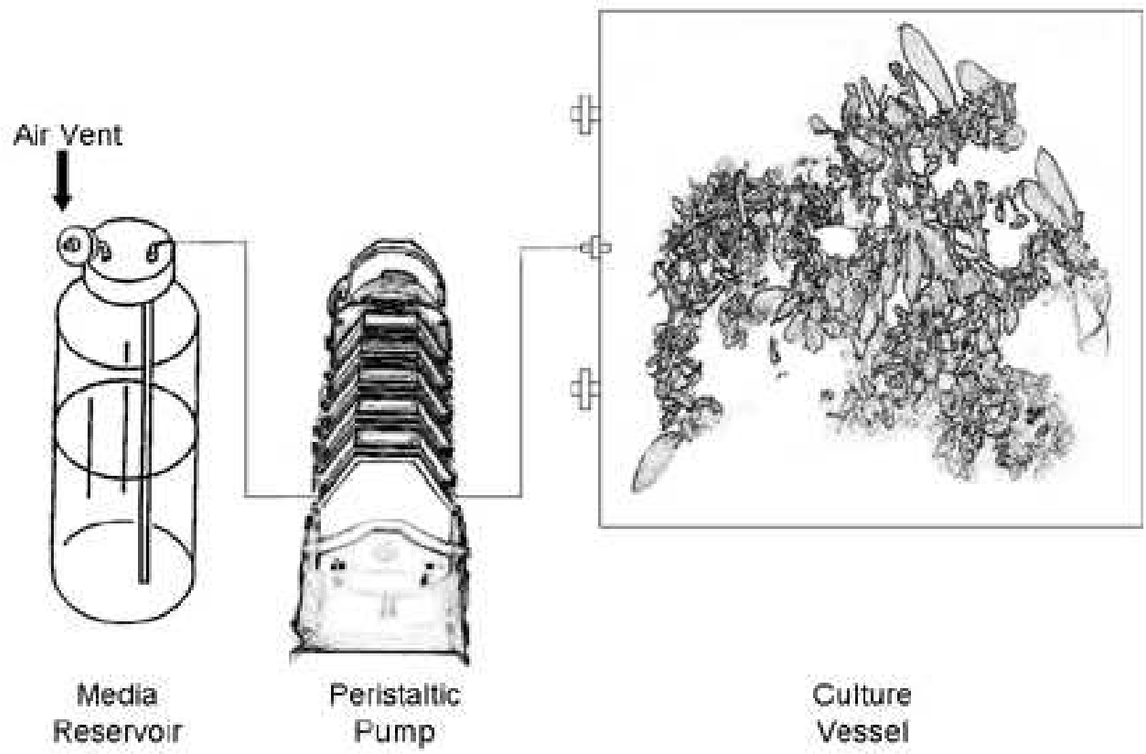

Fig. 1. Diagrammatic representation of automated plant culture system.

with sterile air greatly aided in the formation of flowers and fruits from cucumbers in the APCS (Tisserat & Galletta, 1993).

Flowering is considered a complex, morphological event regulated by a combination of genetic, hormonal, nutritional and environmental factors. In prior studies, the production of fruits from flowers *in vitro* was achieved with a great degree of cultural manipulation (Al-Juboory et al., 1991; Dickens & Van Staden 1985; 1988; Ishioka et al., 1991; Pasqua et al., 1991; Rastogi & Sawhney 1986; Tisserat & Galletta 1993; 1995). This study will investigate a number of the parameters which have been identified as considered important factors to promote flowering and fruiting reports. Specifically, these factors included: cultivar types, vessel size, medium volume, inorganic salt concentrations, sucrose levels, medium pH, growth regulators, and photoperiod. The influence of cv. type and plant density on sterile pea reproductive activity was also investigated. These factors are addressed using peas (*Pisum sativum* L.) as the bioassay species. Peas are identified as a species that quickly flowers and fruits *in vitro* and therefore would be an ideal plant to study reproductive activities *in vitro* (Franklin et al., 2000). An understanding of how these factors affect flowering and fruiting is important to achieve maximum fruiting yields *in vitro.*

2. Materials and methods

2.1 Cultures and media

Pea (cv. 'Oregon Sugar Pod II') plantlets were obtained from one-week-old sterilely germinated seeds. At this time these seedlings were usually one to 2-cm in length and

possessed 2 to 3 leafs and a rudimentary tap root. BM contained MS salts and the following in mg / liter: thiamine·HCl, 0.5; myo-inositol, 100; sucrose, 30,000; and agar, 10,000 (Sigma Chemical Company, St. Louis, MO). The pH was adjusted to 5.7 ± 0.1 with 0.1 N HCl or NaOH before the addition of agar. Liquid BM pH value was adjusted to 5.0 ± 0.1. BM was dispensed in 25-ml aliquots into culture tubes (150 mm H x 25 mm diam.; 55 mm^3 cap.) and baby food containers (76 mm H x 60 mm diam.; 143 mm^3 cap.) (Sigma Chemical Company); and 50-ml aliquots into Magenta (GA 7) polycarbonate containers (70 mm L x 70 mm W x 100 mm H; 365 mm^3 cap.) (Sigma Chemical Company), one-pint Mason jar (130 mm H x 78 mm diam.; 462 mm^3 cap.) (Kerr, Lancaster, PA), 38 x 250 mm culture tubes (250 mm H x 38 mm diam.; 270 mm^3 cap.) (Bellco Glass Inc., Vineland, NJ) and one-quart Mason jar (180 mm H x 91 mm diam.; 925 mm^3 cap.) and 100-ml aliquots were dispensed into ½-gallon Mason jars(250 mm H x 107 mm diam.; 1,850 mm^3 cap.). Vessels were capped with polypropylene lids. The APCS employed a reservoir of a ½-gallon Mason jar containing one-liter liquid BM. All media was sterilized for 15 min at 1.05 kg / cm^2 and 121 °C.

2.2 Automated plant culture system

To determine the influence of vessel type on pea reproductive activity, a single plantlet was cultured in culture tubes, baby food jars, Magenta containers, 1-pint Mason jars, 1-quart Mason jars, 1-quart Mason jar with APCS, 6-liter Bio-Safe container (379 mm L x 170 mm W x 178 mm H; 6000 mm^3 cap.) (Nalgene Co., Rochester, NY) with APCS and a 16.4-liter mega-vessel with APCS. The procedure to construct the APCS has been outlined in detail elsewhere (Tisserat 1996). The APCS vessels employed were a mega-vessel consisting of two interlocking polycarbonate pans (325 mm L x 265 mm W x 600 mm H; 16,400-mm^3 cap.) (Cambro, Huntington Beach, CA), a polycarbonate Bio-Safe container, or a ½-gallon Mason jar. Cultures were soaked 4 times daily for 5 minutes and then the medium was evacuated. The APCS (i.e., in vitro hydroponics system) consisted of two digital programmable timers, a peristaltic pump fitted with "easy-load" pump head (Model L/S-16 Masterflex—Cole Parmer, Chicago, IL), a culture chamber, silicone tubing, and a single medium reservoir (Fig 1). A variety of culture chambers could be employed with this pumping system. Digital timers (Intermatic Inc., Chicago, IL) controlled the operation of the pumps forward and reverse flow in order to fill and drain the culture vessels. Culture vessels were fitted with bacterial hydrophobic air vents to accommodation ventilation and polypropylene spigots were accommodate media filling and removal. In some cases, a layer of glass gravel, 50 mm in depth was added to culture vessels to mimic a loose "soil-type" environment for plant roots. APCS culture vessels were ventilated with 30-min air exchanges at 10 applications per day via air vents. Air provided to the culture systems was from compressed air, pretreated through a charcoal filter and regulated to a 300 ml/min flow rate. Digital timers were controlled ventilation treatments, when applied.

2.3 Experiments

The influence of medium volume on vegetative and reproductive growth was addressed by culturing a single plantlet within ½-gallon Mason jar with an APCS and employing the medium reservoir volumes of 150, 250, 350, 500, or 1000 ml. In subsequent experiments, a test of medium volume employing 1000, 1500, 2000, and 2500-ml medium reservoir volumes was conducted. A comparison was also made employing an APCS and either ½- or full-

strength MS salts using a 1000-ml reservoir volume. The influence of various concentrations of sucrose on pea growth responses was conducted by employing a ½-gallon Mason jar with an APCS. Sucrose levels were tested at 0, 0.5, 1, 1.5, 3, 5, 7.5, or 10 % levels. The influence of pH on the growth of pea seedlings was tested at 4, 5, 5.7, 6, 7, or 8 levels in 1-quart Mason jars containing 50-ml BM. To test the effect of growth regulators on pea seedlings vegetative and reproductive growth, a single plantlet was grown on 0, 0.01, 0.1 and 1.0 mg / l BA, NAA, or GA_3 in 1-quart Mason jars containing 50-ml BM. The influence of co-culture of more than one plantlet per vessel on the growth responses of pea was tested by planting 1, 3 or 5 plantlets in 38 x 250 mm culture tubes, 1-pint jar, 1-quart jar, ½-gallon jar and ½-gallon jar with an APCS. The influence of pea cultivar on the growth responses was tested by culturing plantlets in the APCS of the following cvs.: 'Bush Snappy', 'Oregon Sugar Pod II', 'Super Snappy' and 'Wando'. Photoperiods effects were tested at 8, 12, 16, and 24 h on a single plantlet grown in a 1-quart Mason jar containing 50-ml BM.

Ten replicates/treatment were planted and experiments were repeated at least three times. Following eight weeks incubation, data on culture fresh weight, leaf number, plant height, flower number, and fruit number were recorded. In some cases, plants growing in the APCS were allowed to continue undisturbed for an additional 8 weeks with their media replenished in order to promote continued reproductive activities. Correlation coefficients were calculated to compare fresh weight, leaf length and plant height to culture chamber capacity, medium volume employed and culture chamber height when appropriate. Proportional data was analyzed by Fisher's exact test, and other data was tested by standard analysis of variance and Student-Newman-Keuls multiple range test, when appropriate. Cultures were incubated at a constant 26 ± 1 oC under a 16-hr daily exposure to 70 μmol / m^2 / s cool white fluorescent lamps.

3. Results and discussion

Flowering occurred in a number of culture vessels including 25 x 150 mm culture tubes, Magenta containers, 1-pint Mason jars, 1-quart Mason jars, 1-quart Mason jars with APCS, Bio-safes with APCS and mega-vessels with APCS at the end of 8 wks in culture (Fig. 2). However, the number of flowers produced in the larger vessels (*e.g.* Bio-Safe or mega-vessels) was consistently greater than in the smaller vessels (*e.g.* tubes and 1-pint Mason jars). Fruit formation only occurred from those vessels employing an APCS. More fruits were produced from plantlets grown in the mega-vessels than using any other culture system tested. Regardless of the culture vessel system employed, plantlets readily exhibited rooting and leaf production. There is a relationship between the number of leaves produced and occurrence of flowering and fruiting. Within the APCS, plantlets grew larger and developed more leaves and flowers than plantlets grown in the non-APCS systems. Flowers were usually initiated after plantlets produced 10-20 leaves. Abscission of flowers was common in all the vessels employed. Within the APCS, about 30 leaves were produced before flowers were retained and developed into fruits. A range from 5 to 15 fruits per plantlet was produced in the mega-vessel (Fig. 3A). Fruits were found to achieve sizes of 40 to 50 mm in length after 8 weeks in culture. Fruit as large as 75 to 80 mm in length may be produced eventually. Continued culture of plantlets in the APCS, to 16 weeks, resulted in enlargement of fruits as well as continued initiation of more flowers and fruits (Fig. 3A-C). Pea plantlets could be maintained for several months in the APCS by replacing the medium every 8 weeks. After 16 weeks in culture, hundreds of leaves and flowers were produced

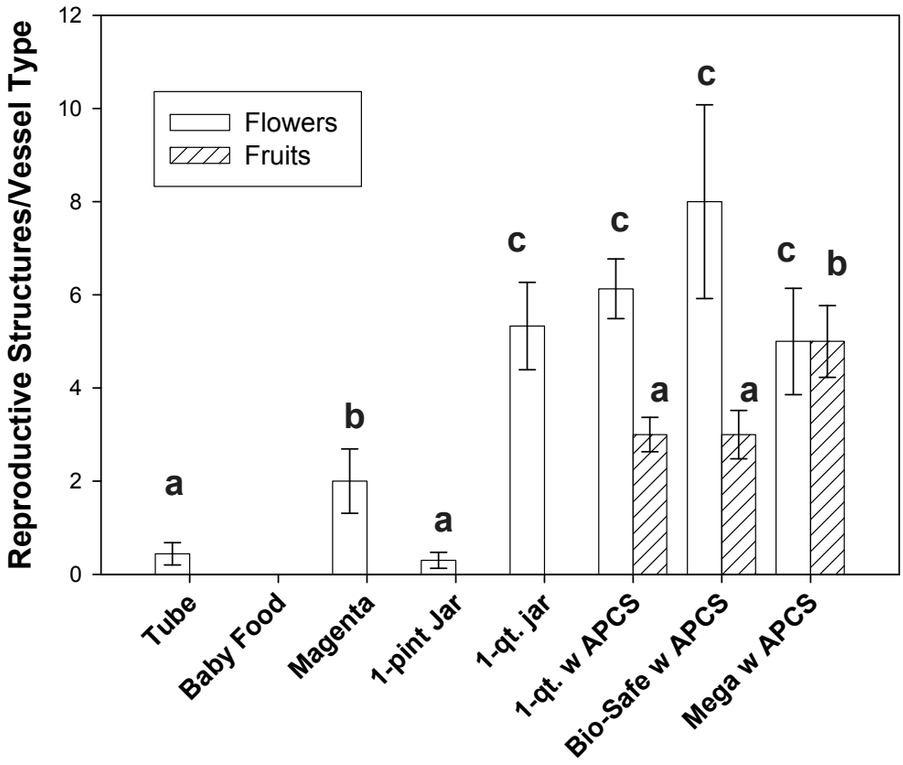

Fig. 2. Reproductive responses from pea plantlet tips in various culture vessels after 8 weeks in culture. Note that fruits were only produced from the larger culture vessel systems with APCS. Data were averaged for 10 replications/treatment. Experiments were repeated 3 times and a single representation is presented. Mean separation by Student-Newman-Keuls multiple range test ($P \leq 0.5$). Columns with the same letter on top were not significantly different.

from each cultured pea plantlet. Leafs were short-lived usually surviving 4 to 10 weeks resulting in a continuous production of leaves from the cultured plantlets. As many as 30 to 40 fruits were obtained from a single plantlet cultured in the mega-vessel after 16 weeks. Fruiting was found to be continuous and non-synchronous with older fruits exhibiting senescence adjacent to neighboring newly initiated fruits on the same plantlet. One to six seeds developed within the fruit pods; however several seeds within a pod may be aborted (Fig. 3B). Nevertheless, some seeds were viable and capable of germination as evidenced by their *in vitro* germination during this study. These same plantlets eventually flowered and fruited themselves. Fruits sometimes dehisced in culture and a single seed may germinate from a fruit while still attached to the parent plantlet (Fig. 3C). These observations confirm the fertility of pea seeds obtained from sterile fruits by other investigators (Franklin et al., 2000; Ochatt et al., 2008).

The influence of varying the medium reservoir volume with 150, 250, 350, 500, or 1000 ml reservoir volume was tested using a ½-gallon Mason jar with APCS. The highest vegetative and reproductive responses were obtained from an APCS employing a 1000 ml volume

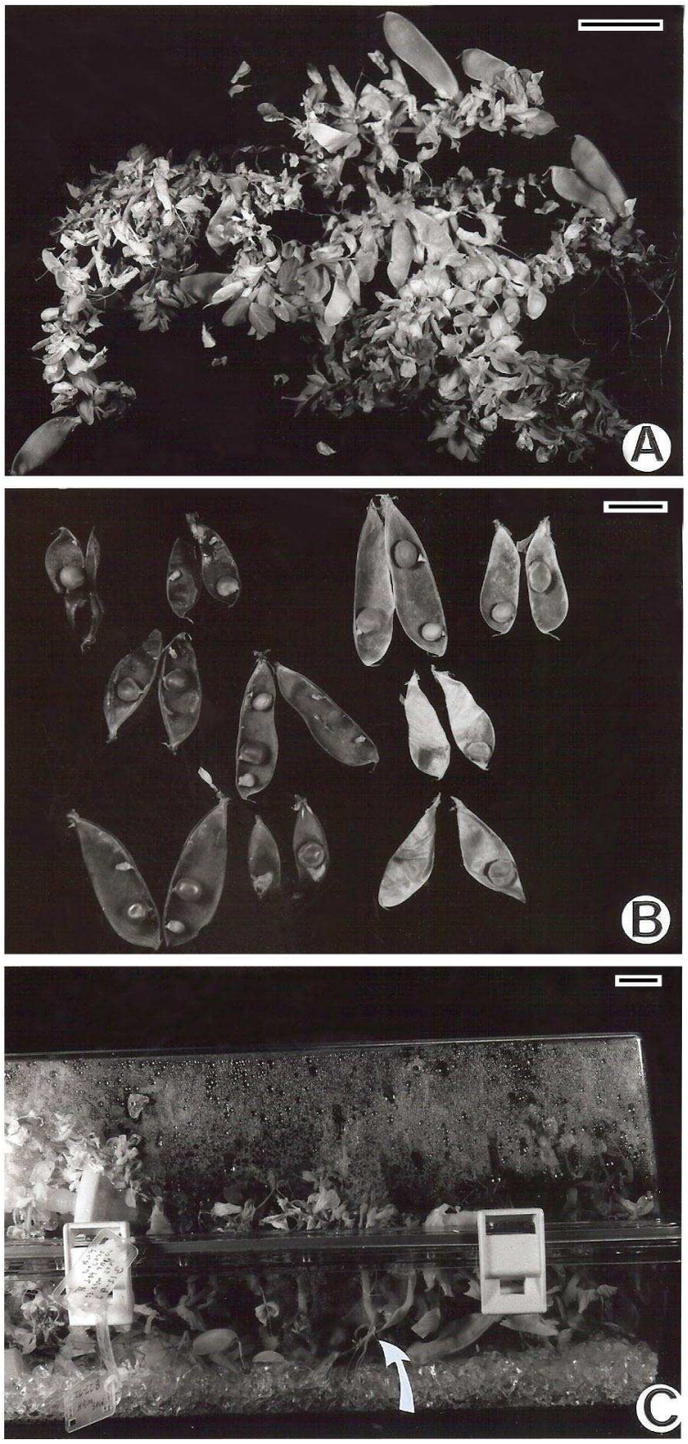

Fig. 3. Reproductive responses from 16-week old pea plantlets *in vitro* grown in an APCS. (A**)** Pea plantlet exhibiting numerous fruits obtained from the mega-APCS. (B) Examples of bisected fruits revealing aborted and non-aborted seeds. (C) Growth responses of pea plantlets in Bio-Safe vessel, note the arrow indicating the precocious germination of seed from a dehisced fruit while still attached to the parent plant. Bar = 10 mm.

reservoir volume (Fig. 4). High positive correlations occurred between media volume employed and culture weight, flower number or fruit number (Fig. 4). However, in subsequent experiments (data not shown), when employing medium volumes larger than 1000 ml such as 1500, 2000 or 2500 ml, no improvement of the vegetative or reproductive responses occurred verses using the 1000 ml medium reservoir volume (data not shown). Apparently, the 1000 ml volume is the limit that the medium volume beneficially provides. These results conform to those made by Dickens & Van Staden (1988) who found that vessel size and medium volume influenced flowering in *Kalanchöe blossfeldiana*. Larger vessels,

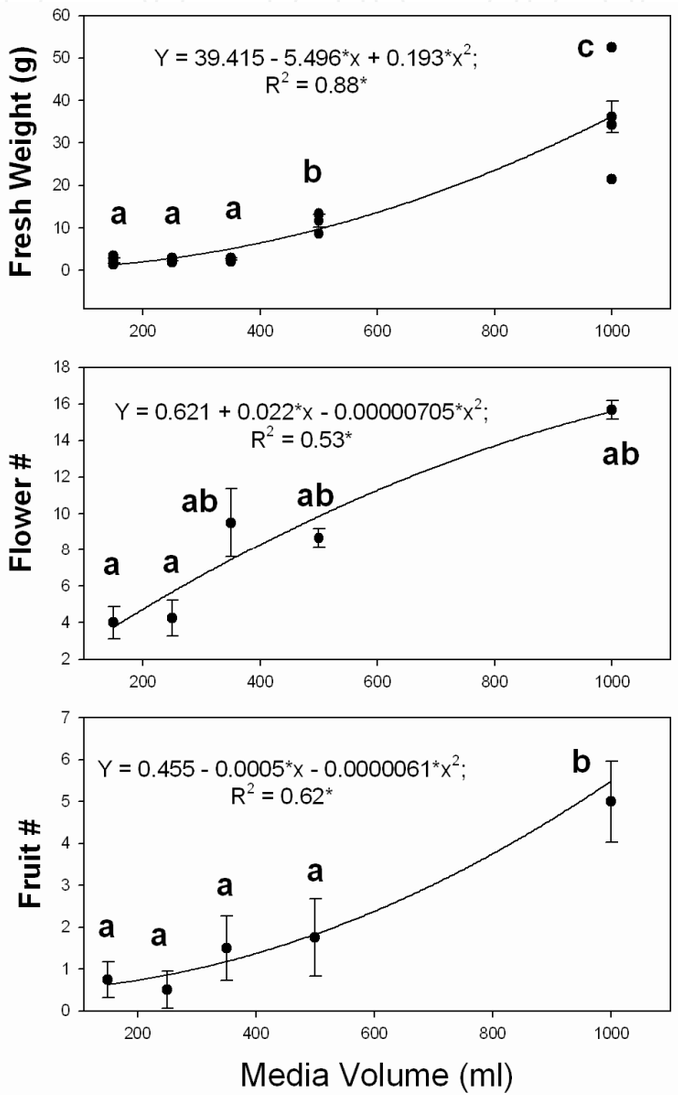

Fig. 4. Growth of Pea seedling plantlets in ½-gallon Mason Jars with APCS employing various media volumes. Regression coefficients (R^2) and regression equations between media volume and fresh weight, flower number or fruit number and media volume are given. All correlations are significant at $P \leq 0.05$ if denoted by asterisk. Letters represent statistical comparisons of mean predicted media volumes. Different letters represent non-overlap of the 95% confidence limits.

100 mm^3 (ml) flasks containing 40 ml medium, gave rise to more flowers than those *Kalanchöe* cultures grown in 50 mm^3 flasks or 40 mm^3 test-tubes containing 20 ml medium. In this study, considerably larger vessels and medium volumes were employed and they enhanced flowering and culture growth. In prior studies, Tisserat & Galletta (1993; 1995) found that an APCS employing large culture chambers (e.g. ≈ ½-gallon Mason jars) allowed for flowering and fruiting from cucumbers and pepper plantlets. This work confirms these previous findings and quantifies the effect of culture chamber size and medium volume on the production of flowers and fruiting.

The concentration of inorganic salts in the medium influenced vegetative and reproductive responses. Nutrient medium containing ½-strength MS inorganic salts produced cultures of significantly less fresh weight and fewer flowers and fruits than cultures produced on medium containing full-strength MS inorganic salts (data not shown). For example, in terms of culture weight on medium containing ½-strength MS salts, 16.2 ±0.8 g fresh weights was produced while on full-strength MS salts, 36.0 ± 9.0 g fresh weight was produced. Similarly, differences in reproductive activity was influenced by inorganic salt concentration; on ½-strength MS salts, 9.3 ± 1.3 flowers and 2.8 ± 0.5 fruits were produced while on full-strength MS salts, 15.7 ± 4.7 flowers and 6.0 ± 0.6 fruits were produced. Based on these observations, it is apparent that vegetative growth and flowering is dependent on inorganic salt concentration. Dickens and Van Staden (1988) suggest that lack of necessary inorganic salts, especially nitrogen, reduces reproductive activity *in vitro*. In contrast, lowering the salt levels stimulated *Pharbitis* plantlets to flower (Ishioka et al., 1991).

Sucrose was found to be a requirement for both vegetative and reproductive activities in pea *in vitro*. The concentration of externally supplied carbohydrates is an important factor for reproductive activity from cultured pea. No reproductive responses were obtained from cultured pea plantlets grown on medium containing 0, 0.5, 1, or 1.5 % sucrose; reproductive responses only occurred on medium containing ≥ 3% sucrose. Highest fresh weight and flowering responses occurred using 3 % sucrose; while the highest fruit production occurred in the 3 and 5 % sucrose levels (Fig. 5). Less vegetative and reproductive responses (with the exception of the fruiting rate at the 5 % sucrose level) occurred as the sucrose levels increased beyond 3 % (Fig. 5). The best concentration for both reproductive and vegetative growth was 3 % sucrose. Similarly, 3 % sucrose has been found to be stimulatory in aiding flowering in other plants (Dickens & Van Staden 1988; Rastogi & Sawhney 1986). However, Ishioka et al., (1991) found that 7.5 % sucrose gave optimum flowering in *Pharbitis* plantlets. These diverse findings suggest that sucrose concentration greatly influences flowering and should be determined empirically for each species cultured.

The effect of pH on either the vegetative or reproductive activities of pea plantlets was not critical. No difference in fresh weights or flowering responses were obtained from cultures grown on any of the pH treatments after 8 weeks in culture (data not shown). These results differ from responses obtained from tobacco floral bud and tomato bud culture where pH greatly influenced flowering (Pasqua et al., 1991; Rastogi & Sawhney, 1986). Presumably, pea plantlets employed in this study were much larger than the tomato bud cultures, and were therefore less affected by media pH.

Testing various concentrations of growth regulators, BA, NAA and GA_3 at concentrations varying from 0.0, 0.01, 0.1 and 1.0 mg / l revealed that best vegetative and reproductive

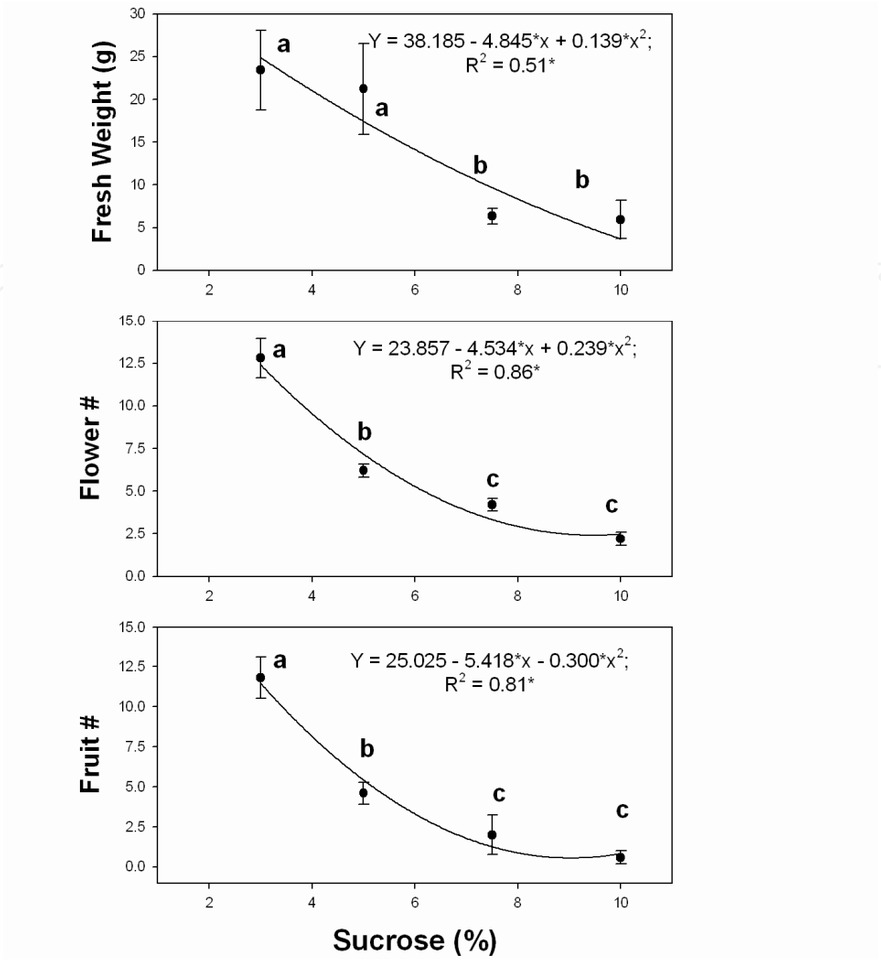

Fig. 5. Growth of Pea seedling plantlets in ½-gallon Mason Jars with APCS employing various sucrose concentrations. Regression coefficients (R^2) and regression equations between media volume and fresh weight, flower number or fruit number and media volume are given. All correlations are significant at $P \leq 0.05$ if denoted by asterisk. Letters represent statistical comparisons of mean predicted media volumes. Different letters represent non-overlap of the 95% confidence limits.

responses occurred from cultures grown on medium devoid of any growth regulators (Table 2). High concentrations of all the growth regulators (*i.e.* 0.1 and 1.0 mg / l) gave decreased flowering responses compared to low concentrations (i.e. 0.01 mg /l). More leafs and flowers were produced from cultures grown on BM without any growth regulators than on medium containing growth regulators. Again this suggests that leaf number is related to flowering. Growth regulators have been found to be beneficial for flowering from immature tissue, organ and shoot cultures grown *in vitro* (Al-Juboory et al., 1991; Rastogi & Sawhney 1986; Ishioka et al., 1991; Lee et al., 1991). In contrast, in this study, larger pea plantlets do not benefit either vegetatively or reproductively from inclusion of exogenous growth regulators. These results were similar to that found for cucumber or pepper where inclusion of growth regulators also repressed flowering *in vitro* (Tisserat & Galletta 1993; 1995).

Treatment (mg / l)	Leaf #	Flower #	Fruits #
0.0 GA_3/NAA/BA	62.8±3.9 a	1.3 ±0.4 a	0.7 ± 0.1 a
0.01 GA_3	52.0 ± 1.9 a	0.7 ± 0.1 b	0.2 ± 0.1 b
0.1 GA_3	49.3 ± 6.4 a	0.1 ± .1 c	0
1.0 GA_3	49.9 ± 5.6 a	0.1 ± 0.1 c	0
0.01 NAA	50.0 ± 2.7 a	0.4 ± 0.4 c	0
0.1 NAA	47.2 ± 5.6 a	0.4 ± 0.2 c	0
1.0 NAA	43.5 ± 2.6 a	0.3 ± 0.1 c	0
0.01 BA	50.5 ± 5.5 a	0.6 ± 0.4 c	0.1 ± 0.1 b
0.1 BA	40.2 ± 4.4 a	0	0
1.0 BA	42.9 ± 7.1 a	0	0

[a]Treatment averages and SE presented. Mean separation within sucrose treatment columns by Student-Newman-Keuls multiple range test ($P \leq 0.05$).

Table 2. Growth responses of pea seedlings on agar medium after 8 weeks in culture[a].

The influence of co-culturing of several plantlets in a single culture vessel on the vegetative and reproductive responses has not been addressed in the literature. The number of co-cultured plantlets per vessel had a profound influence on the vegetative and reproductive responses exhibited by cultured plantlets. High positive correlations occurred for flowers/culture and vessel capacity, fresh weight/culture or leafs/culture when a single shoot was cultured per vessel (Fig. 6). However, when three or five seedlings were employed per vessel less or no correlations occurred. Usually, fewer flowers and fruits were produced from co-cultured plantlets compared to when only a single plantlet was cultured. Clearly, the benefits of employing larger culture vessels are reduced when multiple plantlets are employed.

Pea cv. is important to the number of flowers and fruits produced per plantlet *in vitro* (Table 3). 'Bush Snappy' and 'Oregon Sugar Pod II' gave the highest rates of flowering in 8 weeks, 60 and 60 % respectively, while 'Super Snappy' and 'Wando' exhibited 30 and 20 % flowering, respectively (Table 2). Similarly, these same two cultivars also gave the highest rates of fruit production, 60 and 20 % respectively. 'Super Snappy' and 'Wando' exhibited less flowering and fruiting but manifested larger shoot lengths than 'Bush Snappy' and 'Oregon Sugar Pod II' (Table 2). No difference in terms of the number of leafs produced occurred among the 4 cultivars tested with 23 to 25 leaves/culture usually being produced from all cvs. at the end of 8 weeks. Pea was not influenced by any photoperiod tested in vitro in terms of flowering and fruiting responses in pea (Table 4). Pea is a day-neutral plant and characteristically was unaffected by photoperiod treatments given *in vitro*. Shoot apices of *Pharbitis*, a short-day plant, were unaffected by the photoperiod employed in culture (Ishioka et al., 1991). However, short-day photoperiod was prerequisite for flowering of *Kalanchöe blossfeldiana* and soybean nodal sections (Dickens & Van Staden 1985; 1988). Both *Kalanchöe* and Soybean are short-day plants.

Development of sterile fruits in tissue culture may have some futuristic prospects. Surgery, cancer, AIDs and extreme-allergy patients often require sterile diets to prevent infection complications (Butterweck, 1995; Watson, 2001; Pryke & Taylor, 2008). To supply suitable diets foods must be sterilized through a variety of heat or radiation treatments (Pryke &

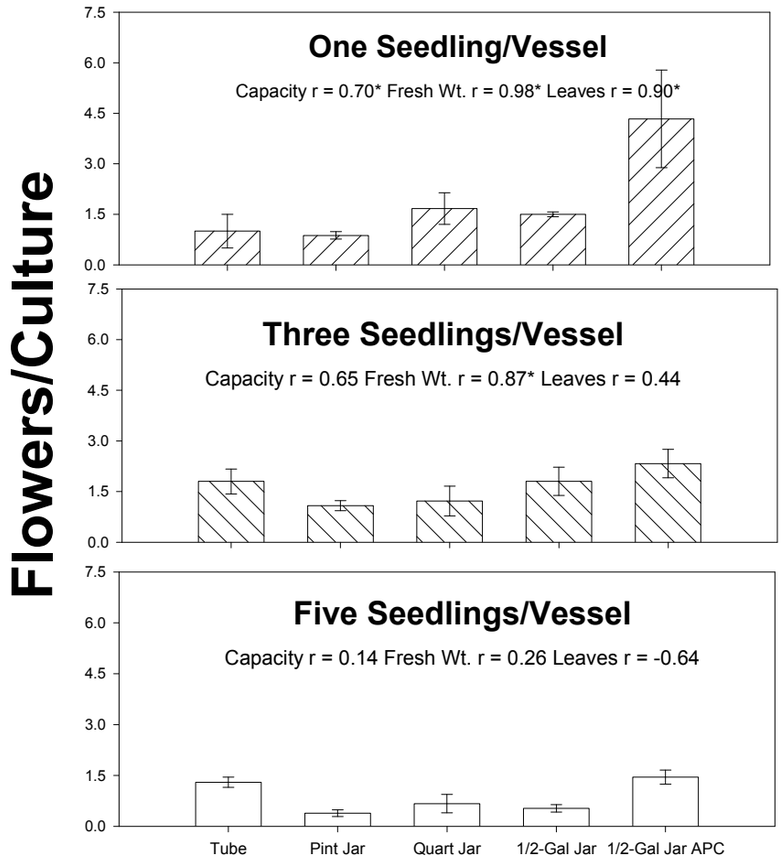

Fig. 6. Relationship between numbers of seedlings/vessel, leaves/culture and culture system cap. (mm^3) to flowers/culture. Correlation coefficients (r) between flowers/culture and culture chamber cap., fresh weight, or leaves produced are given for number of seedlings cultured/vessel. Correlations significant at $P \leq 0.05$ are designated by an asterisk.

Cultivar	Shoot Length (mm)[a]	Leaf #[a]	Flowering %[b]	Fruiting %[b]
Bush Snappy	148.3 ± 8.3 a	24.2 ± 0.8 a	60 a	60 a
Oregon Sugar Pod II	149.0 ± 9.7 a	23.0 ± 0.8 a	60 a	20 b
Super Snappy	167.9 ± 7.3 b	25.5 ± 0.9 a	20 b	0
Wando	169.0 ± 5.3 b	25.5 ± 3.2 a	30 b	10 b

[a]Treatment averages and SE presented. Mean separation within sucrose treatment columns by Student-Newman-Keuls multiple range test ($P \leq 0.05$).
[b]Treatment percentages within columns separated by Fisher's exact test ($P < 0.5$). There were 10 replications per treatment.

Table 3. Response of pea cultivars on the vegetative and reproductive responses in vitro after 8 weeks in culture after 8 weeks in culture.

Photoperiod (h)	Shoot Length (mm)[a]	Leaf #[a]	Flowering %[b]	Fruiting %[b]
8	160.0 ± 7.3 a	32.5 ± 3.1 a	90 a	50 a
12	163.0 ± 1.5 a	39.5 ± 2.9 a	70 a	20 a
16	208.8 ± 13.2 b	40.6 ±1.8 a	80 a	20 a
24	185.5 ± 10.6 b	39.0 ± 3.8 a	90 a	30 a

[a]Treatment averages and SE presented. Mean separation within sucrose treatment columns by Student-Newman-Keuls multiple range test ($P \leq 0.05$).
[b]Treatment percentages within columns separated by Fisher's exact test ($P \leq 0.5$). There were 10 replications per treatment.

Table 4. Influence of photoperiod on the morphogenetic responses in vitro in Oregon Sugar Pod II pea after 8 weeks in culture.

Taylor, 2008). The nutritional value of these processed foods is altered somewhat compared to that of the original freshly harvested produce. Sterile fruits produced in vitro may provide high nutritionally viable produce which physically and chemically resemble that from in vivo sources. This study suggests that viable fruiting structures can be procured employing sterile tissue culture technology. Nevertheless much refining work remains to be preformed to develop edible products. In addition, production of sterile fruits with viable seeds can be a means to shorten generational cycles for faster breeding (Ochatt et al., 2002). It has been previously recognized that plant tissue culture offers an avenue to produce food products (Fu et al., 1999; Stafford, 1991). However, little interest has been expended to develop suitable systems that could accommodate fruits and vegetable production in vitro. Presumably, the increased spatial and nutritional demands for flowering and fruiting required by plants to be achieved contributed toward this. A sterile hydroponics system could be employed to achieve sterile food production.

4. Conclusions

In vitro reproductive activity has certain advantages, such as year round flowering and fruiting in a controlled environment, and disadvantages, such as the inherent difficulty associated with a sterile environment coupled with poor flowering in certain species and absence of fruiting in most species when cultured *in vitro*, when compared to growing plants in soil. Previous *in vitro* studies often utilized small explants such as nodal sections or shoot tips that were entirely medium dependent for their survival and growth. In this study, large plantlets possessing several leaves and a substantial root system achieved both flowering and fruiting within 8 weeks *in vitro*. Pea is an excellent plant to critically study all reproductive events *in vitro*. The only other plant reported in the literature that flowered in as short a time frame as pea is soybeans which produced flowers in 6 weeks and fruits in 10 weeks in culture (Dickens & Van Staden 1985). The APCS differs from the traditional tissue culture vessels by employing an enlarged culture chamber that accommodates for larger plantlet growth and employs a larger medium reservoir that supplies more nutrients for plantlet growth. With the APCS, no external growth regulators were required for reproductive activities (Tisserat & Galletta 1993; 1995).

Prolonged culture growth is obtained (*e.g.* 16 wks) by replacing the medium reservoir without any culture manipulations. The primary factors controlling the pea reproductive

activity were carbohydrate levels and culture vessel size. Absence of or low sucrose levels did not allow cultures to flower. Fruiting could not occur in small vessels (*e.g.* 25 x 150 mm culture tubes or baby food jars). Secondary factors influencing reproductive events were plantlet density, medium volume, inorganic salt concentration, and CV. Factors having no influence on the flowering of pea were pH, growth regulators and photoperiod. Recognition of the merits of these factors will aid in a more through understanding of the lowering and fruiting process *in vitro*. The long term goal of manufacturing sterile in vitro hydroponically grown foods can be obtained.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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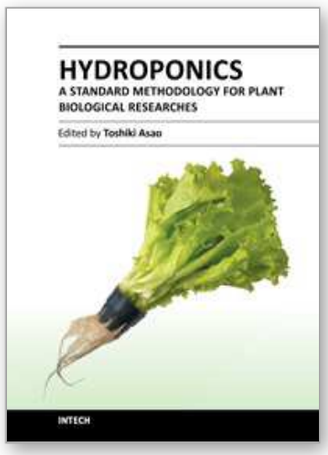

Hydroponics - A Standard Methodology for Plant Biological Researches

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Hydroponics-A standard methodology for plant biological researches provides useful information on the requirements and techniques needs to be considered in order to grow crops successfully in hydroponics. The main focuses of this book are preparation of hydroponic nutrient solution, use of this technique for studying biological aspects and environmental controls, and production of vegetables and ornamentals hydroponically. The first chapter of this book takes a general description of nutrient solution used for hydroponics followed by an outline of in vitro hydroponic culture system for vegetables. Detailed descriptions on use of hydroponics in the context of scientific research into plants responses and tolerance to abiotic stresses and on the problems associated with the reuse of culture solution and means to overcome it are included. Some chapters provides information on the role of hydroponic technique in studying plant-microbe-environment interaction and in various aspects of plant biological research, and also understanding of root uptake of nutrients and thereof role of hydroponics in environmental clean-up of toxic and polluting agents. The last two chapters outlined the hydroponic production of cactus and fruit tree seedlings. Leading research works from around the world are brought together in this book to produce a valuable source of reference for teachers, researcher, and advanced students of biological science and crop production.

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