CHAPTER 29

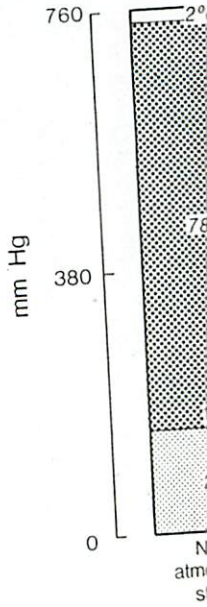
Protocols of Low-Pressure Storage

M.P. Bridgen and *G.L. Staby*

Two forms of tissue culture storage have recently been studied as alternatives to dry storage (Chapter 27) and cryopreservation (Chapter 28). These include low-pressure (hypobaric) and low-oxygen storage (Bridgen and Staby, 1981). The low-pressure system (LPS) functions by decreasing the atmospheric pressure surrounding the tissue cultures, as a result decreasing the partial pressure of all gases that are in contact with the plant material (Fig. 1). The low-oxygen system (LOS) functions at atmospheric pressure (760 mm Hg) by combining an inert gas, such as nitrogen, with oxygen to create the desired partial pressure of oxygen (Fig. 1). Experimental results obtained from plant tissue culture experiments with LPS and LOS are similar regardless of plant part or species used (Table 1).

Table 1. Conclusions Derived from Experiments with Plant Tissue Cultures Subjected to Low-Pressure and Low-Oxygen Treatments

1. Partial pressures of oxygen below 50 mm Hg reduce the rate and the amount of growth of plants stored in vitro.
2. Low partial pressures of oxygen cause similar effects in plant tissue cultures regardless if obtained by LPS or LOS.
3. Both organized and unorganized plant tissues are affected by low partial pressures of oxygen.
4. No phenotypic growth differences are observed after the tissue cultures are removed from storage and then grown to maturity in vivo.



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LITERATURE REVIEW

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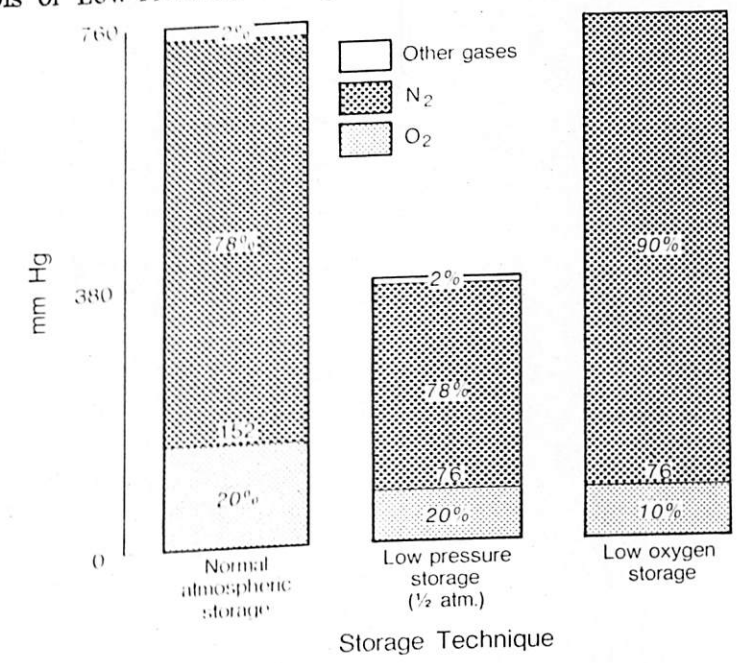

Figure 1. Comparison between normal atmospheric storage, low-pressure storage, and low-oxygen storage.

LITERATURE REVIEW

No universal storage method has been found to be suitable for all plant material in the tissue-cultured state (Caplin, 1959; Withers, 1978). However, short-term storage by low temperatures (4 C) is successful with many plants (Mullin and Schlegel, 1976; Seibert and Wetherbee, 1977; Withers, 1978). Refrigerated storage is already being used for the storage of *Fragaria* spp. meristem plantlets (Mullin and Schlegel, 1976), but this method has several disadvantages that limit its usefulness. The medium still needs periodic replenishment (Mullin and Schlegel, 1976); there is possible cell deterioration through dehydration (Bajaj and Reinert, 1977); selection of plant material may occur (Withers, 1978); and cultures must be removed from storage before cellular damage occurs (Bannier and Steponkus, 1976; Withers, 1978).

Low-pressure storage is useful in extending the shelf life of meat, poultry, shrimp, fish, vegetables, fruits, cut flowers, potted plants, cuttings, and other metabolically active products (Anonymous, 1975). It is also successful in controlling physiological and pathological disorders of many horticultural crops (Anonymous, 1975; Dilley et al., 1975). Low-pressure or hypobaric systems are based on the following principles. First, the commodity must be placed in an atmosphere of controlled temperatures (Anonymous, 1975). Second, the atmosphere must be at reduced pressures so that the partial pressures of each gas within the storage and commodity are reduced proportionately to the pressure

Jacket design by Juanita Elefante-Gordon

causing an increase in the gas exchange in the commodity (Lougheed et al., 1976). Third, a continuous air exchange is used to flush away any toxic vapors released into the storage area and last, high humidity prevents shrinkage, weight loss, and desiccation of the commodity (Anonymous, 1975; Gaffney, 1978).

Previous work has indicated that low pressures may be a potentially useful tool in the long-term storage of plant tissue cultures. It was first shown that normally short-lived seeds such as onion, celery, and cabbage exhibit increased germination after low-pressure storage when compared to atmospheric storage (Lougheed et al., 1976). It was then demonstrated that tomato plant growth was inhibited at low pressures (Rule and Staby, 1981).

In addition to the potential for long-term storage, low pressures also have the added advantage of reducing the activity of culture medium pathogens in aseptic material (Covey and Wells, 1970). Spore germination, mycelial growth, and sporulation of *Penicillium digitatum*, *Alternaria alternata*, *Botrytis cinerea*, *Diplodia natalensis*, and *Sclerotinia sclerotiorum* are reduced under low pressures (Adair, 1971; Apelbaum and Barkai-Golan, 1977). Subatmospheric pressures also have a fungistatic effect on *Penicillium expansum*, *Rhizopus nigricans*, *Aspergillus niger*, *Botrytis alli*, and *Alternaria* sp. (Wu and Salunkhe, 1972).

Low-oxygen storage is the combination of different gases to create a desired atmosphere at atmospheric pressure. Originally reported for the storage of apples and pears under low oxygen and high carbon dioxide, it is still being used in commercial operations for the storage of various fruit crops (Dewey et al., 1969; Smock, 1979).

There are several theories as to why low-pressure storage and low-oxygen storage delay senescence of horticultural crops. One theory is that by decreasing the partial pressure of oxygen in the atmosphere, the amount of CO_2 evolved is also reduced (Kessel and Carr, 1972; Parkinson et al., 1974; Siegel, 1961), and with the low temperatures, respiration is decreased. In addition senescence may also be delayed in low-pressure storage because of the continuous flow of air which flushes away toxic gases such as ethylene that may accumulate (Gamborg and LaRue, 1971; LaRue and Gamborg, 1971).

Most growth studies of plants with oxygen have been related to the measurements of oxygen uptake of CO_2 release in the light. This has allowed the proposal of several theories explaining why low oxygen has an effect on plants growing in vivo. Researchers now know that photosynthesis is increased as O_2 in the atmosphere is reduced (Forrester et al., 1965; Hesketh, 1967; Ludwig and Canvin, 1971; Servaites and Ogren, 1978; Takabe and Akazawa, 1977) by a direct inhibitory effect of O_2 on the RuBP carboxylase of the photosynthetic carbon cycle (Challet and Ogren, 1975). It is also possible that photorespiration decreases as the partial pressure of oxygen is lowered (Ehleringer and Bjorkman, 1977; Forrester et al., 1965; Tregunva et al., 1964). This would inhibit CO_2 production and possibly stimulate CO_2 fixation (Tjepkema and Yocum, 1973; Yentur and Leopold, 1976). Hesketh (1967) found that both the increase in photosynthesis and the decrease in photorespiration are dependent upon species and temperature. Since O_2 is necessary for opening and closing of stomates, as was shown with

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Table 2. Average Fresh W
Chrysanthemums

TREATMENT	
Atmospheric Pressure (mm Hg)	PO_2 (mm H
760	152.0
760	54.0
300	60.8
760	28.1
150	30.4
760	8.4
70	8.0

[a]S.E. = 0.01.
[b]S.E. = 1.81.

Growth of tobacco shoot
of leaves and roots and by

wheat and barley (Akita and Moss, 1973), transpiration can also be affected by low partial pressures of oxygen (Regehr et al., 1975).

The idea that low partial pressures of oxygen may be advantageous for the storage of plant tissue cultures arose from Caplin (1959). He noticed that liquid petrolatum, commonly known as mineral oil, was widely used for the conservation of cultures of various microorganisms. The mineral oil was used to reduce the rate of growth and to decrease the amount of evaporation from the agar medium. Caplin's experiments with carrot tissue cultures demonstrated that the amount of growth under oil or nutrient solution is controlled by the supply of oxygen to the tissue.

The only reported experiments examining low partial pressures of oxygen with plant tissue cultures have been completed by Bridgen and Staby (1981), using low-pressure storage (LPS) and low-oxygen storage (LOS). Differentiated cultures of *Nicotiana tabacum* L. Wisconsin 38 and *Chrysanthemum* x *morifolium* Ramat. Nob Hill and undifferentiated cultures of *Nicotiana tabacum* L. Wisconsin 38 were studied. The exact LPS and LOS procedures are described in the next section; however, the results follow in this section.

Growth of chrysanthemum shoots was measured by fresh weight gain (Table 2), height increases (Fig. 2), and total number of leaves (Table 2). Plantlet growth was not totally inhibited by any of the treatments over the 6 week period; however, there was a difference in the amount of growth among treatments. Treatments having a partial pressure of oxygen (PO_2) of 50 mm Hg or higher were not different from the controls after 6 weeks in storage. Growth of treatments less than 50 mm Hg was less than the controls with plantlets grown at a PO_2 of approximately 8 mm Hg increasing the least over the 6 weeks. Plantlets grown under LOS and LPS at corresponding PO_2 had similar growth patterns.

Table 2. Average Fresh Weight Gain and Number of Leaves of Chrysanthemums after 6 Weeks in Storage

TREATMENT		AVERAGE FRESH WT. GAIN (mg)[a]	AVERAGE NUMBER OF LEAVES[b]
Atmospheric Pressure (mm Hg)	PO_2 (mm Hg)		
760	152.0	383.3	7.58
760	54.0	316.6	6.56
300	60.8	325.0	7.50
760	28.1	151.6	5.78
150	30.4	233.3	6.64
760	8.4	55.0	1.73
70	8.0	50.0	2.29

[a]S.E. = 0.01.
[b]S.E. = 1.81.

Growth of tobacco shoot tips was measured by counting the number of leaves and roots and by measuring plant height. The visual rating

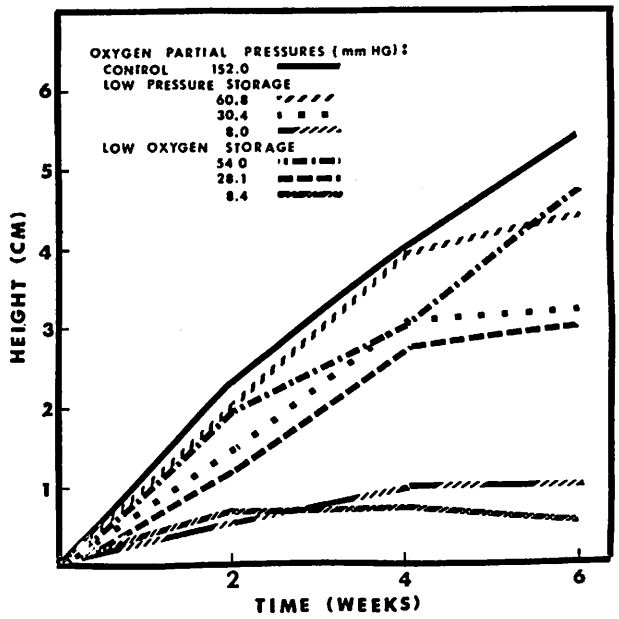

Figure 2. Increase in height of chrysanthemum plants after 6 weeks in storage. S.E. = 1.71.

Table 3. Visual Rating System for Evaluating Tissue Responses

RATING	VISUAL DESCRIPTION
1	≤0.5 cm high, ≤6 leaves
2	0.5–1.0 cm high, 6–10 leaves
3	1.0–3.0 cm high, 10–15 leaves
4	≥3.0 cm high, ≥15 leaves
5	Culture bottle completely filled; could not calculate without opening the bottle

system described in Table 3 was used to express results. Growth trends were similar to those observed for the chrysanthemums; as the PO_2 was reduced, the rate of growth decreased. Similarly, the lower the PO_2, the greater the reduction in growth (Table 4). Medium desiccation was observed in this experiment after 2 weeks only with the LPS treatment which was held at 40 mm Hg. This caused the plantlets to dehydrate in 50% of the bottles and prevented growth measurements of one replication.

Tobacco callus growth was evaluated by measuring the increase in height, width, and length to estimate volume increase (cm^3) from the initial 125 mm^3 masses (Figs. 3 and 4). The growth curves for the callus tissue were similar to differentiated chrysanthemum and tobacco tissue; however, differences among treatments were more evident. Growth decreased as the PO_2 was lowered, and there was no difference between LPS and LOS at similar PO_2.

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Table 4. Visual Rating of

Atmospheric Pressure (mm
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760
300
760
150
760
40

[1]Value represents data fro

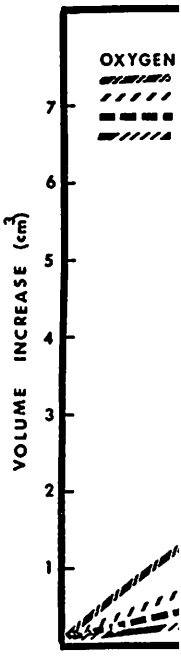

Figure 3. Tobacco callus
2, 4, and 6 weeks.

One-third of the chrysa
flowering following each 6
first transferred onto MS
0.93 µM KIN. Then after
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and long days. After
greenhouse conditions.
treatments flowering, grow

Table 4. Visual Rating of Tobacco Shoots after 6 Weeks in Storage

TREATMENT		
Atmospheric Pressure (mm Hg)	PO_2 (mm Hg)	Rating
760	152.0	3.46
760	48.6	2.97
300	60.8	2.93
760	26.6	2.34
150	30.4	1.92
760	9.1	1.74
40	8.0	1.29[1]
		S.E. = 0.83

[1]Value represents data from only 1 replication.

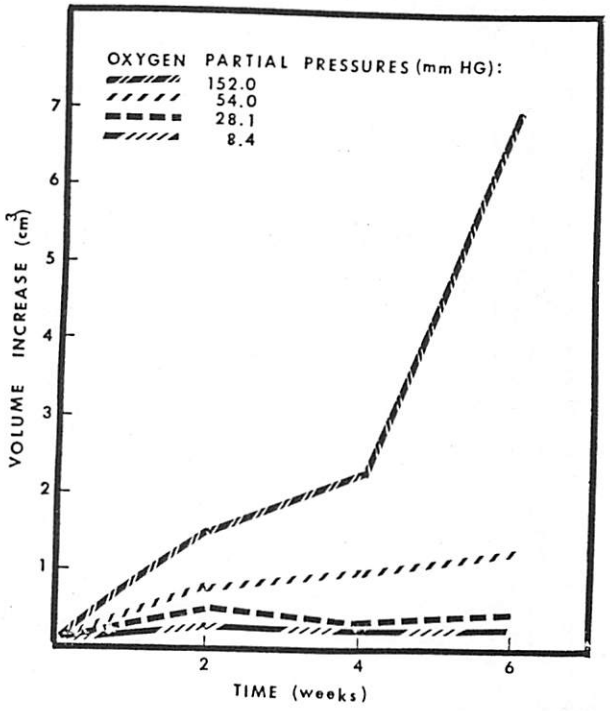

Figure 3. Tobacco callus volume increase after low-oxygen storage for 2, 4, and 6 weeks.

One-third of the chrysanthemum and tobacco plantlets were grown to flowering following each 6 week experiment. To do this plantlets were first transferred onto MS medium supplemented with 1.1 μM IAA and 0.93 μM KIN. Then after 4 weeks these plantlets were potted up in a Metro Mix 200 soil formula and placed in the greenhouse under misting and long days. After 2 weeks plants were grown under standard greenhouse conditions. Little difference was noticed between the treatments flowering, growth habits, and final heights (Tables 5 and 6).

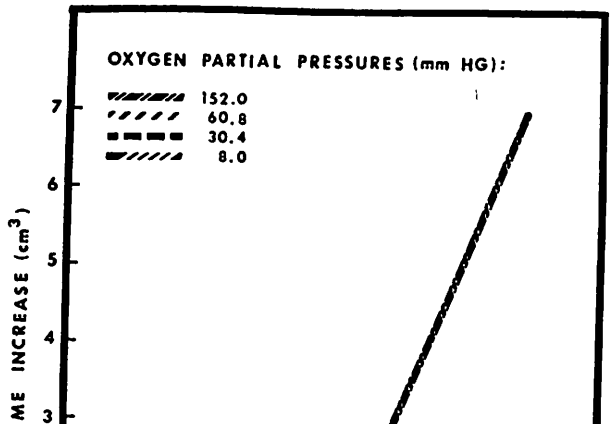

Figure 4. Tobacco callus volume increase after low-pressure storage for 2, 4, and 6 weeks. S.E. = 2.55.

Table 5. Average Height, after 40 Days in the Greenhouse, of Chrysanthemum Plants Which Were Previously Stored for 6 Weeks Under Low Oxygen and Low Pressure Conditions

TREATMENT		
Atmospheric Pressure (mm Hg)	PO_2 (mm Hg)	Height (cm)
760	152.0	32.7
760	54.0	27.0
300	60.8	35.2
760	28.1	32.4
150	30.4	29.7
760	8.4	32.3
70	8.0	27.7
		S.E. = 2.96[1]

[1]S.E. = Standard error.

PROTOCOLS

In Bridgen and Staby's experiments (1981), the plant material was prepared in the following manner. Chrysanthemum plants were grown

Protocols of Low-Pressure

Table 6. Average Height,
Tobacco Plants
Under Low Oxy

Atmospheric Pressure (mm
760
760
300
760
150
760
40

[1]S.E. = Standard error.

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reached a height of 15.0 c
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for 1 min, and then allow
until ready to culture. Th
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were obtained from existing

All cultures were grown
modified MS medium supple
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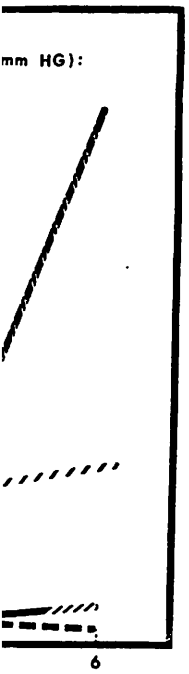

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Hg)	Height (cm)
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	35.2
	32.4
	29.7
	32.3
	27.7
	S.E. = 2.96[1]

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Table 6. Average Height, after 150 Days in the Greenhouse, of Tobacco Plants Which Were Previously Stored for 6 Weeks Under Low Oxygen and Low Pressure Conditions

TREATMENT		
Atmospheric Pressure (mm Hg)	PO_2 (mm Hg)	Height (cm)
760	152.0	49.25
760	48.6	47.75
300	60.8	50.00
760	26.6	44.50
150	30.4	40.30
760	9.1	52.75
40	8.0	48.00
		S.E. = 4.04[1]

[1]S.E. = Standard error.

under greenhouse conditions with 16 hr days, and pinched when they reached a height of 15.0 cm. Lateral bud breaks were then removed, soaked in 1.05% sodium hypochlorite for 15 min, rinsed in 50% ethanol for 1 min, and then allowed to remain in 0.26% sodium hypochlorite until ready to culture. These lateral, vegetative buds were placed in vitro and used as stock plants. Each experiment commenced with 5.0 mm shoot tips removed from the stock plants. Tobacco stock cultures were obtained from existing cultures.

All cultures were grown in 30 ml French square glass bottles on a modified MS medium supplemented with 0.11 μM IAA and 0.93 μM KIN for chrysanthemum sections, 1.1 μM IAA and 0.93 μM KIN for tobacco shoots, and 4.5 μM 2,4-D and 100 ml/l CW for tobacco callus. Culture medium was sterilized using a steam pressure autoclave at 121 C for 12-15 min. Immediately before each experiment, each bottle cap was completely unscrewed and set on top of the bottles to allow adequate moisture exchange.

All experiments were performed in a randomized block design, with each treatment being replicated twice and with 16 bottles of plantlets per treatment. The plant material in each experiment was grown at uniform conditions under 26-28 C with 16 hr daylengths and 2.0-2.2 kilolux light intensity supplied by cool-white fluorescent lights. Each treatment was maintained in a 10 liter desiccator which was placed inside a clear polyethylene bag. The desiccators were scrubbed in 1.05% sodium hypochlorite before the onset of each experiment.

All low-pressure systems were run from a Precision Scientific Model 75 vacuum pump which pulled the air through potassium permanganate filters to remove various hydrocarbons including ethylene (Scott et al., 1970), then through Matheson Model 49 pressure regulators to air flow meters and a water bath, before reaching the desiccators (Fig. 5a). A relative humidity of 94-96% was maintained by passing the atmospheres through the water bath which consisted of 1 liter side-arm Erlenmeyer flasks filled with 850 ml of distilled water. The temperature of this water was raised 3 C above room temperature to allow maximum humi-

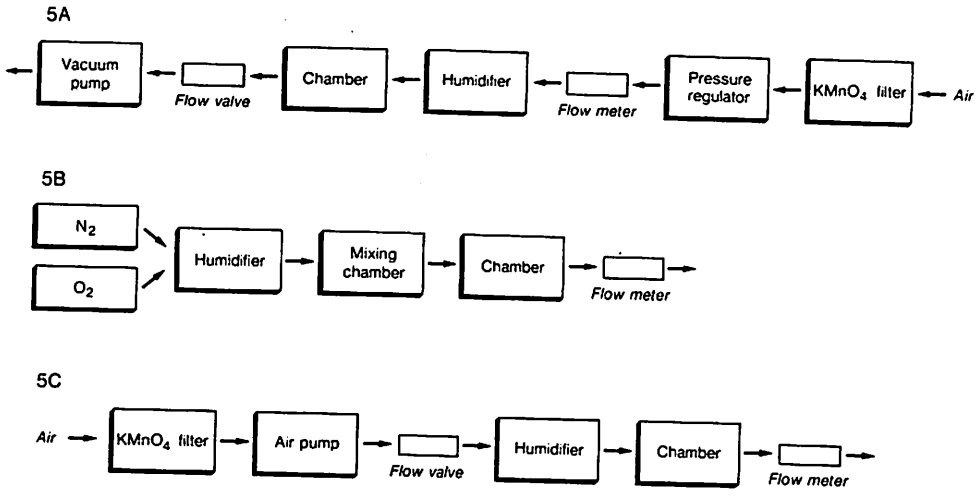

Figure 5. Schematic of low pressure (a), controlled atmosphere (b), and atmospheric pressure systems (c).

dity in the chamber (Gaffney, 1978). The gas flow rates of all treatments were monitored daily along with the pressures of each LPS treatment which were estimated with a mercury manometer.

Controlled atmosphere systems were comprised of various combinations of oxygen and nitrogen, which were humidified to 60-72% by bubbling the gases through a tank of water (Fig. 5b). Gas concentrations were obtained by maintaining constant pressures of each gas and by using glass tube orifices of different sizes to control the percentage of each gas that was entering the system. Gas composition of the atmosphere was measured on a Packer thermal conductivity gas chromatograph at an oven temperature of 100 C and an injector and detector temperature of 170 C. A 3 mm x 90 cm stainless steel column was packed with a 5 Å 60/80 mesh molecular sieve for O_2 and N_2. Initially, each experiment had 3 ml gas samples evacuated for daily analysis; however, after an atmosphere was established, samples were tested weekly.

Controls were set up at atmospheric pressures and atmospheric oxygen concentrations at a relative humidity of approximately 94-96% created by the same system as described for the low pressures. All air was pulled through a potassium permanganate filter and air flows were maintained by a Universal 1.3 amp air pump (Fig. 5c).

Contamination in each experiment was never greater than 10% after the 6 week period. This was due in part to the low-oxygen environments, but also due to the fairly aseptic conditions that were maintained.

FUTURE PROSPECTS

Although the tissue cult very successful to date, s examined before commercia cern, particularly if these plasm preservation, is the tial pressures of oxygen on genotypic variations in the

An aspect of the LPS sy desiccation at low pressure tip experiments and somev grown in liquid medium (Br plasm preservation would l possibly be controlled by growth chamber or by de hour.

Another aspect of LPS would be the effects of There may be additional low-oxygen conditions over

Comparisons should be m determine which one is th The LOS system may be re efficient vacuum pump in Once set up, the LPS shoul

These experiments and t partial pressures of oxygen organized and unorganized lished by using either LPS growth differences. With techniques may be feasible germplasm banks.

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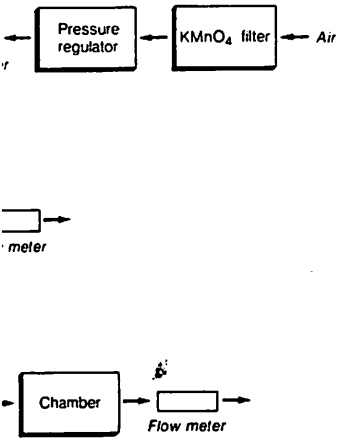

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to the low-oxygen environ-
onditions that were main-

FUTURE PROSPECTS

Although the tissue culture research with LPS and LOS has been very successful to date, several aspects of the procedures should be examined before commercial applications can be made. Of major concern, particularly if these techniques were to be used for plant germplasm preservation, is the examination of long-term effects of low partial pressures of oxygen on the plants. If these low PO_2 cause subtle genotypic variations in the cultures, these systems may not be feasible.

An aspect of the LPS system that should be examined is the medium desiccation at low pressures. This was exhibited in the tobacco shoot tip experiments and somewhat in experiments with tomato root tips grown in liquid medium (Bridgen, 1979). Medium desiccation for germplasm preservation would limit the storage time of cultures and could possibly be controlled by elevating the relative humidity within the growth chamber or by decreasing the number of air exchanges per hour.

Another aspect of LPS and LOS storage systems to be examined would be the effects of C_3 and C_4 plants under the various PO_2. There may be additional advantages to storing C_4 plants under the low-oxygen conditions over C_4 plants.

Comparisons should be made between the LPS and LOS systems to determine which one is the easiest to use and the most economical. The LOS system may be relatively costly on a large scale, whereas an efficient vacuum pump in the LPS system would be less expensive. Once set up, the LPS should be relatively easy to run and monitor.

These experiments and the theories backing them demonstrate that partial pressures of oxygen below 50 mm Hg reduce the amount of both organized and unorganized plant tissue growth. This can be accomplished by using either LPS or LOS and does not create phenotypic growth differences. With these facts in mind, it appears that these techniques may be feasible to use in the future for plant tissue culture germplasm banks.

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