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ORIGINAL PAPER

Tissue culture in synthetic atmospheres: diffusion rate effects on cytokinin-induced callus growth and isoflavonoid production in soybean [*Glycine max* (L.) Merr. cv. Acme]

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Abstract Concentration is one factor that is known to determine how metabolic gases influence the growth and secondary metabolism of plant tissues in culture. How actual gas bioavailability influences these processes has not been studied despite its potential importance in specialized applications. A simple model system, soybean [*Glycine max* (L.) Merr. cv. Acme] callus culture, was selected for experiments because exogenous cytokinin (6-benzylaminopurine; BAP) elicits two types of responses: (1) enhanced callus proliferation, and (2) rapid induction of the isoflavonoid daidzein (7,4′-dihydroxyisoflavone). Synthetic atmospheres supplying metabolic gases with higher or lower bioavailability than in air were created by replacing the nitrogen moiety in standard air with either helium or argon, respectively. Callus was cultured on agar or in liquid shake cultures according to standard procedures. At an optimal cytokinin concentration for stimulation of callus proliferation, 4.4×10^{-7} M BAP, increased diffusion rates for the metabolic gases resulted in greater weight gain in agar cultures. Weight gain was 11% higher for He-treated and 39% lower for Ar-treated cultures than for the nitrogen control. In contrast, there was no significant effect of metabolic gas diffusion rate on daidzein production in either agar or liquid cultures. Apart from the potential application of these synthetic atmospheres for enhancing plant tissue culture growth, they may have unique value for the space program as an effective way of replicating the gas exchange limitations posed for plants by microgravity (Ar atmosphere), and as a countermeasure for this limitation (He atmosphere).

Keywords Buoyancy-driven convection · Gaseous environment · Isoflavonoids · Microgravity · Secondary metabolism · Tissue culture

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Introduction

Metabolic gas concentrations in the atmosphere surrounding tissue cultures are routinely controlled in animal systems. In plant tissue culture,

attention is increasingly given to roles of oxygen, carbon dioxide and ethylene in controlling growth and differentiation (Buddendorf-Joosten and Woltering 1994; Linden et al. 1995; Tate and Payne 1991). Recently, our lab has highlighted the distinction between bulk concentrations and actual bioavailability of gases, most notably in the unusual environment on orbiting spacecraft, where microgravity negates buoyancy-driven convective air movement and leads to symptoms of hypoxia and carbon starvation in plants due to the limitation of gas exchange by the rate of diffusion alone (Porterfield et al. 1997; Musgrave et al. 1998). Plant tissue cultures are usually grown in ambient air, in which the bulk gas, nitrogen (78%), carries the metabolic gases oxygen (21%) and carbon dioxide ($<0.1\%$). Replacement of atmospheric nitrogen with the noble gases helium or argon produces a series of air-like gas mixtures with differing densities (0.4, 1.2, and 1.7 kg/m^3 for He, N_2 and Ar, respectively) and diffusion properties. Even though the concentrations of oxygen and carbon dioxide are the same in these synthetic atmospheres, Fig. 1 shows that using different carrier gases varies oxygen diffusion by as much as 3-fold (comparing He- and Ar-substituted atmospheres).

The experiments described here were designed to investigate how changes in metabolic gas

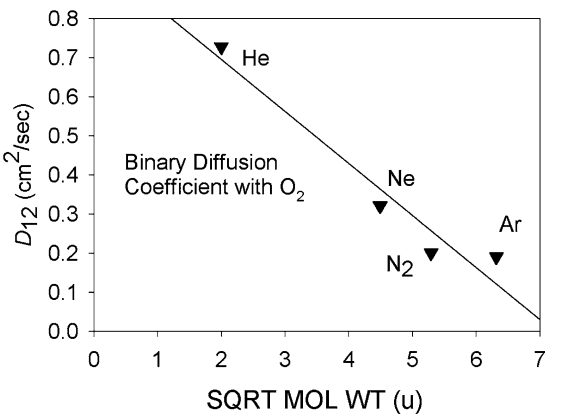

Fig. 1 Binary diffusion coefficients for various gases paired with oxygen were calculated according to Reid and Sherwood (1996) and Lyman et al. (1990). The graph shows how the diffusion coefficient for oxygen moving in a He atmosphere differs from those for movement in N_2 or Ar, based on a relationship with the molecular weight of the gas. Similar relationships hold for the movement of CO_2 in these different carrier gases

bioavailability influence growth and secondary metabolism in a morphologically simple system, soybean [*Glycine max* (L.) Merr.] callus culture. Soybean callus was chosen because its proliferation is stimulated by cytokinin (CK) in a classic manner (Miller 1961), and it also exhibits rapid CK-inducible production of a compound of medicinal value, the isoflavonoid daidzein (7,4′-dihydroxyisoflavone) (Miller 1969, 1972). Recent studies have pointed to daidzein and its glycosylated form, daidzin, as anti-dipsotropic agents, decreasing voluntary intake of ethanol by up to 75% in a rat model of alcoholism (Lin et al. 1996), so factors influencing the production of this compound by tissue cultures are of interest.

The gaseous environment is known to play a role in secondary metabolite production in plant tissue culture. Pan et al. (2000) found that the production of a taxane diterpene in *Taxus chinensis* cultures was increased slightly when treated with 2% CO_2. Bioreactors that recirculated exhaust gases from liquid cultures of *Catharanthus roseus* were shown to have higher concentrations of dissolved CO_2 in the medium and higher production of the alkaloid ajmalicine (Schlatmann et al. 1993). Linden et al. (1995) have noted that cell growth and secondary metabolite productivity of *Artemisia annua* and *Taxus cuspidata* cultures depend on dissolved gas composition in the medium, with interactions between the effects of carbon dioxide, oxygen, and ethylene. Tate and Payne (1991) have proposed that subambient levels of oxygen may reduce both the growth and secondary metabolite levels of cultured cells of *Daucus carota* and *C. roseus*. Collectively, these studies suggest that an increased supply of O_2 or CO_2 to plant tissues in culture results in higher growth and secondary metabolite production.

In contrast to these studies on the effects of metabolic gas concentration on growth and secondary metabolite production of tissue cultures, our interests lie in understanding how the diffusion rate of metabolic gases may influence these processes. Based on the work reviewed above, we hypothesized that increased bioavailability of metabolic gases via enhanced diffusion would allow increased rates of both primary and secondary metabolism. Like low atmospheric pressure growth systems (Musgrave et al. 1988),

synthetic atmospheres that replace the N_2 in our standard atmosphere with He have the potential of enhancing gas exchange in plant tissue cultures. Conversely, an Ar-substituted atmosphere would be expected to slow growth and metabolism due to reduced diffusion by the metabolic gases in it.

This research has application to the long-term exploration agenda of the space program. Because the lack of buoyancy-driven convection in microgravity limits gas exchange, various strategies are being examined that would promote optimal plant growth in this unusual environment. Furthermore, the production of secondary metabolites under spaceflight conditions is an important but rarely studied area of space biology. Detrimental health effects of radiation exposure and gravity unloading during long-term missions might be mitigated by a diet rich in antioxidants or other chemopreventive agents. The ability to stimulate secondary metabolite production through the use of an alternative, inert carrier gas could also provide improved yields of natural products generated on earth using bioreactors.

Materials and methods

Tissue source and assay procedures

Callus for this study was derived from *Glycine max* (L.) Merr. cv. Acme (USDA Soybean Germplasm Collection, Urbana, IL) epicotyl tissue. Dark-grown seedlings (2 d old) were surface sterilized for 10 min in a solution of 10% bleach and 0.1% Tween-80. Following three 5 min rinses in sterile water, the seedlings were dissected to yield epicotyl tissue that was cultured on a growth medium containing 5 mg/l naphthalene acetic acid, 0.5 mg/l 6-benzylaminopurine, 0.4 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 30.0 g/l sucrose, 8.0 g/l agar, 4.3 g/l Murashige–Skoog basal medium, and a pH between 5.7 and 5.8 (modified from Miller 1961 and Smith 1992). Stock callus initiated using this procedure was maintained on the above medium.

Preliminary trials confirmed the dose-response effect of 6-benzylaminopurine (BAP) on soybean cultures in Miller's (1961, 1969) two CK assays: callus weight gain and daidzein production. For these trials, the concentrations of BAP used in both weight gain and daidzein production experiments were 0, 4.4×10^{-9}, 4.4×10^{-8}, 4.4×10^{-7}, 4.4×10^{-6}, 4.4×10^{-5}, and 4.4×10^{-4} M. These levels were chosen because Miller (1969) reported a saturation effect of the cytokinins kinetin and zeatin for the weight gain and daidzein production in a dose-response test using Acme soybean callus, with little difference between 10^{-6} and 10^{-5} M. Data from preliminary trials were used to assign concentrations of BAP for use in gas experiments to assess whether there was any interaction between gas diffusion rate and BAP concentration.

For the callus assays, single 10 ± 2 mg pieces of stock callus were sub-cultured into sterilized 25 ml sample jars containing 10 ml of sterile callus growth medium at the desired CK concentration. Six replicates (jars) were made for each hormone treatment. Jars were placed under continuous fluorescent light (50 μmol/m^2/s, PAR) at 25°C for 21 d, and then fresh weight of each callus was measured. After weighing, each fresh callus was extracted with 95% ethanol using a 1:10 (w/v) ratio, and relative daidzein content was measured as outlined below. For dry weight determinations, callus was dried at 60°C for 48 h prior to weighing.

For Miller's (1969) daidzein production assay, callus (0.57 ± 0.005 g) was mashed using sterile technique, then transferred to a 50 ml Erlenmeyer flask containing 11.4 ml of a sterile liquid medium composed of 30.0 g/l sucrose, 300 mg/l KH_2PO_4, 0.8 mg/l KI, and 2.0 mg/l NAA. BAP was added as described above to achieve the desired hormone levels. Each treatment had four replicates (flasks), and flasks were closed off with cotton plugs. Flasks were placed on a gyratory shaker set to 100 rpm in the dark at 25°C and incubated for 48 h. After this time, 45.6 ml 95% ethanol was added and each flask was transferred to a refrigerator and incubated at 4°C for 24 h. After this time the callus was separated away from the supernatant by centrifuging at 3,000 rpm for 3 min. The differential absorbance of extracts over 200–350 nm with respect to a sample without BAP was read on a Spectronic Genesys 5 spectrophotometer. Using mass spectrometry, Levine

et al. (2001) previously validated ultraviolet (UV) spectra of the isoflavonoids obtained from soybeans in this standard manner (Miller 1969). Therefore, results of the daidzein assay were quantified simply as the absorbance at 262 nm relative to samples not treated with BAP after confirming that the difference spectrum was consistent with known UV spectra of ethanolic daidzein extracts, i.e. with a wide peak around 260 nm, a shallow valley around 240 nm, and a shoulder around 300 nm (Geissman 1962; Mabry et al. 1970; Levine et al. 2001). Because extracts from liquid shake cultures treated with 10^{-4} M BAP exhibited a difference spectrum characteristic of genistein in ethanol [with a narrow peak near 270 nm and a deep valley near 230 nm (Geissman 1962, Mabry et al. 1970; Levine et al. 2001)] rather than daidzein, these data were excluded from the daidzein assay analysis.

Synthetic atmosphere experiments

Three custom-blended gas mixtures (Merriam-Graves Corp., Springfield, MA) composed predominantly (79%) of N_2, Ar or He each were verified by gas chromatography to contain 21% O_2 and 400 ppm CO_2, the concentration of these metabolic gases found in ambient air. For the gas weight-gain experiment, each gas treatment was delivered to a collection of sample jars in gas-tight chambers. After a 10-min initial purge, a rate of 0.5–1 ml/min was maintained through the chambers for the 20-d growth period. Gas chromatography of aliquots from the chamber headspace confirmed that the desired atmosphere was provided by this flow rate. Each chamber was surface sterilized with 95% ethanol, and a 2 cm thick pad of sterilized florist's foam (Smithers Oasis USA, Kent, OH) moistened with sterile water was installed to maintain humidity. In each chamber were placed a total of 18 sample jars, each containing a 10 $\pm$ 2 mg callus. Six jars per BAP treatment were used in each chamber. Chambers were closed and then transferred to the growing conditions described previously. At the end of each 20 d experiment (3 were run in total), the fresh weight of each callus was measured and portions of each callus were placed into 95% ethanol in a ratio of 1:100 (w/v) for later UV spectrophotometry to monitor relative daidzein content as described previously.

For the experiment investigating gas effects on daidzein production in liquid cultures, each gas treatment was delivered to individual flasks via a single 10-outlet manifold connected directly to the gas cylinder. Each manifold outlet was connected to a tubing inlet through a cotton plug via a 0.2 μm Acrodisc syringe filter (Pall Gelman Science Inc., Ann Arbor, MI) and an 18-gauge disposable syringe needle. The appropriate flow rate to achieve the desired experimental conditions (12–15 ml/min) was experimentally determined by gas chromatography as noted above. Four replicates (flasks) were used for each BAP treatment along each manifold, leaving two unused outlets. After culturing, flasks were held under the same conditions as in the preliminary test, and gases were delivered for 48 h. In-line flow meters (between the cylinder and the manifolds) were used to monitor total flow delivered and flow rate was adjusted as needed. Four trials of this experiment were run.

Analysis

Statistical analysis was conducted using the general linear model to carry out one- or two-way analyses of variance in SAS statistical software (SAS Institute Inc., Cary, NC). Individual trials were considered random effects, while effects of hormonal or gas treatments were considered fixed effects. Means separations were conducted using Duncan's multiple range test, with $\alpha = 0.05$ used to determine significance.

Results

The BAP dose responses by the three model systems used in this investigation are shown in Fig. 2. The response of the soybean tissue in the callus proliferation assay was optimal at 10^{-7} and 10^{-6} M BAP (Fig. 2A). Callus weight gain was typified by highly correlated fresh and dry weights across the CK-response range (Fig. 3), confirming that weight gain was due to cell proliferation rather than differential accumulation of water. Response in the two daidzein production assays

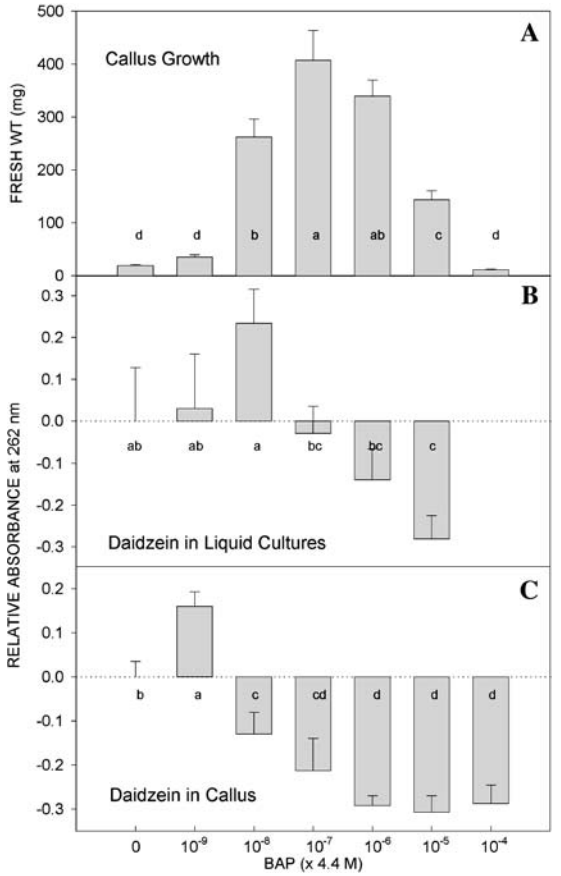

Fig. 2 Dose response of soybean callus cultures to 6-benzylaminopurine (BAP) in three different systems: (**A**) weight gain by callus grown over 21 d, (**B**) daidzein production by callus in liquid shake culture, and (**C**) daidzein production by callus culture grown as in (**A**). Bars represent means ± standard error. Bars marked with the same letter are not significantly different at the 0.05 level according to Duncan's Multiple Range Test

peaked at lower CK concentrations than the callus proliferation assay. Rapid induction (48 h) of daidzein production by liquid shake cultures peaked at 10^{-8} M (Fig. 2B). Callus cultures used in the daidzein production assay (20 d) showed a foreshortened dose-response curve (Fig. 2C), with peak relative absorbance at 10^{-9} M BAP and no significant differences among the lowest levels of absorbance between 10^{-7} and 10^{-4} M BAP. A statistical analysis confirming the power of the CK response in the three assays is given in Table 1.

How conducting these assays in synthetic atmospheres with different carrier gases (He, N_2, Ar) influenced these CK responses is shown in two ANOVA tables (Table 2, for the callus growth assay and Table 3, for the two daidzein production assays). As seen in Table 2, BAP treatment had a highly significant effect ($P = 0.008$) on weight gain of callus cultures. While carrier gas had no effect upon weight gain overall, there was a significant interaction between BAP treatment and carrier gas treatment ($P = 0.035$; Table 2). Separating the interaction between BAP treatment and gas treatment revealed that carrier gas had a highly significant effect on weight gain only in cultures treated with 10^{-7} M BAP (Table 2). In particular, Ar-treated cultures had significantly lower growth than He-treated cultures (Fig. 4).

The effect of BAP in the daidzein assay in liquid shake cultures run in synthetic atmospheres was significant at the $P = 0.07$ level, with

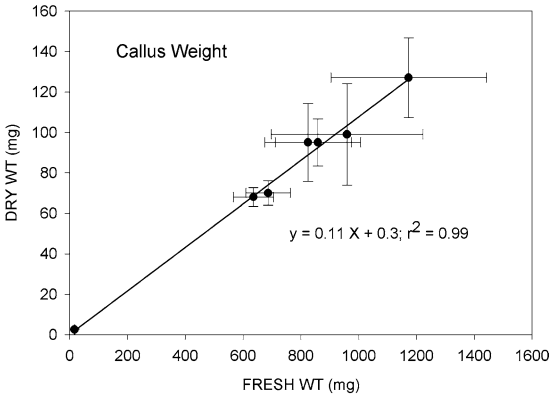

Fig. 3 Weight of soybean callus following drying at 60°C for 48 h was linearly related to fresh weight of the same samples over a range of CK-induced growth responses. Data shown are means ± standard error; $n = 5$

Table 1 Analysis of variance for dose-responsiveness of weight gain and relative daidzein production to 6-benzylaminopurine in callus and liquid shake cultures of soybean

Culture type	Effect on	*n*	*F*	*P*-value
Callus	Weight gain	6	33.41	< 0.001[a]
Liquid shake	Daidzein production	4	3.40	0.024[a]
Callus	Daidzein production	6	15.93	< 0.001[a]

For all tables, *n* = Number of samples per level of treatment, *F* = *F*-ratio for one-way ANOVA, *P*-value = probability that differences in means were due to chance

[a] Significant at the 0.05 level

Table 2 Analysis of variance for weight gain of soybean callus cultures grown under different levels of 6-benzylaminopurine and gas mixtures of differing diffusion rates (n = 4–6)

Factor	F	P-value
BAP	127.28	0.0078[a]
Carrier gas	3.02	0.2485
BAP × Carrier gas	7.96	0.0346[a]
Carrier gas:No BAP	0.05	0.9536
Carrier gas:10^{-7} M BAP	17.07	< 0.001[a]
Carrier gas:10^{-4} M BAP	0.12	0.8848

[a] Significant at the 0.05 level

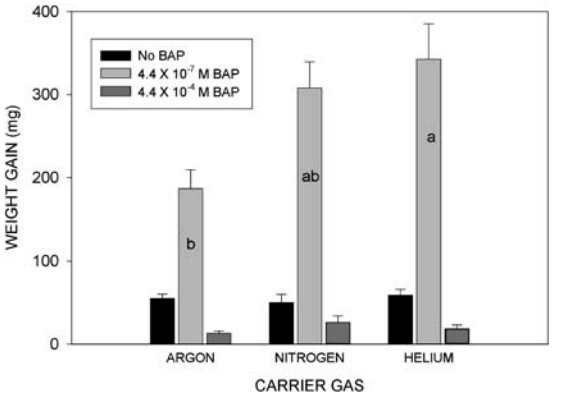

Fig. 4 Weight gain of soybean callus cultures grown under atmospheres of altered diffusion rates and three levels of 6-benzylaminopurine. Bars represent means ± standard error. Data from two trials were pooled. Bars with the same letter are not significantly different at the 0.05 level according to Duncan's Multiple Range Test

untreated cultures having lower relative daidzein content than those treated with 10^{-8} M BAP (Table 3). There was no significant effect of gas treatment on relative daidzein content in either the liquid shake or callus cultures. In contrast to the results with the callus growth assay, for the daidzein production assays, there was not a significant interaction between gas treatment and BAP treatment regardless of whether the callus or liquid shake culture system was used (Table 3).

Discussion

The prediction that carrier gas would influence proliferation rate in soybean callus cultures was confirmed by the present study (Fig. 4). While there was no significant effect of carrier gas on weight gain in callus grown on medium containing no BAP or 10^{-4} M BAP, there was a highly significant effect for callus grown on 10^{-7} M BAP. While CK is known to promote cell division, at high concentrations it can also inhibit respiration (Miller 1979). At 10^{-7} M BAP, presumably neither cell division nor cellular respiration was limiting. Under these conditions the diffusion rate of the metabolic gases (as determined by the carrier gas) played a major role in callus proliferation, with an 11% increase in cumulative weight gain for the He-treated and a 39% decrease for the Ar-treated cultures, compared to that for N_2-treated cultures.

As observed by Schreiner (1968) for linear growth rate of *Neurospora crassa* hyphae grown for 100 h under atmospheres containing oxygen and various noble gases, the cumulative weight gain in soybean cultures grown under 10^{-7} M BAP showed a good fit to a line inversely proportional to the square root of the molecular mass of the carrier gas (Fig. 5A). At pressures below that of 30 Earth atmospheres (3,000 kPa), the square root of the molecular weight is used to calculate diffusion coefficients for pairs of gases (such as O_2 diffusing in He, etc.; Reid and Sherwood 1996; Lyman et al. 1990) (Fig. 1), explaining the linear relationship observed between this parameter and growth measures in both systems. A better fit (r^2 = 0.82) was obtained when callus growth was plotted against the respective densities of the 21% O_2, 79% carrier gas mixtures (Dudley and Chai 1996) (Fig. 5B). Although Schreiner (1968) claimed that gas density did not account for the effect on growth rate seen in the fungus, he based his argument on

Table 3 Analysis of variance for the daidzein production assay in soybean callus and liquid shake cultures grown under different levels of 6-benzylaminopurine and gas mixtures of differing diffusion rates

Culture type	Factor	n	F	P-value
Callus	BAP	3–6	1.25	0.4453
	Carrier gas		0.55	0.6462
	BAP × Carrier gas		0.63	0.6657
Liquid shake	BAP	4	13.46	0.0669
	Carrier gas		0.26	0.7861
	BAP × Carrier gas		2.27	0.2194

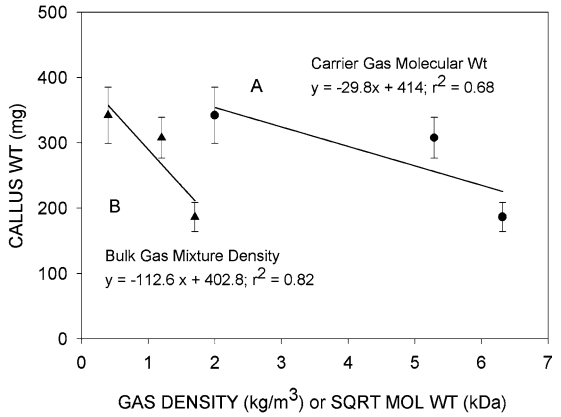

Fig. 5 Weight gain of soybean callus cultures grown under nonlimiting concentrations of BAP is inversely proportional to the square root of the molecular mass of the carrier gas (**A**). A better regression results when the weight gain is plotted against the density of the gas mixtures (21% O_2 combined with the respective carrier gases) (**B**). Data shown are means ± standard errors of data pooled from two trials

growth in variable gas mixtures at extremely high pressures, when other factors come into play. But at pressures below 3,000 kPa, his data confirm that reducing mean free path length for metabolic gas diffusion by varying either the type of carrier gas or total pressure of the system gave similar reductions in growth. Also of note in Fig. 5 is the similar *y*-intercept for both regression equations, which suggests a maximal growth response in a hypothetical low-pressure atmosphere with no carrier gas to interfere with the movement of the metabolic gases. Indeed, Musgrave et al. (1988) reported enhanced growth by mungbean seedlings at one-fifth atmospheric pressure (compared to that at ambient pressure), even though both treatments had experienced the same partial pressures of oxygen.

The results of the daidzein assay did not support the prediction that enhanced diffusion of metabolic gases would increase secondary metabolism. One possible explanation for the lack of effect of carrier gas on secondary metabolism in the liquid culture system is that the resistance of the liquid medium to gas diffusion plays a more limiting role in bioavailability of gases to the cells than does the resistance of the carrier gas to diffusion. However, in the present study, callus cultures also showed no significant differences in relative daidzein content between gas treatments (Table 3). These cultures had no liquid resistance to diffusion and were treated with gases for 20 d, a point at which both Pan et al. (2000) and Schlatmann et al. (1993) observed differences between gas treatments for secondary metabolites in *T. chinensis* and *C. roseus*, respectively. The most likely explanation for the absence of an effect of gas diffusion rate in the daidzein assay is that differences in diffusion rates between argon, nitrogen, and helium do not change bioavailability of oxygen enough to be relevant to isoflavonoid metabolism. Tisserat et al. (2002) noted that production of thymol in *Thymus sativa* (thyme) was not significantly different between cultured plants treated with 10% oxygen and those treated with ambient levels of oxygen when carbon dioxide was not limiting. They also noted that piperitenone oxide production in *Mentha* sp. (mint) was not significantly different between cultured plants treated with 21, 32, or 43% oxygen when carbon dioxide was not limiting.

The foreshortened daidzein accumulation curve in callus cultures (Fig. 2C) suggests a tradeoff between primary and secondary metabolism. Maximum response in the daidzein production assay by callus cultures was found in callus treated with 10^{-9} M BAP, which was a concentration still too low to promote significant increases in fresh mass (Fig. 2A). Van der Plas et al. (1995) drew a similar conclusion based on observations of two apparently separate developmental programs in *Morinda citrifolia*, one favoring cell division and the other favoring anthraquinone production. Similarly, Kim et al. (2003) compared liquid- and gas-phase reactors for culturing plant tissues and have shown that an aeration scheme that maximized growth rate did not result in optimal secondary metabolite production.

Reductions in growth of soybean callus cultures in gas environments of reduced diffusion rate but neither limited nor inhibited by their CK supply (Fig. 4) support previous studies, which suggested that reduced diffusion of metabolically active gases may be a limiting factor to plant growth under spaceflight conditions (for example, see Porterfield 2002). This underscores the importance of proper ventilation for plant growth

chambers used in spaceflight experiments, and provides preliminary evidence that slowing gas diffusion rate may provide a suitable earth-based model for understanding plant growth in environments with reduced buoyancy-driven convection. Recently, Kuang et al. (2006) reported similarities between the ultrastructural features of Brassica and wheat grown in microgravity on the Mir space station and those grown on the ground in a synthetic atmosphere that substituted Ar for the N_2 in a normal atmosphere. The reduced diffusion rate of the Ar-substituted atmosphere retarded development in a manner similar to what had been observed in spaceflight material: protein bodies were smaller than in the control treatment, and starch grains were retained later in seed development (Kuang et al. 2000; Musgrave et al. 2000). With regard to microgravity effects on secondary metabolism, Levine et al. (2001) have previously noted that microgravity alters distribution but not overall production of isoflavonoids in dark grown soybean seedlings. The lack of effect of gas diffusion rate on production of daidzein that we report here in both liquid shake cultures and callus cultures of soybean is consistent with their results.

The physically variable gas mixtures used in our synthetic atmospheres offer a unique tool for studying the role of diffusion rate in different metabolic processes. By studying growth and secondary metabolism that is triggered by CK application and by using the morphologically simple callus system we have minimized uncertainties caused by comparisons between different types of plant material. The finding that callus growth is diffusion-limited under optimal CK concentrations has important implications for controlled atmosphere applications in tissue culture work. Furthermore, the He-substituted atmosphere, already in use for medical applications as ''heliox'' (Gupta and Cheifetz 2005), may also serve as a countermeasure strategy for growing plant materials in a microgravity setting, since diffusion gains possible with a He-based atmosphere would counteract the lack of buoyancy-driven convection.

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