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Effects of Sucrose, Inoculum Density, Auxins, and Aeration Volume on Cell Growth of *Gymnema sylvestre*

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To improve the cell protocol for *Gymnema sylvestre*, we investigated the influence of initial sucrose concentration, inoculum density, and optimal concentrations of auxins (IBA and NAA) in flask cultures, as well as the role of aeration volume in bioreactor cultures. Cell growth was enhanced 9-fold when the medium was supplemented with 3% sucrose versus a sucrose-free environment. Increasing the inoculum density to 60 g (wet weight) L^{-1}, but no further, greatly improved the growth of these cultures. All concentrations of IBA proved inhibitory while supplementation with 5 mg L^{-1} NAA was associated with significantly higher dry-cell weights. In our bioreactor cultures, a step-wise increase in aeration volume from 0.05 to 0.40 vvm was optimal for cell growth. Although biomass (i.e., fresh weight) accumulated in the bioreactor up until Day 20, the dry-cell weights increased 10-fold, but only through Day 15. The internal dynamics of our culture media indicated that sucrose was preferentially utilized and that its concentration steeply decreased at the log phase. In contrast, both glucose and fructose supplies were exhausted only at the beginning of the declining phase. Our findings suggest that a 15-d culture period is optimal for *G. sylvestre* cell growth in a bioreactor.

Keywords: bioreactor, cell culture, *Gymnema sylvestre*

Gymnema sylvestre R. Br., a member of the Ascelpidiaceae family, contains acidic glycosides and anthroquinones with antidiabetic, antisweetener, and anti-inflammatory activities. Although the most medicinally important compound in this species is gymnemic acid, one of the D-glucuronides of a hexahydroxyolean-12-ene, only trace amounts of this component are contained within. Commercial and conventional propagation of *G. sylvestre* is greatly impeded by reduced seed viability, inherently low germination rates, and the depressed rooting ability of vegetative cuttings. Likewise, the gymnemic acid content is affected by geographic location, climate, and seasonal variations, and field-grown plants are not a sustainable source for this active ingredient. Therefore, one must seek alternative propagation methods. In this regard, a cell suspension culture system would be beneficial in accelerating the large-scale multiplication, improvement, and conservation of that species.

The optimization and scale-up of plant cultures necessitates an understanding of substrate and inoculum requirements for the promotion of cell growth (Leng et al., 2004; Sivakumar et al., 2005). The main limiting factors are nitrogen source (Rho and Andre, 1991; Wroblewski et al., 1995; Liu and Zhong, 1997), supplies of phosphate and trace ions (Curtis et al., 1991), condition of the medium (Lee and Shuler, 2000), inoculum density (Hahn and Paek, 2005), and CO_2 accumulation (Hohe et al., 1999; Thanh et al., 2006). For most plant cell lines, sucrose is an important carbon and energy source, and its initial concentration can affect such parameters as growth rate and the yield of secondary metabolites (Zhong and Yoshida, 1995; Chen et al., 1997; Shohael et al., 2006). A critical minimum for inoculum size is another determinant of culture success, while its density can influence metabolite formation (Wang et al., 1997), hairy root growth (Kanokwaree and Doran, 1997), and somatic embryogenesis (Mavituna and Buyukalaca, 1996). Aeration volume also increases root production (Hofer, 1996; Kino-Oka et al., 1999). For example, in a liquid-phase reactor, greater root hair density increases the demand for oxygen by 10-fold (Ramakrishnan and Curtis, 1995; Williams and Doran, 1999; Heo et al., 2006), making additional aeration necessary for root proliferation.

In attempts to optimize culturing conditions, most research has focused on auxin sensitivity by hairy roots. Although the synthetic auxin 2,4-D has been extensively used in plant cell cultures, this member of the chlorophenoxy acid group can have deleterious consequences to human health (Manoharan et al., 2005). Therefore, tests are needed that determine the efficacy of other synthetic auxins, e.g., NAA and IBA, in culturing systems. Here, we have studied the effects of initial sucrose concentration, inoculum size, and concentrations and types of auxins in flask culture as well as the ideal aeration volume needed in bioreactor culture to promote cell growth of *G. sylvestre*.

MATERIALS AND METHODS

Flask Culture

Using young leaves of *G. sylvestre*, we initiated callus formation in culture tubes (23×150 mm) containing an MS medium supplemented with 20 g L^{-1} sucrose, 0.44 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.23 mg L^{-1} 6- benzyladenine (BA), and 2.3 g L^{-1} Gelrite (Duchefa, The Netherlands). The media pH was adjusted to 5.8 before autoclaving at 121°C. Cultures were maintained at 25 ± 2°C under a 16-h photoperiod and with a light intensity of 40 μmol m^{-2} s^{-1}. These cell suspensions were sub-cultured at 2-week intervals. The cultures (6 g wet weight, WW) were transferred to 400-mL conical flasks containing 100 mL of the liquid medium, and were grown for 15 d in the dark on a gyratory shaker (95 rpm) at 20 ± 2°C. Except for

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the assessment of inoculum density, all trials were conducted with 6 g (WW) of inoculum per 100 mL of media. Although the test compositions varied, each experimental medium was supplemented with 1 mg L^{-1} of 2,4-D, 0.1 mg L^{-1} kinetin, and 30 g L^{-1} sucrose, except for trials that focused on individual auxins and sucrose concentrations. The sub-cultured cells were transferred at Day 15 to a testing medium, at an initial cell density of 60 g (WW) L^{-1}. For our sucrose experiments, the various MS media were supplemented with 10, 30, 50, 70, or 90 g L^{-1} sucrose. Evaluations of inoculum density were conducted on media inoculated with 20, 40, 60, 80, 100, or 120 g (WW) L^{-1} of cells. To investigate the effect of substituting another auxin for 2, 4-D, we added either NAA or IBA at rates of 1, 3, 5, 7, or 9 mg L^{-1}.

Bioreactor Culture

Bioreactor cultures were maintained for 15 d in a dark room at 20 ± 2°C. Each 3-L balloon-type bioreactor contained 2 L of an MS medium (working volume) supplemented with 30 g L^{-1} sucrose, 5 mg L^{-1} NAA, and 0.1 mg L^{-1} kinetin. After 60 g (WW) L^{-1} of cells were added, the medium was aerated with sparges, and the airflow rate was adjusted during the cultivation period to a homogenous mixing state. To examine the influence of aeration volume on cell growth, several levels were tested: 0.05, 0.10, 0.20, 0.30, or a progressive treatment of 0.05 to 0.40 vvm. For that last scenario, the initial aeration volume was set at 0.05 vvm, then increased in a step-wise manner to 0.1, 0.2, or 0.4 vvm according to the pattern of cell growth.

Growth Measurements

Samples were harvested at regular intervals by passing the collected supernatant through Advantec filters (Toyo Roshi Kaisha, Japan). Fresh weights were recorded gravimetrically after the cells were first lyophilized under vacuum (DOA-P104-AA; Gast, USA). Dry weights were then determined after the cells were oven-dried at 60°C for 48 h. The growth indices and rates (Martinez and Park, 1993) of these suspension cultures were calculated with the following equations:

Growth index = Final fresh cell weight/ Initial inoculum fresh cell weight; and

Growth rate = [(Final cell weight) – (Initial cell weight)]/ (Initial cell weight)/ (Culture period, in days).

Estimation of Physico-Chemical Properties and Composition of the Culture Media

The electrical conductivity and pH of the media were determined at regular intervals with a pH/ conductivity level 1 (Inolab; WTW, Germany), via Tetracon and Sentix 41 sensors, respectively. To estimate the change in sugar compositions, 50 mL samples were collected regularly during the run, and the supernatants were passed through a 0.45 μM membrane filter (13 mm PVDF syringe filter; Whatman, USA). Sugar concentrations were determined by a Waters 2690 HPLC (USA) equipped with a Waters Coregel 87C carbohydrate column (300 X 7.8 mm; flow rate, 1.0 mL min^{-1}) and using a Waters 410 RI detector (Refractometer Differential, USA).

RESULTS AND DISCUSSION

Effect of Sucrose on Cell Growth

The highest fresh and dry weights were attained in media containing 3% sucrose (Table 1). Based on the cell growth index, dry-cell weights were 5- and 9-fold greater in the presence of 1% and 3% sucrose, respectively, than on the sucrose-free medium. However, despite sucrose being an indisputably important carbon and energy source, elevating its concentration to 5, 7, or 9% resulted in dry-weight reductions of 38.7, 19.4, and 34.9%, respectively, compared with the response to 3% sucrose. This decline in performance might be attributed to the inhibition of nutrient uptake as the osmotic potential was enhanced and the medium became more viscous. However, Do and Cormier (1991) have shown in *Vitis vinifera* that a higher concentration of sucrose can act as an osmotic agent, with mannitol having a similar effect on growth. Additionally, this retardation in growth could be caused by a cessation in the cell cycle when nutrients are limited and sucrose concentrations are higher (Gould et al., 1981; Wu et al., 2006).

Effect of Inoculum Density on Cell Growth

Inoculum density contributes to the success of plant cell cultures by modulating the metabolism of the suspension. Such cultures generally begin with a high threshold for cell concentrations, below which growth does not occur. Here, we noted that inoculum size had a great influence on the growth profile of *G. sylvestre* suspension cultures (Fig. 1A), with dry-cell weights increasing significantly when the initial density was 60 g L^{-1} of inoculum. No further enhancement was achieved above that level, and growth rates, in fact, showed a steep decline at densities of 80 or 100 g L^{-1} (Fig. 1B). Interestingly, however, the maximum fresh and dry weights were attained with an inoculum density of 120 g L^{-1}. Similar fluctuations in growth rate patterns have been reported by Tanaka (1987) and Schlatmann et al. (1994). Earlier studies with oil palm suspension cultures demonstrated that dry-cell weights were highest from an inoculum density of 5 to 15 g L^{-1} (de Touchet et al., 1991); this varying effect of density on growth has also been demonstrated in *Atropa belladonna* (Kanokwaree and Doran, 1997), *Catharanthus roseus* (Contin et al., 1998), and *Panax notoginseng* (Hu et al., 2001).

Table 1. Effect of sucrose concentrations on cell growth of *G. sylvestre* after 3 weeks in flask culture. Values not followed by the same letter within a column indicate significant differences at $P < 0.05$ (DMRT). Data are means ± S.E. (n=6).

Sucrose (%)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth index
0	28.4 e	1.5 d	1.42
1	146.4 b	6.9 c	7.32
3	192.8 a	12.9 a	9.64
5	88.7 c	7.8 c	4.44
7	91.9 c	10.4 b	4.60
9	60.3 d	8.4 c	3.02

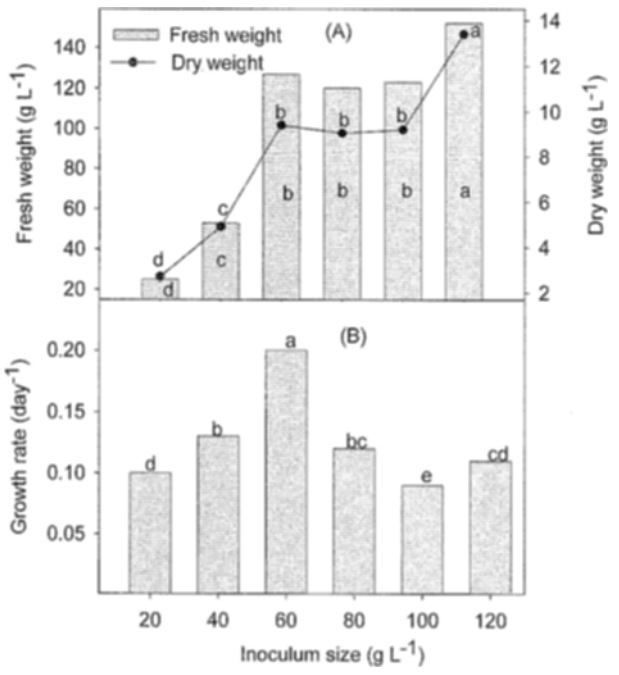


Figure 1. Effect of inoculum size on weights of fresh and dry cells (**A**) and growth rate (**B**) of *G. sylvestre* after 3 weeks in flask culture. Means marked by the same letter in the same parameter are not significantly different at <P 0.05 (DMRT). Values are means ± S.E. (n=6).

Table 2. Effects of IBA and NAA on cell growth of *G. sylvestre* after 3 weeks in flask culture. Values not followed by the same letter within a column indicate significant differences at P <0.05 (DMRT). ns (non significant), * and ** denote significance at P <0.05 or 0.01 respectively. Data are means ± S.E. (n=6).

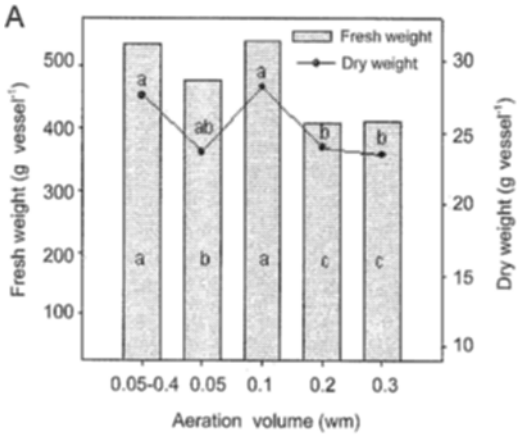
PGR (A)	Concentration (mg L^{-1}) (B)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	% Dry weight
Control	0	220.1 ab	11.1 ab	5.1 d
IBA	1	168.2 cde	9.7 bcd	5.8 bc
	3	189.7 bcd	10.5 abc	5.5 bcd
	5	200.5 bc	10.5 abc	5.2 cd
	7	146.4 e	8.6 cde	6.0 b
	9	110.4 f	7.2 e	6.5 a
NAA	1	215.7 ab	10.6 abc	4.9 d
	3	238.4 a	11.8 ab	5.0 d
	5	242.5 a	11.9 a	4.9 d
	7	199.3 bcd	10.4 abc	5.2 cd
	9	166.6 de	8.2 de	4.9 d
Significance				
PGR (A)		**	*	**
Concentration (B)		**	**	*
A×B		ns	ns	ns

Effects of IBA and NAA on Cell Growth

Auxins are responsible for maintaining plant cell- and tissue-culture systems, and are associated with the promotion of growth, callus proliferation, rooting, and morphological diversity (Dodemann et al., 1997; Chan et al., 2004; Kim et al., 2004). We also observed their significant effect on cell growth (Table 2). For example, compared with the control, the addition of 1, 3, 5, 7, or 9 mg L^{-1} IBA to the medium decreased dry-cell weights by 13, 5, 5, 23, and 35%, respectively. In contrast, dry weights increased 7% in the medium containing 5 mg L^{-1} NAA. However, at higher concentrations, e.g., 7 and 9 mg L^{-1} NAA, those weights were diminished by 6 and 26%, respectively, compared with the control. Thus, higher concentrations of NAA as well as the addition of any IBA to the media was inhibitory. Our results on the positive effects of NAA on cell growth agree with those reported by Leonardi et al. (2001), but contradicted the findings of Cuenca and Amo-Marco (2000) in their research on *Salvia* species.

Effect of Aeration Volume on Cell Growth in Bioreactor Cultures

For the large-scale, bioreactor cultivation of plant cells/tissues, a reliable supply of oxygen is a very important factor (Jeong et al., 2006). Here, an aeration volume of 0.1 vvm



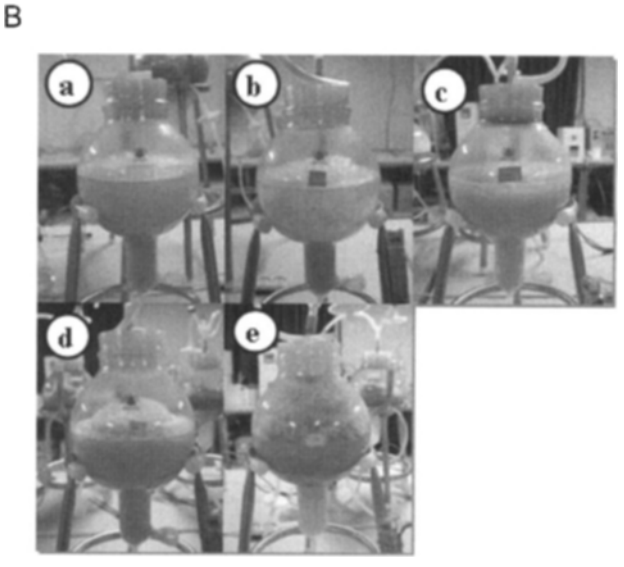


Figure 2. Effect of aeration volume on growth of *G. sylvestre* cells after 15 d in bioreactor culture. (**A**) Fresh and dry weights. Means marked by the same letter in the same parameter are not significantly different at <P 0.05 (DMRT). Values are means ± S.E. (n=3). (**B**) 0.05-0.40 vvm (a), 0.05 vvm (b), 0.10 vvm (c), 0.20 vvm (d), and 0.30 vvm (e)

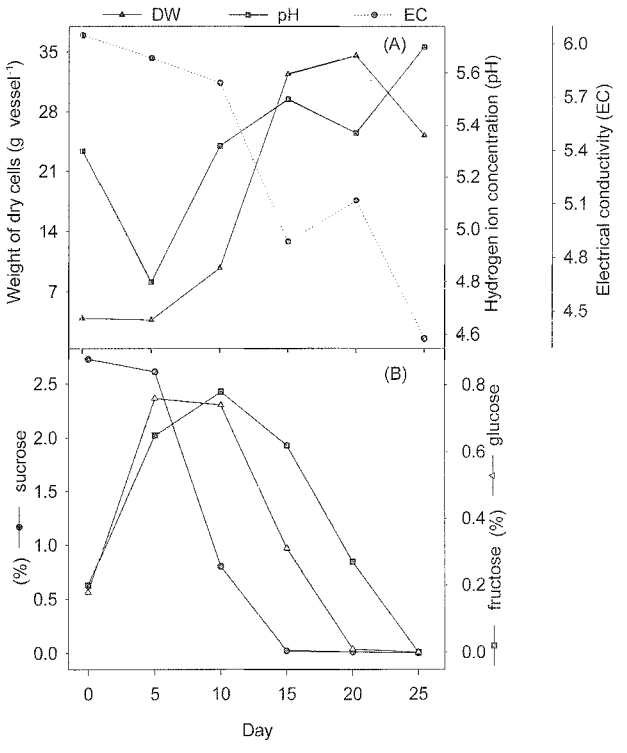


Figure 3. Changes in dry-cell weights, medium pH, and EC (A), and concentrations of sucrose, fructose, and glucose in the medium (B) during bioreactor culture of *G. sylvestre*.

was the most highly correlated with improved cell growth (Fig. 2A-E). Although our step-wise treatment of 0.05 to 0.40 vvm produced an initial effect equal to that achieved with 0.1 vvm, continuous aeration at that lower volume for 15 d resulted in dry-weight increases of 17, 24, and 27% over those recorded from aeration treatments of 0.05, 0.20, and 0.30 vvm, respectively. These observations are in agreement with those reported by Kanokwaree and Doran (1997) and Cui et al. (2000).

We also investigated cell-growth profiles and changes in pH, EC, and concentrations of sucrose, glucose, and fructose in the bioreactor culturing system (Fig. 3A, B). The first 5 d of cultivation were considered the lag phase, with Days 10 through 20 being the log phase. Afterward, the dynamics for this growth pattern shifted briefly to the stationary phase before moving rapidly into the declining phase at Day 25 d. At the peak of the log phase (Day 20), the weights of the dry cells had increased by 8-fold. A similar pattern of growth behavior has been observed in *Perilla frutescens* cell cultures (Zhong et al., 1992).

The decline phase corresponded to an increase in pH and a marginal decline in electrical conductivity (Fig. 3A). During this period, the entire carbon source for the culture medium was exhausted (Fig. 3B). Although alterations in media pH generally are related to the utilization of sugars (Thom and Komor, 1984), variations in EC can be attributed to cell growth as developmental events deplete the media salts. In our experiments, an interesting pattern of monosaccharide utilization was noted over the cultivation period. Concentrations of glucose and fructose were enhanced at the start of the log phase (Day 10), while the level of disaccharide (sucrose) was simultaneously being depleted. By the end of the log phase (Day 20), only fructose was detectable. Sucrose apparently was being hydrolyzed to glucose and fructose by extracellular and/or cell wall-bound invertase during the initial period of cultivation, thereby elevating the concentrations of those two monosaccharides. Thus, the preference by these *Gymnema* cell cultures was largely tilted toward sucrose particularly early on. Similar observations have been made with *Coleus blumei* (Martinez and Park, 1993) and *C. roseus* (Merillon et al., 1984).

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