Plant Res. 1(2) 98~104(1998)

Effect of Media Components and Phytohormones on *in vitro* Frond Proliferation of *Lemna gibba* G3 and 24 Additional *Lemna gibba* Strains

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ABSTRACT

The effects of basal media, sucrose and phytohormone concentrations, and gelling agent combinations on *in vitro* frond proliferation of *Lemna gibba* G3 and 24 additional *Lemna gibba* strains were examined. Frond proliferation was equivalent on Schenk and Hidebrandt, Murashige and Skoog, Nitsch and Nitsch, and Gamborg' s B5 media and poor on Murashige and Skoog medium in the absence of benzyladenine. With the addition of benzyladenine, Schenk and Hildebrandt and Gamborg' s B5 were superior and equivalent. The addition of benzyladenine increased equally frond proliferation at either 1 or 10μM, however at 10μM fronds were severely curled or fused. Benzyladenine and thidiazuron suppressed root growth but kinetin was found to greatly enhance root growth. Gibberellic acid inhibited frond proliferation. Frond proliferation was significantly different on the four sucrose concentrations of 0, 1, 3, and 8% Among them, 3% sucrose was found to be superior. The reduced frond size observed in cultures grown on 8% sucrose could be explained by showing medium osmotic potential in excess of frond water potential. Gelling agents also varied significantly in their ability to promote frond proliferation with 0.25% Gelrite or a mixture of 0.15% Gelrite and 0.4% agar. Proliferation of 25 *Lemna gibba* strains on medium near optimal for *Lemna gibba* G3 showed a six-fold variation across strains with *Lemna gibba* G3 placing in the top 5 fastest proliferating strains.

Key words: duckweed, frond proliferation, media components, PGRS effect.

INTRODUCTION

Duckweeds are the sole members of the monocotyledonous family, *Lemnaceae*. All 32 species are small, free-floating, aquatic plants that rapidly propagate themselves via asexual proliferation. Hillman(1961) recognized that their growth habit, small size, and ease of aseptic culture gave them great potential as model plants for the study of whole plant development and biochemistry. In actuality, the ensuing thirty-five years has seen only modest work on the genetic study of duckweed development and molecular biology. Only a small number of duckweed mutants have been identified and characterized(Slovin and Cohen, 1988). A concerted effort to establish and extensive mutant collection required to undertake mutant analysis of development has never materialized. Alternatively the critical cell and molecular technologies needed for molecular genetics analyses of developmental processes, e. g. cell and tissue culture, gene transfer, cloning and characterization, have seen only limited development (Okubara and Tobin, 1991; Chaloukoval and Smart, 1994).

To further the development of duckeed as a whole-plant, model system, we have focused our initial efforts on development of gene transfer and ancillary cell and tissue culture methods. Although a large number of whole plant proliferation studies have been done across many species and strains within species(Landolt and Kandeler, 1987), duckeed tissue and cell culture research is limited. Chang and co-workers reported callus formation from fronds of *Lemna perpusilla*(Chang and Hsing, 1978), and *Lemna gibba*(Chang and Chiu, 1976, 1978).

Slovin and Cohen(1985) reported callus proliferation from meristematic tissue in *L. gibba* G3. More recently, we have undertaken an extensive study of medium requirements for callus formation in *L. gibba* G3(Moon and Stomp, 1997). The results presented here are an extension of our callus induction, proliferation and frond regeneration work, with emphasis on optimization of medium parameters for frond proliferation.

MATERIALS AND METHODS

Duckweed plants used in these experiments were produced from a *Lemna gibba* G3 culture provided by Dr. J. P. Slovin, USDA, Beltsville, MD or were produced from *Lemna gibba* strains in the *Lemnaceae* Germplasm Collection transferred to us from Dr. Elias Landolt, ETH, Zurich. Frond stock cultures were maintained on liquid Murashige and Skoog medium (Murashige and Skoog, 1962), with 2% sucrose in the absence of phytohormones. Fully expanded fronds were harvested for use in experimental treatments two week after stock cultures were transferred to new medium. All media components were combined and autoclaved at 121℃ for 20minutes, and cooled media were poured into 100mm disposable plastic petri dishes. The exception was gibberellic acid which was filtered sterilized and added to cooled medium prior to pouring. Incubation of stock cultures and experimental treatments was at 23℃, under a 16hr light photoperiod of approximately 40μmol $m^{-2}s^{-1}$ illumination provided by Gro-Lux fluorescent lights.

Data for all experiments consisted of fresh weights of the duckweed plants proliferated from single fronds plated ventral side down on treatment media. Data was taken four weeks after plating on treatment media. Completely randomized disigns were used for all experiments with four duckweed fronds plated on each petri dish of treatment, with each dish serving as a replication(four observations per replication). The numbers of replications used per experment varied and are noted in the experimental descriptions below. Data were analyzed using SAS variance procedures(SAS Institute, Cary, NC) to determine treatment differences and means were separated using Duncan's multiple range test. Tests of significance were conducted at the α=0.05 level.

Effect of basal media and benzyladenine

The purpose of this full factorial experiment was to test the ability of four basal media, each with three levels of BA to promote frond proliferation. The four basal media were: SH(Schenk and Hidebrandt, 1972), NN(Nitsch and Nitsch, 1969), MS(Murashige and Skoog, 1962), and B5(Gamborg et al., 1968). The three benzyladenine(BA) concentrations used were 0, 1, and 10μM. All 12media/BA combinations contained 3% sucrose and 1.5g/L Gelrite plus 4g/L Difco Bacto agar. Five replications of this experiment were done, resulting in 20 observations per treatment.

Effect of cytokinins and gibberellic acid

The purpose of this experiment was to test the ability of cytokinin and gibberellic acid(GA_3) to promote frond proliferation. The five cytokinins: kinetin(KN), BA, thidiazuron(TDZ), N6-[2-isopentenyl] adenine(2-iP), and zeatin(ZN), and GA_3 were tested separately, each at 1μM concentration. The basal medium of all 6 treatments was SH with 3% sucrose and 1.5g/L Gelrite plus 4g/L Difco Bacto agar. Five replications of this experiment were done, resulting in 20 observations per treatment.

Effect of sucrose

The purpose of this experiment was to test the ability of different sucrose levels to promote frond proliferation. Four sucrose concentrations: 0, 1, 3, and 8%(w/v), were tested separately in SH basal medium with 1 μM BA and solidified with 1.5g/L Gelrite plus 4g/L Difco Bacto agar. Five replications of this experiment were done, resulting in 20 observations per treatment. A second experiment was conducted to measure the water potential of duckweed stock plants. The standard dye method of Knipling(1967) was used. Plant samples of 0.5 grams each were immersed in 5.0ml of a graded series of sucrose concentrations ranging from 0.2 to 0.45 molal. The samples were incubated for eight hours at room temperature to reach equilibration with the sucrose solution before determining the relative

density of the sucrose incubation solutions to the original standard sucrose solutions. Approximate water potentials for duckweeds and medium containing different sucrose concentrations were calculated following Salisbury and Ross(1985). Fronds, grown on basal medium at two different sucrose concentrations, 0.2 and 4%, were also stained for starch accumulation(Johansen, 1940).

Effect of gelling agent

The purpose of this experiment was to test ability of two gelling agents, Gelrite and Difco Bacto agar, to promote frond proliferation. The two gelling agents were tested individually 2.5g/L Gelrite or 10g/L agar, and in combination of 1.5g/L Gelrite plus 4g/L agar or lg/L Gelrite.

Extrapolation of *L. gibba* G3 medium preference to 24 additional *L. gibba* strains

The purpose of this expreiment was to determine the variability of *L. gibba* strains in their response to proliferation medium optimized for *L. gibba* G3. The basal medium consisted of SH with 1μM BA, 3% sucrose and 1% Difco Bacto agar. Twelve fronds(four fronds per plate and 3 replications or plates per strain) from twenty-five *L. gibba* strains, *L. gibba* G3 and 24 other strains, were plated onto the medium and incubated for 4 weeks under standard temperature and light conditions. Means are for 12 observations.

RESULTS AND DISCUSSION

Effect of basal media and benzyladenine

Frond proliferation was significantly affected by basal medium composition and by BA concentration(Table 1). Averaged across all BA concentrations, SH and B5 media were superior and showed statistically equivalent frond proliferation. The poorest basal medium was clearly MS. Averaged across all media, the effcet of BA concentration was significant with 10μM BA darkened. The reduction in root developmint may have more complex regulation. White(1936) showed that increasing medium nitrated concentration from 5-100g/L(as calcium and magnesium nitrates), concentrations of nitrogen orders of magnitude lower than those we used, decreased root development to a small but significant extent in *Lemna*.

Effect of cytokinins and gibberellic acid

Frond proliferation was significantly promoted by the presence of BA in the culture medium(Table 2). No significant difference in frond proliferation was detected among the

Table 2. Effect of five cytokinins and gibberellic acid on proliferation of *Lemna gibba* G3 fronds cultured on solid SH basal medium with 3% sucrose after four weeks in culture

Phytohormone(1μM)	Fresh weight of fronds(g)*
Kinetin	0.48 b
Benzyladenine	0.68 a
Thidiazuron	0.44 b
N6 -[2-isopenteny1] adenine	0.44 b
Zeatin	0.46 b
Gibberellic acid	0.33 c

*Means followed by the same letter are not different at the α=0.05 level according to Duncan' s multiple range test

Table 1. Effect of solid basal media and basal medium with two different concentrations of benzyladenine on proliferation of *Lemna gibba* G3 fronds after four weeks in culture

Basal medium[1]	Benzyladenine Concentrations (μM) 0	1.0	10.0	All*
SH	0.42	0.79	0.79	0.66a
MS	0.13	0.22	0.38	0.25c
NN	0.41	0.46	0.49	0.45b
B5	0.40	0.73	0.76	0.64a
A11*	0.34c	0.55b	0.60a	

[1]Basal media: SH=Schenk&Hildebrandt; MS=Murashige&Skoog; NN=Nitsch&Nitsch; B5=Gamborg' s B5
*Means within a column or row followed by the same letter are not different at the α=0.05 level according to Duncan' s multiple range test.

other four cytokinins, and with these four cytokinins, frond proliferation was statistically equivalent to that seen with SH basal medium without cytokinin. This result stand in contrast to those of Oota(1965) who found kinetin active in frond proliferation in that fronds grown on 10 μM kinetin proliferated to a greater extent than those culture in the absence of the cytokinin. In the presence of gibberellic acid frond proliferation was significantly inhibited relative to all cytokinin treatments and was somewhat less than that observed with SH basal medium in the absence of added phytohormones.

Oota(1965) found that growth on gibberellin at a concentration comparable to that used here was approximately equivalent to that of fronds grown in the absence of any exogenous phytohormones.

Visual observations of frond morphology, root development and frond senescence acrose all treatments indicated that these three variables were affected by cytokinin type and the presence of GA_3. Frond curling and irregularly shaped fronds were frequently observed in cultures containing either BA or TDZ. Fronds cultured on the other cytokinins did not show obvious morphological differences, with the exception of slight differences in frond size. Media containing 2-iP or KN produced fronds of somewhat larger size whereas fronds cultured on media containing GA_3 or ZN were slightly smaller by comparison. Root development was suppressed by the presence of BA or TDZ in the culturing media, whereas KN enhanced root growth greatly. Frond senescence was greatest on media containing BA and TDZ whereas senescence was absent in frond cultured on GA_3 containing medium.

Table 3. Effect of sucrose concentration on frond proliferation of *Lamna gibba* G3 cultured on solid SH basal medium containing 1 μM BA and 3% sucrose after four weeks in culture

Sucrose(%)	Fresh weight of fronds (g)*
0.0	0.01 d
0.1	0.48 b
3.0	0.75 a
8.0	0.33 c

*Means followed by the same letter are not different at the α=0.05 level according to Duncan' s multiple range test

Effect of sucrose

Frond proliferation was significantly affected by sucrose concentration (Table 3). Without sucrose, frond proliferation was poor, fronds were pale green or yellow-green in color and frond senescence occurred earlier.

Sucrose concentrations of 1% or 3% greatly enhanced proliferation with the highest proliferation and root development obtained on 3% sucrose. The higher sucrose concentration of 8% produced significantly fewer fronds over the four week subculture time and these fronds were smaller, curled and dark green with short, thick roots. The smaller frond size of duckweed grown on 8% sucrose was similar to observations made in previous experiments when *L. gibba* G3 fronds were grown on different sucrose concentrations up to 4%, the treatment in which frond size was reduced (data not shown). To test the hypothesis that excess osmotic potential at 4%(and presumably 8%) sucrose resulted in inhibition of cell expansion due to loss of frond cell turgor, osmotic potential of duckweed stock culture fronds grown on Hoaglands basal medium with 1% sucrose was determined. The osmotic potential of the duckweed plants was approximately -6.4 to -7.0 bars; the osmotic potential of the 4% sucrose medium was calculated to be- 8.0 bars. This difference in osmotic potential could limit tugor pressure needed to drive cell expansion resulting decreased frond size. In addition to smaller frond size at 4% sucrose concentration, iodine staining revealed enormous accumulation of starch.

Effect of gelling agent

Frond proliferation varied significantly with gelling agent(Table 4). Gelrite alone at 2.5g/L or in combination with agar (1.5g/L Gelrite and 4g/L agar) were clearly superior in suporting frond proliferation relative to the other combination of Gelrite/agar or agar alone. As the Gelrite concentration increased frond vitrification also increased and was frequently observed at 0.25% Gelrite. Root development was promoted by the presence of Gelrite, either alone or in combination with agar. Frond senescence was more prevalent in media containing Gelrite.

Table 4. Effect of Gelrite and Difco Bacto agar and two combinations of gelling agents on frond proliferation of *L. gibba* G3 cultured on solid SH basal medium containing 1μM BA and 3% sucrose after four weeks in culture

Gelling Agents	Fresh Weight of Fronds(g)*
10.0g/L Bacto agar	0.48 c
2.5g/L Gelrite	0.87 a
1.5g/L Gelrite+4.0g/L Bacto agar	0.85 a
6.0g/L Bacto agar+1.0g/L Gelrite	0.73 b

*Means followed by the same letter are not different at the α=0.05 level according to Duncan' s multiple range test

This result is consistent with those of Oota(1965) who showed that frond proliferation was promoted by inclusion of cytokinin in the medium. The promoitive effect of BA was not observed with NN medium. In the absence of BA, frond proliferation occurs readily. The statistical equivalence of three media: B5, NN and SH in the absence of BA can be explained by the lack of BA promotion of frond proliferation observed with NN basal medium.

Frond morphology, color, senescence and root development were all affected by basal medium composition and BA concentration. Frond curling and frond fusion varied directly with BA concentration and was greatest on media with 10 μM BA. Root development was inhibited by BA, with 10 μM supporting virtually no root growth. More curled fronds and smaller frond size was observed on MS medium relative to the other basal media.

Media total nitrogen contents most probably account for the major differences observed in frond color, senescence, and root development. The nitrogen content of the basal media as ratios of NH_4^+/NO_3^-(mM) are: SH 0/24; MS 20/40; B5 2/24; and NN 9/18, with MS containing apporximately 2.5-fold more nitrogen than the other media tested. Frond color acrose media varied from yellow-green to dark green, with MS supporting the darkest green fronds, those on B5 were pale green or yellow-green, while those on NN or SH were pale green. On solid media, proliferation resulted in stacks of fronds, some with little contact with the medium. Senescence, detected by complete yellowing of fronds, was observed starting about three weeks after transfer. No frond senescence was observed on MS medium. The dark green color and lack of senescence on MS medium most probably resulted from the high level of nitrogen in this medium. This result in similar to that of white(1936) who showed that as medium nitorgen increased frond color plus 6g/L

Table 5. Frond proliferation of 25 *Lemna gibba* strains on solid SH basal medium with 1 μM benzyladenine and 3% sucrose after four weeks in culture

Strain[1] number	Country of Origin	Fresh weight of Fronds(g)*	Strain[1] number	Country of Origin	Fresh weight of Fronds(g)*
8703	Japan	0.59 a	7641	Israel	0.31 cdef
8405	France	0.59 a	7107	Germany	0.28 defg
8655	Argentina	0.53 a	6861	Italy	0.28 defg
7021	Spain	0.50 ab	8418	Spain	0.23 defg
G3	-	0.50 ab	7135	Netherlands	0.23 defg
7937	Australia	0.40 bc	7262	Chile	0.22 fgh
8273	Italy	0.38 cd	7810	England	0.20 ghi
8682	Saudi Arabia	0.38 cd	7784	Ethiopia	0.19 ghi
7533	Ukraine	0.38 cd	7932	Ireland	0.17 ghi
8384	Morocco	0.35 cd	8637	Tanzania	0.17 ghi
7198	Portugal	0.33 cde	7257	Kenya	0.17 ghi
8291	Iran	0.33 cde	8678	India	0.17 ghi
7641	Israel	0.31 cdef	8760	Czechoslovakia	0.10 i
7107	Germany	0.28 defg			

[1]Strain number is designation in Lamnaceae Germplasm Collection

*Means followed by the same letter are not different at the α=0.05 level according to Duncan' s multiple range test

agar. The basal medium of the four treatments was SH with 3% sucrose and no phytohormones. Five replications of this experiment were done, resuliting in 20 observations per treatment.

Extrapolation of *L. gibba* G3 medium preference to 24 additional *L. gibba* strains

Frond proliferation varied six-fold across the 25 strains of *L. gibba* showing considerable within species variation(Table 5). These 25 strains were originally collected at various geographical locations around the world and vary somewhat in frond size. No correlation was seen between proliferation ranking and geographical origin or frond size.

Our results represent the first reported screening of plant tissue culture media for supporting *Lemna gibba* proliferation. By identifying the most active cytokinin, benzyladenine, and optimizing its concentration as well as that of sucrose and gelling agents, we have developed a culturing mileau which rapidly proliferates duckweed. The systematic screening of *L. gibba* strains has revealed large differences in within species growth rates which could be exploited in transformation experiments. Bergmann and Stomp(1994) have suggested that fast growth rates could increase the frequency of Agrobacterium-mediated gene transfer. This idea remains to be tested in *Lemna*. The combination of our callus regeneration method and optimization of frond proliferation is a complete system upon which to begin gene transfer work.

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