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CELL BIOLOGY AND MORPHOGENESIS

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Callus induction and regeneration in *Spirodela* and *Lemna*

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Abstract The development of tissue culture systems in duckweeds has, to date, been limited to species of the genus *Lemna*. We report here the establishment of an efficient tissue culture cycle (callus induction, callus growth and plant regeneration) for *Spirodela oligorrhiza* Hegelm SP, *Spirodela punctata* 8717 and *Lemna gibba* var. *Hurfeish*. Significant differences were found among the three duckweed species pertaining to carbohydrate and phytohormone requirements for callus induction, callus growth and frond regeneration. In vitro incubation with poorly assimilated carbohydrates such as galactose (*S. oligorrhiza* SP and *L. gibba* var. *Hurfeish*) and sorbitol (*S. punctata* 8717) as sole carbon source yielded high levels of callus induction on phytohormone-supplemented medium. Sorbitol is required for optimal callus growth of *S. oligorrhiza* SP and *S. punctata* 8717, while sucrose is required for callus growth of *L. gibba* var. *Hurfeish*. Sucrose either alone (*S. oligorrhiza* SP, *L. gibba* var. *Hurfeish*) or in addition to sorbitol (*S. punctata* 8717) is required for frond regeneration.

Keywords Duckweed · Galactose · Sorbitol · Tissue culture

Abbreviations *ABA:* (±)-Abscisic acid · *BA:* N^6-Benzyladenine · *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *Dicamba:* 3,6-Dichloro-2-methoxybenzoic acid · *2iP:* N^6-(2-Isopentenyl)adenine · *NAA:* α-Naphthaleneacetic acid · *PCA: p*-Chlorophenoxy acetic acid · *Picloram:* 4-Amino-3,5,6-trichloropicolinic acid · *TDZ:* Thidiazuron

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Introduction

The *Lemnaceae* (duckweeds) are a widespread family of free-floating monocotyledonous plants growing in freshwater habitats (Landolt 1986a). *Lemnaceae* reproduce primarily by vegetative means with high rates of biomass accumulation (Landolt and Kandeler 1987; Tillberg et al. 1979). A protein content as high as 45% of the dry weight (Chang et al. 1977; Porat et al. 1979), an excellent tolerance for a variety of toxic substances (Landolt and Kandeler 1987) and a rapid uptake of nutrients make *Lemnaceae* an ideal choice for the development of efficient, plant-based, gene-expression systems.

The leaf-like frond of the *Lemnaceae* is a composite of tissues with minor differentiation. Tissue below the epidermis consists of a photosynthesizing parenchyma, partly interrupted by large intercellular air spaces, while adventitious rhizoid(s) are located on the abaxial face. Daughter fronds originate from meristematic tissue deep inside two lateral pouches of the mother frond. Each daughter frond emerging from a pouch already contains two or more new generations of daughter fronds.

Attempts to achieve stable transformation of *Lemnaceae* species have been only partially successful, primarily because of the limited availability of efficient callus induction and regeneration protocols. Sporadic information exists, restricted to the genus *Lemna*, describing callus induction and frond regeneration in *L. gibba* G3 (Chang and Chiu 1976, 1978; Slovin and Cohen 1985), *L. minor* (Frick 1991) and *L. perpusilla* 6746 (Chang and Hsing 1978). There is also a comparative account of the effects of sucrose, phytohormones and light conditions on callus induction, growth and frond regeneration in *L. gibba* G3 (Moon and Stomp 1997) leading to the successful *Agrobacterium*-mediated genetic transformation of *L. gibba* G3 and *L. minor* expressing β-glucuronidase and *nptII* (Yamamoto et al. 2001). However, no information is available on tissue culture of duckweeds of the genus *Spirodela*. The genus *Spirodela* appears to be the most primitive among the *Lemnaceae* and has the smallest genome size in the family (Landolt 1986b). Plants of this genus are widely distributed worldwide and have been extensively used in molecular, biochemical and photosynthesis research (Avni et al. 1991; Ghirardi et al. 1993; Jansen et al. 1996).

We describe herein, efficient and reproducible protocols for callus induction and plant regeneration in three duckweed species: *Spirodela oligorrhiza* SP, *Spirodela punctata* 8717 and *Lemna gibba* var. *Hurfeish*, the latter a local isolate having markedly short roots. The different metabolic competence of intact plants versus callus to assimilate exogenous carbohydrate supplements in support of heterotrophic growth in vitro is successfully exploited in defining an optimal regimen for callus induction and plant regeneration.

Materials and methods

Plant material and growth conditions

The strain of *Spirodela oligorrhiza* (Kurz) Hegelm SP used in this study was isolated by D. Rimon in 1964 (Rimon 1964) from a pond in the Sharon Plain, Israel. An axenic clone was produced, and this has been cultivated in our laboratory for 40 years. *Spirodela punctata* 8717 was isolated by E. Landolt and erroneously labeled as *Lemna disperma* (personal communication). The strain used in this investigation was obtained from him and has been maintained in our laboratory for 10 years. *Lemna gibba* var. (Galilee) *Hurfeish* was isolated in The Galilee, Israel, by D. Porat (Porat et al. 1979). This strain was obtained from him and has been maintained in our laboratory for 20 years. Stock plants of *S. oligorrhiza* SP, *S. punctata* 8717 and *L. gibba* var. *Hurfeish* were routinely maintained on autotrophic half-strength Hutner's medium (Posner 1967; SP medium) at 26°C under continuous white florescent light (30 μmol/m^2 per second) in a 1–3% CO_2-enriched atmosphere. Two-to three-week-old axenic cultures with nearly confluent growth on the surface were used as explant donor material for callus induction.

Frond proliferation and callus induction

Several basal salt media (Duchefa, The Netherlands)—Murashige and Skoog (MS) (Murashige and Skoog 1962), Gamborg's B5 (Gamborg et al. 1968), McCown Woody Plant (Lloyd and McCown 1980; WP medium) and Nitsch medium (Nitsch 1972)—were evaluated for their ability to support plant proliferation and callus induction. The specific concentrations (w/v) of carbohydrates (Sigma, St. Louis, Mo.) and phytohormones (Duchefa, The Netherlands) used are indicated for individual experiments. The pH of the media was adjusted to 5.8 prior to addition of 0.45% agargel (product no. A3301; Sigma). Media (including the carbohydrates) were autoclaved at 120°C, at 1.2 atm for 20 min. Following autoclaving, media were supplemented with filter-sterilized (0.2-μm pore size; Schleicher and Schuell) phytohormones, as required, and dispensed in 90×15-mm polystyrene petri dishes (approx. 40 ml per dish). Mature, 2- to 5-day-old fully expanded mother fronds (*S. oligorrhiza*, 5–8 mm; *S. punctata*, 2–4 mm; *L. gibba*, 7–10 mm), with rhizoids removed or shortened, were manually separated from the young daughter fronds and used as explant source for the induction of callus. The frond explants (about 30–35 per dish) were placed with their adaxial surfaces in contact with the medium. All in vitro cultures were maintained at 24±2°C under continuous white florescent light (30 μmol/m^2 per second). Frond explants turning either white or yellow after 2 weeks of culture were scored as senescent and discarded, whereas those producing daughter fronds were carried to the next stage. These latter explants were periodically observed under a light microscope for positive callusing response.

Plant regeneration

Calli (4–8 mm in diameter) were transferred to regeneration media and scored visually for plant regeneration. A few weeks later, fronds were initiated. After rhizoid initiation, regenerated plants were removed and transferred to SP liquid medium containing 1% sucrose. The regenerating plants were propagated in the growth room under continuous fluorescence light (30 μmol/m^2 per second), at 26°C, in a 1–3% CO_2-enriched atmosphere.

Statistical analyses

All experiments were repeated at least three times with 150–200 fronds cultured on each medium. For the regeneration studies, a minimum of 90–100 calli for *S. oligorrhiza* SP and *S. punctata* 8717, and 15–20 calli for *L. gibba* var. *Hurfeish*, were cultured for each treatment per experiment. Error bars represent standard deviations of three or more experiments and are shown in all graphs. In all of the photographed figures, the scale bar represents 1.0 mm.

Results

Spirodela oligorrhiza SP

The explant mother fronds of *S. oligorrhiza* SP were precultured for 2 weeks on WP medium or B5 medium, each containing 1.5% galactose, 50 mg/l dicamba (an auxin which induces callus in several monocotyledons; Przetakiewicz and Orczyk, 2003) and 2 mg/l BA. Callus was successfully induced in 3–4 weeks following transfer to WP medium containing 2% sorbitol and 1% maltose, 5 mg/l PCA and 2 mg/l 2iP. No callus induction was obtained on B5 medium. When this protocol, was followed, about 91–95% of the explants initially cultured on WP medium gave rise to callus. One to five separate callus initiations were obtained per single frond explant (Table 1). These calli initiations grew slowly on the same medium with periodic transfers every 3–4 weeks

Table 1 Induction of callus from frond explants of *Spirodela oligorrhiza* SP

	Number of fronds initially cultured[a]	Number of fronds transferred[b]	Number of fronds forming callus
Experiment 1	771	725	703 (91%)
Experiment 2	315	300	297 (94%)
Experiment 3	525	500	498 (95%)

[a] Callus was induced from fronds cultured for 2 weeks on McCown Woody Plant (WP) medium, 1.5% galactose, 50 mg/l dicamba and 2 mg/l BA
[b] WP medium, 2% sorbitol, 1% maltose, 5 mg/l PCA and 2 mg/l 2iP

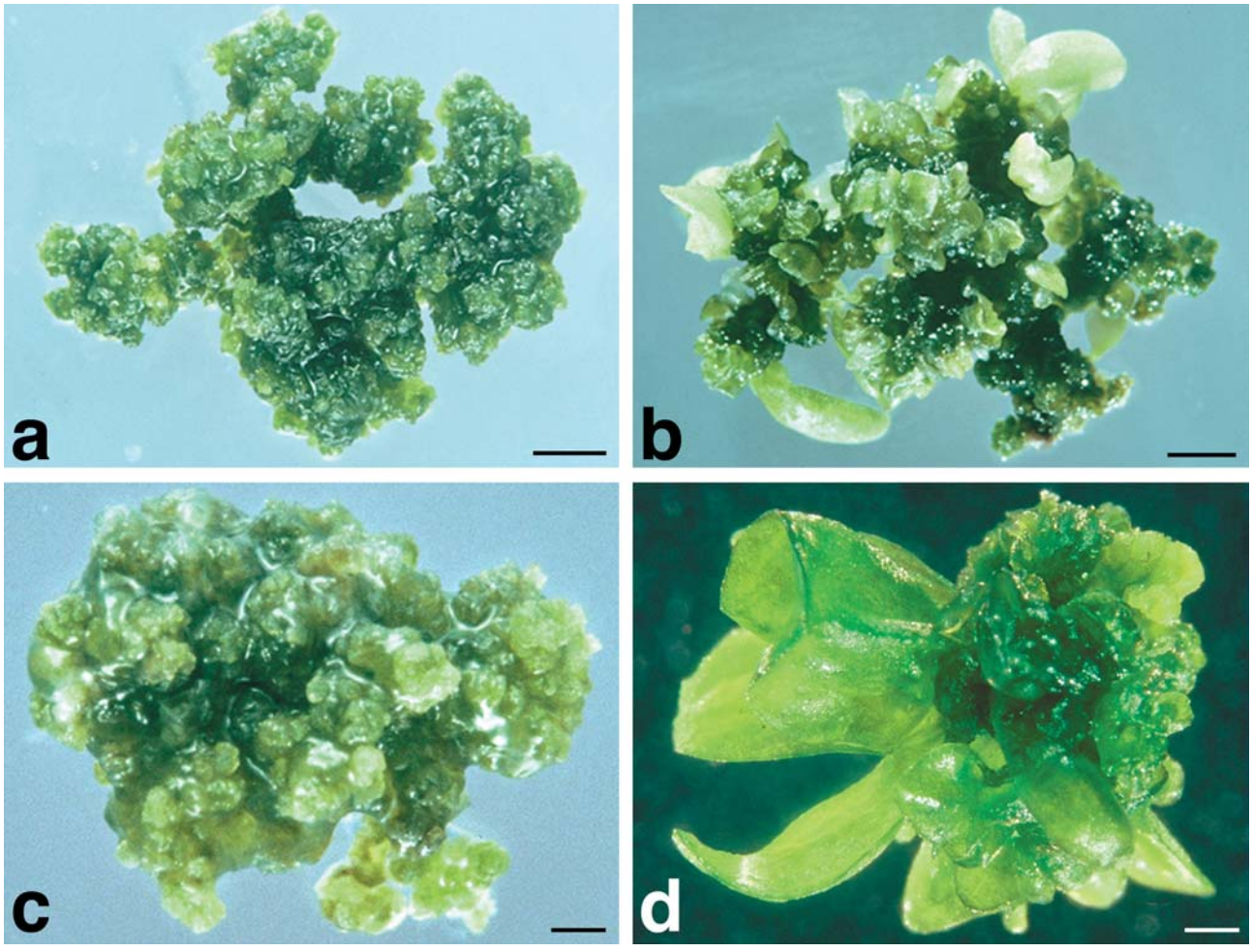

Fig. 1 **a, c** Callus after three successive transfers in species-specific growth medium, **b**, **d** plant regeneration following 4–5 weeks on the species-specific regeneration medium. **a**, **b** *Spirodela oligorrhiza* SP, **c**, **d** *S. punctata* 8717

(Fig. 1a). The presence of conventional sugars such as sucrose, glucose and fructose in the callus growth medium resulted in 100% mortality of the callus cultures.

Regeneration of *S. oligorrhiza* SP plants was obtained on WP medium supplemented with 0.5% sucrose and 1 mg/l TDZ. Regenerating fronds (Fig. 1b) became visibly distinct within 4–5 weeks, and plants could be separated following an additional 3–4 weeks. In over 30 experiments, averaging 150 calli per experiment, we consistently found that 90–100% of the calli regenerated into plants using this medium.

Spirodela punctata 8717

The choice of carbohydrate supplements also had significant effect on *S. punctata* 8717 callus induction and its subsequent growth. As shown in Fig. 2, 92% (± 5%) of the frond explants that were cultured on half-strength MS basal salt medium containing B5 vitamins, 1% sorbitol, 3.5 mg/l 2,4-D, 15 mg/l dicamba and 2 mg/l 2iP formed callus, with one to five separate calli developing per explant within 6–7 weeks. When sucrose (1%) was substituted for sorbitol in this same medium, 56% (±17%) of the explant fronds formed small pieces of unorganized

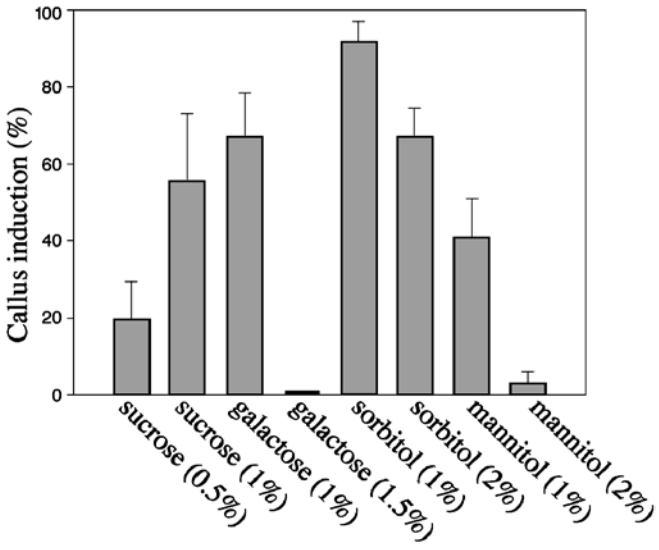

Fig. 2 Effects of carbohydrates on callus induction in *S. punctata* 8717. Frond explants were cultured on half-strength MS basal salt medium containing B5 vitamins, 3.5 mg/l 2,4-D, 15 mg/l dicamba, 2 mg/l 2iP and various concentrations of different carbohydrates

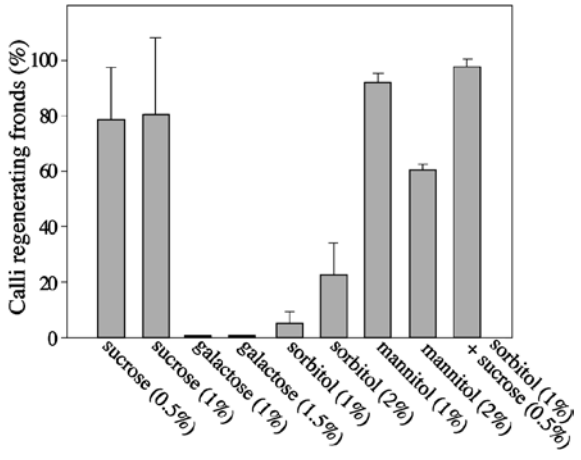

Fig. 3 Regeneration of *S. punctata* 8717 callus in the presence of different sugars

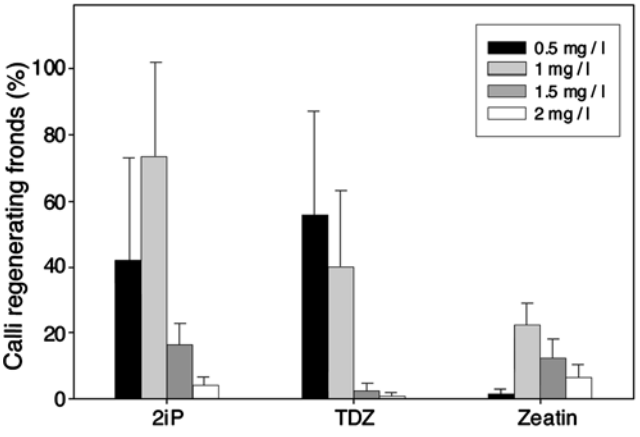

Fig. 4 Effect of hormone additives (2iP, TDZ and zeatin) on regeneration of *S. punctata* 8717 plants from calli

callus-like tissue often mixed with partially differentiated frond-like structures. This tissue was difficult to grow in long-term culture, becoming necrotic upon subsequent transfers. When 1% galactose was substituted for sorbitol, induction of callus formation occurred in 68% (±11%) of the explants, while 1.5% galactose caused complete frond death. Finally, mannitol, as a sorbitol replacement, induced *S. punctata* callus less effectively than the other carbohydrates tested.

WP medium, supplemented with 2% sorbitol, 1 mg/l 2,4-D, 5 mg/l NAA and 0.5 mg/l TDZ, supported optimal growth and long-term maintenance of *S. punctata* 8717 callus cultures. The callus morphology was green, compact and consisted of small, connected amorphous masses occasionally mixed with partially differentiated frond-like structures. A 3-week growth period, repeated two to three times with transfer to fresh medium each time, effectively killed the partially differentiated tissue, resulting in true friable callus (Fig. 1c). Sucrose, galactose and mannitol did not support the growth of *S. punctata* 8717 callus.

Plant regeneration from callus was studied in the presence of different sugars. Sucrose alone (0.5% or 1.0%) supported a regeneration rate of about 80% but with a high standard deviation (19–28%). Galactose (1.0% or 1.5%) completely inhibited regeneration, while sorbitol (1.0% or 2.0%) was poorly supportive. When 1% mannitol served as the carbon source, 93% (±3%) of the calli regenerated; however, 2% mannitol was less efficient (61±2%). The highest and most consistent values of regeneration (98±3%) were obtained when the medium contained 0.5% sucrose plus 1% sorbitol (Fig. 3).

Three hormones (2iP, TDZ, zeatin) were tested for their ability to induce regeneration of *S. punctata* 8717 (Fig. 4). The highest levels of regeneration (74±28%) were obtained with 1.0 mg/l 2iP. *S. punctata* 8717 calli regenerated within 5–8 weeks when cultured on WP medium containing 1.0% sorbitol, 0.5% sucrose and 1 mg/l 2iP, with 20–50 regenerants produced per callus. An example of *S. punctata* 8717 regenerating from callus tissue is shown in Fig. 1d.

Lemna gibba var. *Hurfeish*

Preliminary experiments with *L. gibba* var. *Hurfeish* suggested the utility of B5 medium fortified with 1% sucrose, 50 mg/l dicamba and 2 mg/l BA for callus induction. The procedure involved transferring explant mother fronds every 2 weeks for five or more times on the same medium. Calli were induced from meristematic regions on the ventral surface of the explant frond. They grew slowly and were visible only after 6–8 weeks of culture. However, the efficiency of callus induction by this procedure was never more than 10–12%, irrespective of auxin type(s) and concentration(s).

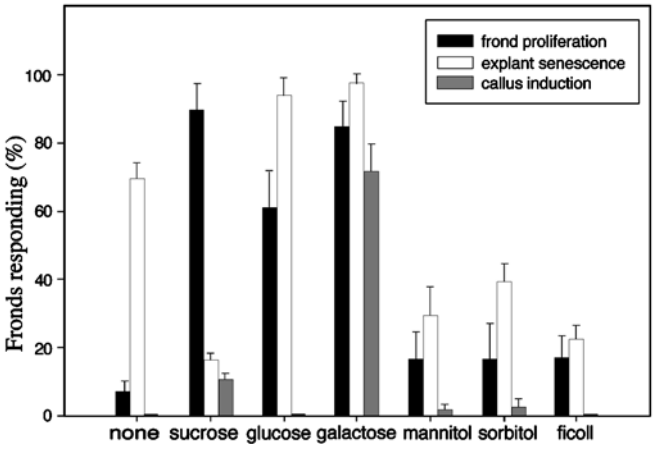

Fig. 5 Effects of carbohydrates on callus induction in *Lemna gibba* var. *Hurfeish*. Frond explants were precultured for 4 weeks on B5 medium containing 50 mg/l dicamba, 2 mg/l BA and a carbohydrate supplement (1%, w/v) as indicated, followed by transfer to B5 medium containing 1% sucrose, 50 mg/l dicamba and 2 mg/l BA for callus induction. Frond proliferation and senescence were scored after 2 weeks on the first medium, and callus induction was scored after 3 weeks following transfer to the second medium

In order to improve the efficiency of callus induction, the effects of carbon sources (1% each) on the proliferation and senescence of explant fronds were examined (Fig. 5). Carbohydrate-free media showed very low levels of plant proliferation and resulted in the loss of more than 65–75% of the plants when cultured on B5 medium containing 50 mg/l dicamba and 2 mg/l BA. The addition of sucrose led to a high proliferation level (more than 90%) and low senescence level but did not improve the rate of callus induction. Glucose supported frond proliferation for only three transfers; moreover, more than 90% of the fronds senesced and died. In any case, glucose did not support callus induction. Galactose (1–3%) caused fast bleaching and senescence of the mother frond and rhizoid within 10–12 days. However, the daughter fronds remained green to pale brown even following 2–3 weeks of further culture (although they too eventually died if maintained on galactose.) This 4- to 5-week-old material can be used for callus production if transferred to B5 medium containing 1% sucrose, 50 mg/l dicamba and 2 mg/l BA (Fig. 6a). In this case, a dramatic increase (to

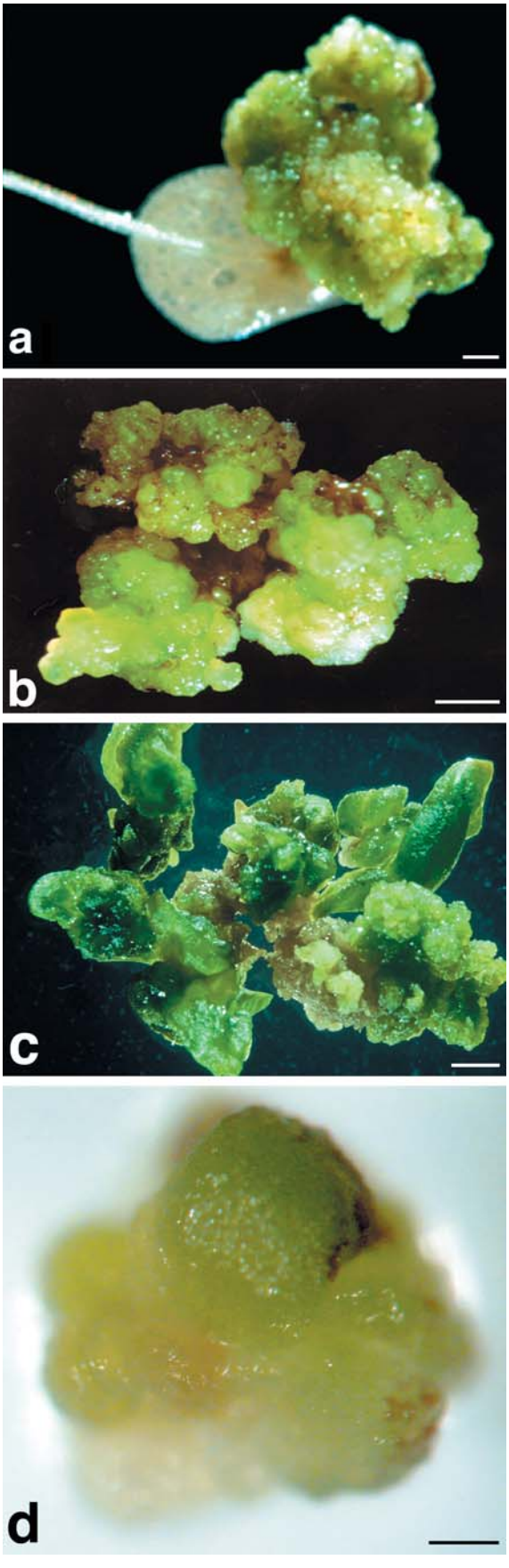

Fig. 6a–d *Lemna gibba* var. *Hurfeish*. **a** Frond explants were precultured for 2 weeks on B5 medium containing 1.5% galactose, 50 mg/l dicamba and 2 mg/l BA to induce callus formation. Note the senescent mother frond. Following induction, the bleached explant was transferred to B5 medium containing 1% sucrose, 50 mg/l dicamba and 2 mg/l BA. The picture shows green calli arising from the ventrally located meristematic regions at the base of the frond explant after 4 weeks in sucrose-containing medium. **b** Callus that was separated from the bleached frond explant and grown for an additional 4 weeks on B5 medium containing 1% sucrose, 10 mg/l PCA, 2 mg/l picloram and 2 mg/l 2iP. **c** Plants regenerating from callus incubated for 2 weeks on B5 medium containing 1% sucrose and 1 mg/l TDZ. **d** Callus 3–4 weeks after induction from an isolated flower on B5 medium containing 1% sucrose, 40 mg/l dicamba and 2 mg/l BA

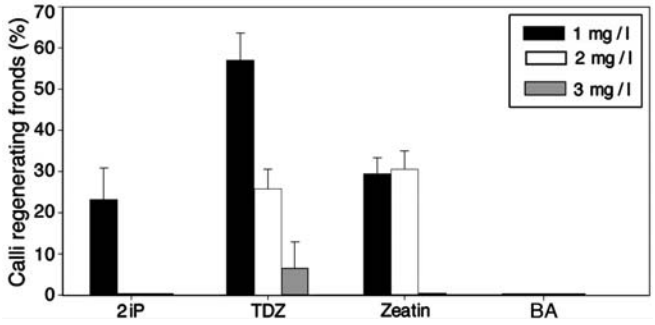

Fig. 7 Effect of hormone additives (2iP, TDZ, zeatin and BA) on frond regeneration in *Lemna gibba* var. *Hurfeish*. The highest level of regeneration was obtained with 1.0 mg/l of TDZ

about 75%) of explant fronds forming calli results (Fig. 5). Finally, ficoll, sorbitol and mannitol promoted neither frond proliferation nor callus induction.

Lemna gibba var. *Hurfeish* calli proved difficult to proliferate on a medium containing high concentrations of dicamba. Following various trials, we eventually found that B5 medium with 1% sucrose, 10 mg/l PCA, 2 mg/l picloram and 2 mg/l 2iP was optimal for callus growth. The callus grew as a watery mixture of pale-yellow and light- to dark-green sectors (Fig. 6b) and doubled its mass in about 10–12 days.

Frond regeneration started from the callus surface on cytokinin-containing media after 10–12 days of culture. Plant regeneration (Fig. 6c) was accomplished 2 weeks later. The regenerating frequency on B5 basal medium containing 1% sucrose and 1 mg/l TDZ was about 55%; on 1 mg/l 2iP, about 25%; on 1 mg/l and 2 mg/l zeatin, about 30%; and on BA (1–3 mg/l), zero (all calli died within 10–12 days of culture) (Fig. 7).

Flowers consist of different tissues that develop from a meristematic zone. We thus attempted to induce callus from flower tissue. Flowering can be induced with a high efficiency (greater than 90%) in older, crowded, *Lemna gibba* var. *Hurfeish* plants when 1 mg/l ABA is added to the normal growth medium (however, flowering was never witnessed in the *Spirodela* species used in this study.) Upon induction, one to two flowers emerged from a pocket of the *L. gibba* var. *Hurfeish* mother frond. Flowers were isolated and incubated on callus induction medium (B5 medium with 1% sucrose and various concentrations of BA and dicamba; not shown). Fifty flowers were treated at each hormonal combination. The only combination that produced more than an occasional callus was 40 mg/l dicamba and 2 mg/l BA, and on this medium, 6–8% of the flowers formed calli after 3 weeks

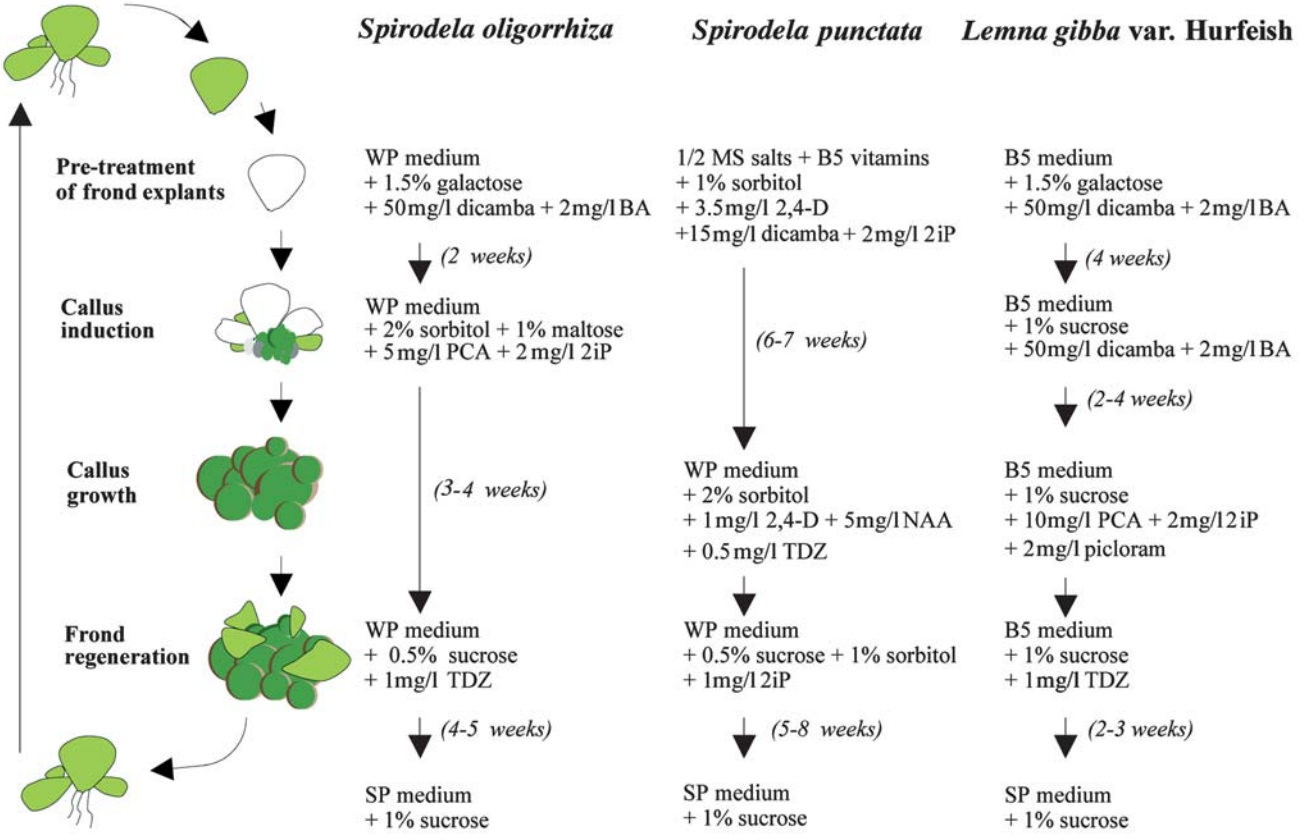

Fig. 8 Tissue culture cycle in *Spirodela oligorrhiza* SP, *S. punctata* 8717 and *Lemna gibba* var. *Hurfeish*. Schematic representation and comparison of callus induction, growth and plant regeneration

(Fig. 6d). However, the rate of growth and efficiency of regeneration were low and further work with this flower-derived callus was discontinued.

Cell-culture cycle

Figure 8 summarizes the various media constituents and effect of carbohydrate supplements on callus induction, growth and regeneration in *S. oligorrhiza* SP, *S. punctata* 8717 and *L. gibba* var. *Hurfeish*. Plants regenerated from the callus cultures of all three species were, in the great majority of cases, true to type in terms of growth rate, frond size and shape, etc. These vegetatively propagated regenerants remained true to type through the duration of the study (100–200 biomass doublings, more than 20 months).

Discussion

Sexual reproduction in duckweed has been observed in nature and can be induced under laboratory conditions, however, these plants propagate primarily by rapid vegetative growth (Landolt 1986a). We sought a strategy to break the rapid vegetative growth cycle of explant fronds and direct meristematic potential in favor of callus induction. This was approached by using carbohydrates that poorly support the growth of the plants. In addition, the selection of only the mother frond as the explant material (plucked free of daughter fronds) further minimized plant propagation and allowed maximum exposure of the meristematic zones.

Sucrose, by virtue of being the most abundant sugar translocated in the phloem of many plant species (Strickland et al. 1987), is often assumed to be the best carbon source for in vitro cultures. However, abundant experimental evidence exists that callus growth and morphogenesis can be significantly influenced by other carbohydrate supplements as well (Swedlund and Locy 1993; Jeanin et al. 1995; Jain et al. 1997; Lemos and Baker 1998). In the duckweed, *Lemna minor* calli have been shown to utilize galactose and sorbitol but not to assimilate glucose and fructose. In contrast, Frick (1991) showed that *L. minor* plants efficiently assimilate glucose, fructose, mannitol and starch, but their growth is inhibited by galactose. Galactose also has been shown to arrest the growth of *L. gibba* plants (Decock et al. 1979). The differential carbohydrate utilization for growth by intact plants and explant calli in *L. minor* was traced to differences in enzymatic activities of sucrose synthase and alkaline and acidic invertase (Frick 1994).

We applied the above strategy concerning differential growth in vivo and in vitro to the callus *induction* stage. We found that the same concentration of galactose that inhibited plant propagation in *Spirodela oligorrhiza* SP and *Lemna gibba* var. *Hurfeish* promoted their callus induction, while the same concentration of sorbitol that inhibited propagation of *S. punctata* 8717 plants promoted the induction of callus in this species. We assume that an essential effect of the unassimilated sugar is the physiological destruction of the explant mother frond, which presumably acts as a strong sink for the added hormones. The *duration* of culture on galactose- or sorbitol-supplemented medium was also critical: excessive frond proliferation occurred in *S. oligorrhiza* SP if the incubation period was shorter than 2–3 weeks; for *L. gibba* var. *Hurfeish*, 4–5 weeks; and for *S. punctata* 8717, 6–7 weeks. However, longer incubation times than those indicated resulted in mother frond death for all three species.

In the case of *L. gibba* G3, a strong requirement for sucrose was found for callus induction, growth and regeneration (Moon and Stomp 1997), although, in a later study (Yamamoto et al. 2001), regeneration proceeded in the absence of any carbohydrate. We found that for *L. gibba* var. *Hurfeish*, culture on galactose-supplemented medium is required for efficient callus induction, while sucrose is required only for callus growth and regeneration. As for *S. oligorrhiza* SP and *S. punctata* 8717, sucrose was found to be inhibitory for callus induction and growth but required for regeneration. Furthermore, in *Spirodela*, species-specific differences with respect to the utilization of galactose were also discernible. From the above, it is apparent that among duckweeds, during the tissue culture cycle, requirements for carbohydrates are species-specific.

In conclusion, we describe efficient and reproducible protocols for callus induction and regeneration in *Spirodela oligorrhiza* SP, *S. punctata* 8717 and *Lemna gibba* var. *Hurfeish*. The frequency of callus induction and frond regeneration in phytohormone-supplemented media can be substantially improved through the use of poorly assimilated carbon sources.

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References

Avni A, Mehta RA, Mattoo AK, Greenberg BM, Chattoo BB, Heller D, Edelman M (1991) Nucleotide sequence of the *Spirodela oligorrhiza* chloroplast *psb*A gene coding for the D1 (32 kDa) photosystem II protein. Plant Mol Biol 17:919–921

Chang SM, Yang CC, Sung SC (1977) The cultivation and the nutritional value of *Lemnaceae*. Bull Inst Chem Acad Sin 24:19–30

Chang WC, Chiu PL (1976) Induction of callus from fronds of duckweed (*Lemna gibba* L.). Bot Bull Acad Sin 17:106–109

Chang WC, Chiu PL (1978) Regeneration of *Lemna gibba* G3 through callus cultures. Z Pflanzenphysiol 89:91–94

Chang WC, Hsing YI (1978) Callus formation and regeneration of frond-like structures in *Lemna perpusilla* 6746 on a defined medium. Plant Sci Lett 13:133–136

Decock PC, Cheshire MV, Mundie CM, Inkson RHE (1979) The effect of galactose on the growth of *Lemna*. New Phytol 82:679–685

Frick H (1991) Callogenesis and carbohydrate utilization in *Lemna minor*. J Plant Physiol 137:397–401

Frick H (1994) Heterotrophy in *Lemnaceae*. J Plant Physiol 144:189–193

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Ghirardi ML, Mahajan S, Sopory SK, Edelman M, Mattoo AK (1993) Photosystem II reaction center particles from *Spirodela* stroma lamellae. J Biol Chem 268:5357–5360

Jain RK, Davey MR, Cocking EC, Wu R (1997) Carbohydrate and osmotic requirements for high frequency plant regeneration from protoplast derived colonies of *indica* and *japonica* rice varieties. J Exp Bot 48:751–758

Jansen MAK, Babu TS, Heller D, Mattoo AK, Edelman M (1996) Ultraviolet-B effects on *Spirodela oligorrhiza*: induction of different protection mechanisms. Plant Sci 115:217–223

Jeannin G, Bronner R, Hahne G (1995) Somatic embryogenesis and organogenesis induced on the immature zygotic embryo of sunflower (*Helianthus annuus* L.) cultivated in vitro: role of sugar. Plant Cell Rep 15:200–204

Landolt E (1986a) The family of *Lemnaceae*—a monographic study, vol 1. Veroff Geobot Inst ETH, Stiftung Rubel, Zurich, pp 13–31

Landolt E (1986b) The family of *Lemnaceae*—a monographic study, vol 1. Veroff Geobot Inst ETH, Stiftung Rubel, Zurich, pp 417–435

Landolt E, Kandeler R (1987) The family of *Lemnaceae*—a monographic study, vol 2. Veroff Geobot Inst ETH, Stiftung Rubel, Zurich, pp 65–69

Lemos EEP, Baker DE (1998) Shoot regeneration in response to carbon source on internodal explants of *Annona muricata* L. Plant Growth Regul 25:105–112

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Int Plant Propagation Soc Proc 30:421–427

Moon HK, Stomp A-M (1997) Effects of medium components and light on callus induction, growth, and frond regeneration in *Lemna gibba* (duckweed). In Vitro 33:20–25

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nitsch JP (1972) Haploid plants from pollen. Z Pflanzenzuecht 67:3–18

Porat D, Hepher B, Koton A (1979) Duckweeds as an aquatic crop: evaluation of clones for aquaculture. Aquat Bot 7:273–278

Posner HB (1967) Aquatic vascular plants. In: Witt FA, Wessels NK (eds) Methods in developmental biology. Crowell, New York, pp 301–317

Przetakiewicz A, Orczyk W (2003) The effect of auxin on plant regeneration of wheat, barley and *Triticale*. Plant Cell Tissue Organ Cult 73:245–256

Rimon D (1964) Bud initiation in *Spirodela oligorrhiza*. Isr J Bot 13:24–39

Slovin JP, Cohen JD (1985) Generation of variant lines of *Lemna gibba* G3 via tissue culture, for use in selecting auxin metabolism mutants. Plant Physiol Suppl Am Soc Plant Physiol 77:11

Strickland GS, Nichol JW, McCall CM, Stuart DA (1987) Effect of carbohydrate source on alfalfa somatic embryogenesis. Plant Sci 48:113–121

Swedlund B, Locy RD (1993) Sorbitol as the primary carbon source for the growth of embryogenic callus of maize. Plant Physiol 103:1339–1346

Tillberg E, Holmvall M, Ericsson T (1979) Growth cycles in *Lemna gibba* cultures and their effects on growth rate and ultrastructure. Physiol Plant 46:5–12

Yamamoto YT, Rajbhandari N, Lin X, Bergmann BA, Nishimura Y, Stomp A-M (2001) Genetic transformation of duckweed *Lemna gibba* and *Lemna minor*. In Vitro 37:349–353