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Callus Induction and Frond Regeneration in *Spirodela polyrhiza*

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Abstract

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Spirodela polyrhiza belongs to the family Lemnaceae (duckweed), which is a group of small aquatic plants offering an attractive plant expression system for the production of recombinant protein. No frond regeneration protocol has been established in this species yet. An efficient protocol for plant regeneration through organogenesis has been developed in *Spirodela polyrhiza* for the first time. Calli were successfully induced from 92% of explants on Murashige and Skoog (MS) medium with 10 μM naphthaleneacetic acid, 2 μM thidiazuron, 1μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% sucrose. MS medium containing 1% (m/v) sorbitol and 1 μM 2,4-D supported long lasting growth (at least 5 months) of 98% of calli. Plants regenerated from 92% of calli on Schenk and Hildebrandt (SH) medium with 10 μM zeatin and 1% (m/v) sucrose. The protocol for frond regeneration could be a good basis for transgenic engineering of *S. polyrhiza*.

Keywords: duckweed; regeneration; tissue culture

Duckweed (Lemnaceae) includes 38 species in five genera: *Spirodela*, *Lemna*, *Landoltia*, *Wolffia* and *Wolfiella*, among them, *Spirodela* is the most ancestral (Wang *et al*. 2014). All duckweeds are small aquatic free-floating plants, widely distributed on the surface of still or slow-flowing water (Tang *et al*. 2014). Being the world's smallest angiosperms, the leaves of duckweeds are small, often not exceeding 5 mm in length (except *S. polyrhiza* that can be up to 1.5 cm) (Wang *et al*. 2015). The biomass of Lemnaceae doubles in 48 hours under controlled and axenic conditions (Burns *et al*. 2015; Stomp 2005). Plants of duckweed proliferate mainly through vegetative reproduction, two daughter plants bud off from the adult plant (Stomp 2005; Demirezen *et al*. 2007).

Today, thanks to its rapid growth, short life span, simple axenic culture on liquid medium and high content of protein, plants of Lemnaceae are of great interest as an ideal and efficient plant expression system for production of foreign target proteins including pharmaceuticals and diagnostic reagents (Cox *et al*. 2006; Nguyen *et al*. 2012). The commercial application of Lemnaceae for a plant expression system requires an efficient callus induction, plant regeneration and genetic transformation technology (Chhabra *et al*. 2011; Nguyen *et al*. 2012). Due to the limitation of the genus *Lemna*, among the 38 species of Lemnaceae, a frond regeneration system from callus has so far been established in *L. gibba*, *L. minor*, *L. perpusilla*, *S. oligorrhiza* and *W. arrhiza* (Li *et al*. 2004; Cox *et al*. 2006; Vunsh *et al*. 2007; Rival *et al*. 2008; Chhabra *et al*. 2011; Nguyen *et al*. 2012; Khvatkov *et al*. 2015).

Being the most primitive, *S. polyrhiza*, which has the smallest genome size in the family Lemnaceae, is the most frequently studied as an ideal system in biochemical research for bioremediation and carbon cycle (Wang *et al*. 2012, 2015; Kuehdorf *et al*. 2014; Tang *et al*. 2014; Olah *et al*. 2015; Xu *et al*. 2015).

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However, no information on a frond regeneration system is available in *S. polyrhiza*.

The main aim of this work is to develop an efficient and reproducible system for frond regeneration in *S. polyrhiza* plants through organogenesis.

MATERIAL AND METHODS

Plant material and growth conditions. The strain of *S. polyrhiza* was isolated in 2011 from a rice field in Tiantangzhai, Hubei, China. Axenic fronds have been isolated and preserved in our laboratory for 4 years. Axenic cultures of *S. polyrhiza* were maintained in 100 ml flasks containing 30 ml of Schenk and Hildebrandt (SH) medium (Tang *et al*. 2006), with 1% sucrose under 16/8 h photoperiod at 25/23°C (30–50 μmol photons/m^2/s).

Callus induction. Effects of various combinations of plant growth regulators (naphthaleneacetic acid (NAA), thidiazuron (TDZ), and 2,4-dichlorophenoxyacetic acid (2,4-D); 0–50 μM, Table 1) and sugars (sorbitol, sucrose, maltose, galactose, mannose and glucose at the 3% concentration, Table 2) in the basal MS medium (Murashige & Skoog 1962) on callus induction were investigated. 3–4 day fronds were surgically injured with four incisions in the meristematic portions to obtain 0.5 × 0.5 cm explants. All cultures of *S. polyrhiza* explants were maintained under 16/8 h photoperiod at 25/23°C (30–0 μmol photons/m^2/s).

Callus growth. Calli 4–8 mm in diameter, which were previously cultured for 4 weeks on the most effective callus induction medium (MS medium + 3% sucrose + 10 μM NAA + 2 μM TDZ + 1 μM 2,4-D) were transferred to MS media enriched with different sugars (at the 1% concentration) and with/without plant growth regulators (10 μM NAA, 2 μM TDZ or 1 μM 2,4-D, Table 3). The effect of media composition on callus growth was analysed under 16/8 h photoperiod at 25/23°C (30–50 μmol photons/m^2/s).

Frond regeneration. Calli, cultivated for 3–4 weeks on the medium which was evaluated as the best in terms of callus growth (MS medium + 1% sorbitol +1 μM 2,4-D), were transferred to SH media with 3% sucrose and various concentrations of three plant growth regulators (PGRs) (5–15 μM 6-(γ,γ-dimethylallylamino)purine (2iP), 5–15 μM TDZ and 5–15 μM zeatin (ZT), Table 4). Frond regeneration was investigated under 16/8 h photoperiod at 25/23°C (30–50 μmol photons/m^2/s).

Statistical analyses. The callus induction culture, growth and regeneration experiments were conducted with three replicates at least, each containing 25 explants, calli, regenerated calli and regenerants, respectively, and all experiments were repeated three times. Results are presented as means ± standard deviations. All data were subjected to one-way analysis of variance (ANOVA) and comparisons of means were made by Student's *t*-test at a 5% level of probability. One-way ANOVA and Student's *t*-test were performed using SPSS 10.0.

RESULTS

Callus induction. The types and concentrations of PGRs had a significant ($P \leq 0.05$) influence on callus induction (Table 1). The most successful callus induction was observed within 3–4 weeks on a callus induction medium (CIM) with 10 μM NAA, 2 μM TDZ, 1 μM 2,4-D, and 3% sucrose, where 92% of explants produced moderately growing callus (Table 1). If sucrose was replaced with maltose, glucose, mannitol or galactose, calli were induced at the rate

Table 1. Effects of PGRs on callus induction in *Spirodela polyrhiza* using MS with 3% sucrose

PGRs (μM)			Percent callus induction from explants of *S. polyrhiza*
NAA	TDZ	2,4-D	
0	0	0	0 ± 1.3^b
5	1	1	10 ± 1.2^b
5	2	1	14 ± 1.1^a
5	5	1	11 ± 1.4^c
10	1	1	45 ± 2.0^b
10	2	1	92 ± 1.7^a
10	5	1	51 ± 2.2^b
15	1	1	34 ± 1.3^a
15	2	1	31 ± 1.4^a
15	5	1	23 ± 0.8^b
20	1	2	32 ± 1.5^a
20	2	2	25 ± 1.5^c
20	5	2	26 ± 2.4^b
50	1	2	21 ± 1.8^a
50	2	2	13 ± 0.8^c
50	5	2	19 ± 1.2^c

PGR – plant growth regulator; NAA– naphthaleneacetic acid; TDZ – thidiazuron; 2,4-D – 2,4-dichlorophenoxyacetic acid; values are means ± SD of three replicates; values followed by different letters in the column are significantly different at $P \leq 0.05$ according to Fisher's LSD

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Table 2. Effect of sugars and PGRs on callus induction in *Spirodela polyrhiza*

Sugar (m/v)						PGRs (μM)			Percent callus induction
Sucrose	maltose	glucose	mannose	galactose	sorbitol	NAA	TDZ	2,4-D	
3%	–	–	–	–	–	10	1	1	45 ± 1.3[b]
3%	–	–	–	–	–	10	2	1	92 ± 1.5[a]
3%	–	–	–	–	–	10	5	1	51 ± 1.1[b]
–	3%	–	–	–	–	10	1	1	45 ± 1.5[b]
–	3%	–	–	–	–	10	2	1	91 ± 1.5[a]
–	3%	–	–	–	–	10	5	1	51 ± 2.17[b]
–	–	3%	–	–	–	10	1	1	38 ± 0.9[b]
–	–	3%	–	–	–	10	2	1	90 ± 0.6[a]
–	–	3%	–	–	–	10	5	1	37 ± 1.1[b]
–	–	–	3%	–	–	10	1	1	36 ± 0.5[b]
–	–	–	3%	–	–	10	2	1	81 ± 0.8[a]
–	–	–	3%	–	–	10	5	1	43 ± 0.6[b]
–	–	–	–	3%	–	10	1	1	41 ± 0.9[b]
–	–	–	–	3%	–	10	2	1	82 ± 1.5[a]
–	–	–	–	3%	–	10	5	1	36 ± 0.7[b]
–	–	–	–	–	3%	10	1	1	3 ± 1.95[b]
–	–	–	–	–	3%	10	2	1	6 ± 1.65[a]
–	–	–	–	–	3%	10	5	1	3 ± 2.17[b]

PGR – plant growth regulator; NAA– naphthaleneacetic acid; TDZ – thidiazuron; 2,4-D – 2,4-dichlorophenoxyacetic acid; values are means ± SD of three replicates; values followed by different letters in the column are significantly different at $P \leq 0.05$ according to Fisher's LSD

of 91%, 90%, 81% or 82%, respectively (Table 2). On the other hand, sorbitol did not initiate the callus formation (Table 2). However, repeated subculture on CIM containing various sugars at a 3% concentration resulted in 100% mortality of callus cultures within 3–4 weeks regardless of the type sugar used.

Callus growth. Callus growth medium (CGM) with 1% sorbitol and 1 μM 2,4-D supported the growth of

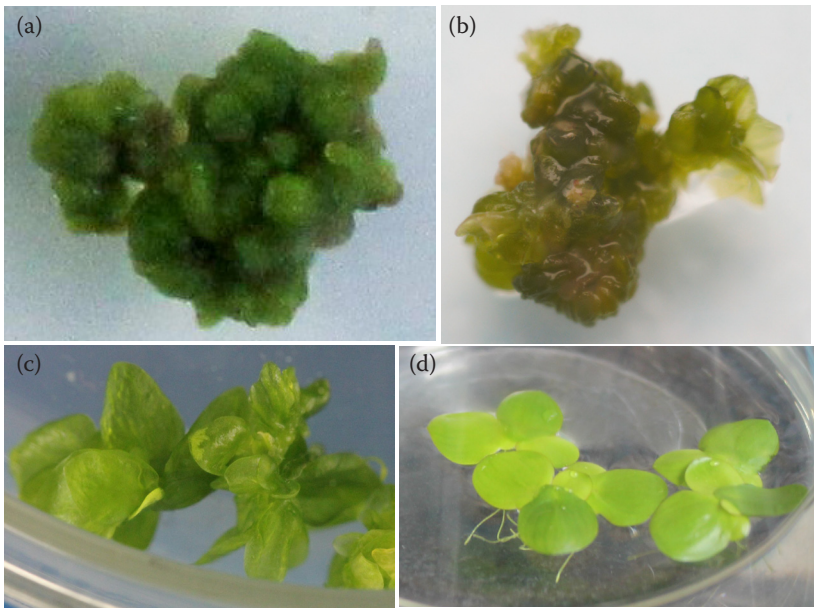

Figure 1. Calli induction and frond regeneration from the culture of *Spirodela polyrhiza*: (a) callus was cultured for 3–4 weeks on MS medium containing 1% sorbitol and 1 μM 2,4-D, (b) callus was induced for 2–3 weeks on SH medium with 1% sucrose plus 10 μM zeatin, (c) regenerated fronds were cultured for 4–5 weeks on SH medium supplemented with 1% sucrose and 10 μM zeatin, (d) formation of whole *S. polyrhiza* plants on growth regulator-free SH liquid medium after regenerated fronds were transferred to SH liquid medium within 1–2 weeks

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Table 3. Effect of different sugars on the growth of *Spirodela polyrhiza* callus

Sugar (m/v)						PGRs (μM)			Percent callus growth
Sorbitol	sucrose	maltose	galactose	mannose	glucose	NAA	TDZ	2,4-D	
1%	–	–	–	–	–	10	2	1	34 ± 1.3[b]
1%	–	–	–	–	–	0	0	1	98 ± 1.5[a]
1%	–	–	–	–	–	10	0	0	31 ± 0.9[b]
1%	–	–	–	–	–	0	2	0	32 ± 0.8[b]
–	1%	–	–	–	–	10	2	1	31 ± 0.9[b]
–	1%	–	–	–	–	0	0	1	95 ± 1.3[a]
–	1%	–	–	–	–	10	0	0	33 ± 0.9[b]
–	1%	–	–	–	–	0	2	0	35 ± 0.7[b]
–	–	1%	–	–	–	10	2	1	15 ± 0.7[b]
–	–	1%	–	–	–	0	0	1	59 ± 0.8[a]
–	–	1%	–	–	–	10	0	0	22 ± 1.1[b]
–	–	1%	–	–	–	0	2	0	26 ± 0.5[b]
–	–	–	1%	–	–	10	2	1	32 ± 0.8[a]
–	–	–	1%	–	–	0	0	1	31 ± 0.6[b]
–	–	–	1%	–	–	10	0	0	29 ± 0.9[b]
–	–	–	1%	–	–	0	2	0	34 ± 1.5[a]
–	–	–	–	1%	–	10	2	1	32 ± 0.9[b]
–	–	–	–	1%	–	0	0	1	35 ± 0.8[a]
–	–	–	–	1%	–	10	0	0	26 ± 1.3[b]
–	–	–	–	1%	–	0	2	0	23 ± 0.5[b]
–	–	–	–	–	1%	10	2	1	28 ± 1.9[b]
–	–	–	–	–	1%	0	0	1	35 ± 1.6[a]
–	–	–	–	–	1%	10	0	0	36 ± 1.5[a]
–	–	–	–	–	1%	0	2	0	32 ± 2.1[b]

PGR – plant growth regulator; NAA– naphthaleneacetic acid; TDZ – thidiazuron; 2,4-D – 2,4-dichlorophenoxyacetic acid; values are means ± SD of three replicates; values followed by different letters in the column are significantly different at $P \leq 0.05$ according to Fisher's LSD

98% of calli, and prevented them from browning and growth cessation (Table 3). The callus morphology was green, compact and consisted of small, connected amorphous masses (Figure 1a). Moreover, the above-mentioned medium supported callus growth for over 5 months (Figure 1b). If sorbitol in CGM was replaced by different sugars, the efficiency of callus growth decreased. Namely, 1% sucrose and 1% maltose supported the growth of 95% and 59% of calli, respectively. In contrast, glucose, mannitol and galactose promoted callus growth far less effectively than maltose (Table 3).

Frond regeneration. The formation of frond-like cluster structures was observed within 2–3 weeks after transferring calli from CGM (MS + 1% sorbitol + 1μM 2,4-D) to a frond regeneration medium (FRM, Figure 1b). The highest levels of *S. polyrhiza* frond regeneration (92%) were obtained within 4–5 weeks on FRM with 10.0 μM zeatin and 3% sucrose (Table 4). Moderate regeneration (58%) was observed on FRM with 10 μM TDZ and 3% sucrose. In contrast, 5.0–15 μM of iP poorly supported frond regeneration (Table 4). Regenerated fronds became visibly distinct within 4–5 weeks (Figure 1c). Fronds with a sufficiently developed root system were separated from their supporting nodules after an additional subculture for 1–2 weeks on SH liquid medium without PGRs (Figure 1d).

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Table 4. Effects of different PGRs on frond regeneration in *Spirodela polyrhiza*

PGRs (μM)			Percent frond regeneration
2ip	TDZ	ZT	
5	–	–	20 ± 1.2[b]
10	–	–	15 ± 1.1[a]
15	–	–	13 ± 1.4[c]
–	5	–	30 ± 0.7[a]
–	10	–	58 ± 2.2[b]
	15	–	25 ± 1.2[a]
–	–	5	31 ± 1.4[a]
–	–	10	92 ± 0.9[b]
–	–	15	51 ± 1.3[a]

PGR – plant growth regulator; 2ip – 6-(γ,γ-dimethylallylamino) purine (2iP); TDZ – thidiazuron; ZT – zeatin; values are means ± SD of three replicates; values followed by different letters in the column are significantly different at $P \leq 0.05$ according to Fisher's LSD

DISCUSSION

So far, among 38 species of duckweed, a successful frond regeneration system was established in *S. oligorrhiza*, *S. punctata*, *L. gibba* var. *Hurfeish*, *L. minor* and *W. arrhiza*, when sugar supplements and specific PGRs were critical determinants for callus induction, callus growth and frond regeneration (Li *et al*. 2004; Cox *et al*. 2006; Vunsh *et al*. 2007; Rival *et al*. 2008; Chhabra *et al*. 2011; Nguyen *et al*. 2012; Khvatkov *et al*. 2015).

Effects of sugars on callus induction. It was reported that requirements for sugars for callus induction in duckweed were species-specific (Li *et al*. 2004; Khvatkov *et al*. 2015). *S. oligorrhiza* SP callus was successfully induced from 91–95% of the explants on CIM with 2% sorbitol plus 1% maltose; in the case of *W. arrhiza*, CIM containing 0.7% sorbitol plus 0.7% mannitol and 2.6% glucose supported callus induction; callus was induced at the rate of over 95% of the *S. punctata* explants on CIM with 1% sorbitol; frond explants were precultured for 2 weeks on CIM with 1% sucrose to induce callus formation in *L. gibba* var. *Hurfeish* (Li *et al*. 2004; Khvatkov *et al*. 2015). Our results proved that *S. polyrhiza* callus was induced from 92% of the explants on CIM containing 3% sucrose. Maltose and glucose (3%) supported callus induction from 91% and 90% of the explants, respectively. Mannitol and galactose (3%) could promote callus formation at the rate of 81% and 82%, respectively.

Effects of sugars on callus growth. Previous reports proved that 2% sorbitol plus 1% maltose poorly supported the callus growth in *S. oligorrhiza*, and the presence of conventional sugars such as sucrose, glucose and fructose in the callus growth medium resulted in 100% mortality of the *S. oligorrhiza* callus cultures. Otherwise, 2% sorbitol was required for optimal callus growth of *S. punctata*, while 1% sucrose supported callus growth of *L. gibba* var. *Hurfeish*, and 3% sucrose was critical for *L. minor* callus growth (Li *et al*. 2004; Khvatkov *et al*. 2015). Our results confirmed that CGM with 1% sorbitol supported the long-term growth of *S. polyrhiza* callus, but CGM containing 1% sucrose could gradually result in the mortality of the callus within 6 weeks.

Effects of PGRs on callus induction. Among duckweeds, callus induction was significantly influenced by the type and concentration of PGRs (Moon & Stomp 1997; Moon & Yang 2002; Khvatkov *et al*. 2015). *S. oligorrhiza* SP callus was induced from 91–95% of the explants on CIM with 5 mg/l PCA and 2 mg/l 2iP. In the case of *W. arrhiza*, CIM containing 5.0 mg/l 2,4-D supported callus induction. Callus was induced from 95% of the *S. punctata* explants on CIM with 3.5 mg/l 2, 4-D plus 2 mg/l 2iP. CIM with 2 mg/l BA was able to induce callus formation in *L. gibba* var. *Hurfeish*. Our results proved that *S. polyrhiza* callus could be induced on CIM containing 10 μM NAA, 2 μM TDZ and 1 μM 2,4-D at the rate of 92%.

Effects of PGRs on frond regeneration. 90–100% of the calli regenerated into plants using FRM with 1 mg/l TDZ in *S. oligorrhiza* SP (Li *et al*. 2004). PGR-free FGM promoted frond regeneration in *W. arrhiza* (Khvatkov *et al*. 2015). The *L. gibba* var. *Hurfeish* frond regenerating frequency on FRM with 1 mg/l TDZ was about 55% (Li *et al*. 2004). As for *S. punctata*, the 95–98% plant regeneration rate was obtained on FRM with 1 mg/l 2ip (Li *et al*. 2004). It was previously reported that *L. gibba* G3 and *W. arrhiza* fronds could regenerate in a PGR-free medium (Moon & Yang 2002; Khvatkov *et al*. 2015). Our results proved that it took at least 8 weeks for *S. polyrhiza* fronds to regenerate from callus in a PGR-free SH medium at the rate of 30% (data not shown). Further experiments confirmed that *S. polyrhiza* fronds could regenerate at the rate of less than 60% in 5–6 weeks on FRM containing 10 μM TDZ and at the rate of over 90% in 3–4 weeks on FRM with10 μM zeatin.

In conclusion, it was for the first time when we established an efficient and reproducible protocol for frond regeneration from callus in *S. polyrhiza*. Callus induction was obtained from 92% of explants on CIM (MS + 10 μM NAA + 2 μM TDZ + 1 μM 2,4-D + 3% sucrose + 0.8% agar), while CGM medium (MS + 1% sorbitol + 1μM 2,4-D) supported the long-term

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growth of *S. polyrhiza* callus, frond regeneration from callus was obtained at the rate of 92% on FRM (SH + 10 µM zeatin + 1% sucrose) within 3–4 weeks. The development of a frond regeneration protocol is the first step towards *in vitro* technology in *S. polyrhiza* to use in gene transfer technology.

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