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Research Article

Establishment of a novel plant regeneration system from suspension-derived callus in the halophytic *Leymus chinensis* (Trin.)

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Abstract The establishment of cell suspension culture and plant regeneration of the halophytic *Leymus chinensis* (Trin.) are described in this study for the first time. Callus induction solid medium containing Murashige and Shoog (MS) basic salt, 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 5.0 mg l^{-1} L-glutamic acid with 30.0 g l^{-1} sucrose and 4.0 g l^{-1} gelrite for solidification induced the highest rate of cell division in Type 1 callus among calli of various types. Liquid medium with the same hormone distribution was therefore, used for cell suspension culture from Type 1 callus. Over a 30 d suspension culture at 100 rpm, great amounts of biomass were accumulated, with 71.07% average daily increment and 22.32-fold total fresh weight increment. Comparison of before and after suspension culture, the distribution of different size callus pieces and the maintenance of callus type were basically unaltered, but a slight increase in relative water contents was observed. To induce the potential of plant regeneration, the directly transferring on plant regeneration solid medium containing MS basic salt, 0.2 mg l^{-1} α-naphthalene acetic acid (NAA), 2.0 mg l^{-1} kinetin (Kn), and 2.0 g l^{-1} casamino acid and indirectly transferring were simultaneously performed. Even now growth rates of suspension-derived callus on solid medium were approximately half of those of Type 1 callus, but faster somatic embryogenesis was observed. Rooting of all regenerated shoots was successfully performed on half-strength MS medium. All plants appeared phenotypically normal.

Keywords *Leymus chinensis*, Cell suspension culture, Plant regeneration, Somatic embryogenesis, Halophytic plant

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Abbreviations

MS Murashige and Shoog
2,4-D 2,4-Dichlorophenoxyacetic acid
NAA α-Naphthalene acetic acid
Kn Kinetin
SD Standard deviation

Introduction

The halophyte *Leymus chinensis* (Trin.), a perennial rhizome grass placed in tribe Gramineae, is an important forage crop widely grown throughout northern China, Mongolia and Siberia (Huang et al. 2004). Because of its intrinsic tolerance of highly alkaline-sodic soil conditions (Jin et al. 2006), *L. chinensis* is used as a soil-binding plant to protect soil from desertification. Its high vegetative productivity, good palatability and protein content, make the species a major forage product to meet the needs of grazing (Shu et al. 2005). However, climate changes, overgrazing and reclamation of grassland result in severe deterioration of the grassland ecosystem, including *L. chinensis* grassland. Under the pressure of low sexual reproductivity (Huang et al. 2004) and relatively chilling-sensitivity, the great demands for genetic improvement of physiological traits become urgent need in this grass. Plant cell culture technologies are introduced at the end of 1960s as a possible tool for both studying and producing plant secondary metabolites (Mulabagal and Tsay 2004). Until now, plant cell culture technology has been widely used for the commercial processing, especially in the generation of recombinant proteins and the industrial production of secondary metabolites (Dicosmo and Misawa 1995). These secondary metabolites include taxol, morphine

and codeine, ginsenosides, berberine, diosgenin, capsaicin and so on (Hunter and Kilby 1990). Like animal cells, plant cells are more and more commonly used in studying the morphological, physiological and molecular responses to stress toxicity, the mechanisms of ion transport and related ion signaling pathways (Keppler et al. 1989; Murata et al. 2000; Janisch and Schempp 2004; Kim et al. 2007). Protoplasts isolated from the cells provide a novel system as single cells instead of complex tissues (Briskin and Leonard 1979). In addition, understanding of physiological characteristics and gene expression patterns of halophytic plants under stress has been believed as a good way of understanding of stress-specific signaling cascades and activate adaptive transcriptional and translational responses. In comparison to routine callus culture, plant cell culture facilitates the rapid cell division and cell generations of various cell lines and stable and direct somatic embryogenesis. Moreover, due to a large surface for exposure to transforming agent and effective selection of transformants, embryogenic cells from suspension culture have been used for gene transformation and gene manipulation (Hall 1991). It has also been thought that the unicellular origin of somatic embryos could reduce the likelihood of chimerism (Deo et al. 2010). Many studies have been reported for several graminaceous species. In rice, reliable and efficient protocols for the establishment of cell suspension cultures have been developed, and transgenic, somatic hybrid, and cybrid plants have been obtained by using protoplasts isolated from suspension cells (Shimamoto et al. 1989; Yang et al. 1989). In barley, successful plant regeneration from suspension cell-derived protoplasts was reported (Jähne et al. 1991), and transgenic barley plants have been successfully produced by direct gene transfer to suspension cell-derived protoplasts (Funatsuki et al. 1995). For the halophytic *L. chinensis*, little studies about cell suspension cultures and suspension-derived plant regeneration has been reported. To the authors' knowledge, even tissue culture information about this grass is not much (Liu et al. 2004; Kim et al. 2005). Based on the previous studies in our lab, we optimize the conditions of tissue culture in this grass, and investigate callus induction from various explants including mature seeds, leaf tissue and root tissue (Sun and Hong 2009; Sun and Hong 2010). Here, we describe the establishment of cell suspension cultures from Type 1 callus, which was previously obtained from callus tissue culture using mature seeds and leaf base segments of *L. chinensis* LcWT07 plants as explants (Sun and Hong 2010). Using liquid medium with the same hormone distribution as callus induction solid medium used in our previous study, the growth patterns of the cultures, such as relative growth rates, relative water contents, and the distribution of different size cell aggregates, were examined during a range of culture durations. The culture conditions are also being used by this author for gene transformation in this grass by *Agrobacterium* -mediated transformation.

Materials and methods

Plant materials

Mature seeds of salt-tolerant *Leymus chinensis* (Trin.) were obtained from the natural grassland in Siping, Jilin, China; these seeds have been optimized for environment by many years of natural evolution. Mature seeds were de-husked and surface-sterilized with 70% ethanol for 1 min, and then 5% sodium hypochlorite for 20 min using a method modified from Sun and Hong (2010). The sterilized seeds were rinsed five times with sterile water and prepared for primary callus initiation as explants.

Callus initiation on solid medium

The mature seed explants were inoculated on callus induction solid medium supplemented with MS basic salt, 2.0 mg l^{-1} 2,4-D and 5.0 mg l^{-1} L-glutamic acid, and 30.0 g l^{-1} sucrose and 4.0 g l^{-1} gelrite for solidification. Callus induction medium was adjusted pH value to 5.8 prior to the addition of gelrite, and sterilized by autoclaving at 120℃ and 1.4 kg cm^{-2} for 20 min. Callus initiation from mature seeds of LcWT07 plants was induced on the solid medium described above at 25℃ in darkness with 60% humidity with 20 d-subculture. According to the callus growth status, Type 1 callus were selected and used for suspension cultures after a 2-month culture based on the criterion described by Sun and Hong (2010).

Proliferation and callus type maintenance on suspension cultures

Approximately 0.5 g of Type 1 callus was placed in 100 ml of callus induction liquid medium in a 250 ml Erlenmeyer flask (Iwaki Glass, Japan) capped with foil and agitated on an orbital shaker at 100 rpm. The liquid medium was the same as solid medium except agar was omitted. The pH value of liquid medium was also adjusted to 5.8 and sterilized by autoclaving at 120℃ and 1.4 kg cm^{-2} for 20 min. After 15 d of suspension culture, the cells were allowed to settle to the bottom of the flask and 50 ml of the supernatant was removed and replaced with an equal volume of fresh medium. At the additional 15 d of suspension culture, the

suspension cells were passed through a 500 μm stainless steel mesh filter and the filtrate was collected. The cells within the filtrate were washed three times with sterile water to remove residual sugar on the cell furface and dried on sterile 100 mm Whatman filter paper in a clean bench for 20 min. Then, the cells were prepared for growth status evaluation, proliferation on callus induction solid medium and plant regeneration on plant regeneration solid medium.

For fresh cell weight measurement, the suspension cells were filtered through Whatman filter paper under vacuum and washed three times with distilled water to remove residual sugar on the cell surface. Then, the cells were transferred to a pre-weighted Petri dish and weighted. For dry cell weight measurement, the cells in Petri dish were transferred into a dry machine for drying at 60℃ for 2 days and the dry cell weight was then measured. Each experiment was repeated three times.

Plant regeneration from suspension-derived callus and rooting

The suspension cells were transferred on plant regeneration solid medium in 90 mm × 15 mm Petri dishes for plant regeneration. Solid medium containing MS basic salt, 0.2 mg l^{-1} α-naphthalene acetic acid (NAA), 2.0 mg l^{-1} kinetin (Kn), and 2.0 g l^{-1} casamino acid, were used for plant regeneration. Plant regeneration medium was adjusted pH value to 5.8 prior to the addition of gelrite, and sterilized by autoclaving at 120℃ and 1.4 kg cm^{-2} for 20 min. The cultures were maintained at 25℃ in darkness for the first 1 week then incubated at 25℃ under low light intensity (25 μE m^{-2} s^{-1}) and a 16/8 h (light/darkness) photoperiod for another 2 weeks. During plant regeneration stage, a 20 d-subculture was collected. After the first subculture of plant regeneration solid medium, suspension cell-derived embryos were transferred to high light intensity (70 μE m^{-2} s^{-1}) and a 16/8 h (light/darkness) photoperiod at 25℃.

Regenerated shoots were transferred onto rooting solid medium containing half-strenght MS basic salt. The cultures were maintained at 25℃ under high light intensity (70 μE m^{-2} s^{-1}) and a 16/8 h (light/darkness) photoperiod. Well-rooted plantlets were removed from rooting solid medium, removed from culture medium and then transplanted to pots containing a mixture of sterilized soil and vermiculite (3:1) in a greenhouse conditions.

Statistical analysis

Statistically significant differences between means were determined by two-way analysis of variance (ANOVA) using Duncan's multiple-range test (Duncan 1955). A *P* value of less than 0.05 was considered significant.

Results

Callus initiation and proliferation on solid medium

Mature seeds of LcWT07 plants were inoculated on callus induction solid medium containing 2.0 mg l^{-1} 2,4-D and 5.0 mg l^{-1} L-glutamic acid, and callus initiation occurred within 2 weeks after transferring onto callus induction medium (Fig. 1A). Based on the difference of callus types,

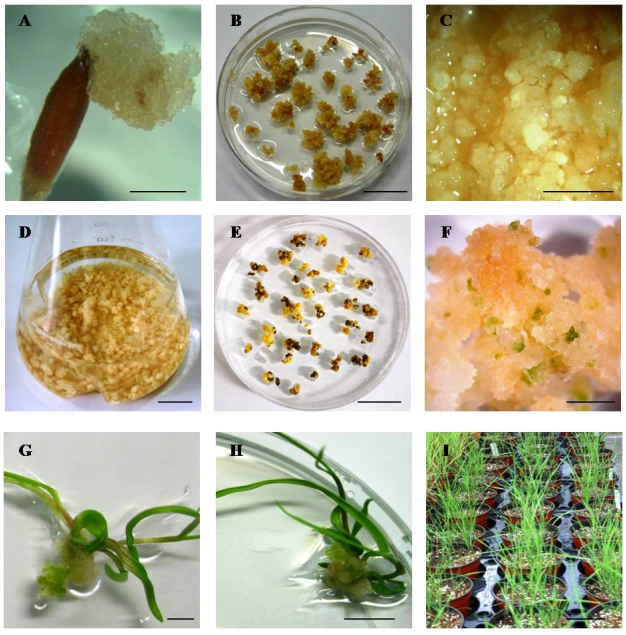

Fig. 1 Callus proliferation and plant regeneration from suspension-derived callus in halophytic LcWT07 *L. chinensis* plants. A. Callus induction from mature seeds within 2 weeks cultured on callus induction solid medium containing 2.0 mg l^{-1} 2,4-D and 5.0 mg l^{-1} L-glutamic acid. B. Callus proliferation of Type 1 callus cultured on callus induction solid medium containing 2.0 mg l^{-1} 2,4-D and 5.0 mg l^{-1} L-glutamic acid. C. Cell division from Type 1 callus after transferred into callus induction liquid medium containing 2.0 mg l^{-1} 2,4-D and 5.0 mg l^{-1} L-glutamic acid. D. Suspension cell aggregates after 30 d suspension culture in callus induction liquid medium containing 2.0 mg l^{-1} 2,4-D and 5.0 mg l^{-1} L-glutamic acid. E. The necrosis of cell aggregates when transferred onto plant regeneration solid medium containing 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} Kn, and 2.0 g l^{-1} casamino acid. F. Suspension-derived callus with some green spots on plant regeneration solid medium containing 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} Kn, and 2.0 g l^{-1} casamino acid. G. Shoot regeneration from suspension-derived callus on plant regeneration solid medium containing 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} Kn, and 2.0 g l^{-1} casamino acid. H. Rooting of regenerated shoots on rooting solid medium containing half-strength MS basic salts. I. Plants from plant regeneration after 2 month culture under greenhouse conditions. Scale bar: 2 mm (A, C, D, F, G, H); 20 mm (B, E); 60 mm (I)

the growth rates were remarkably different: Type 1 callus, yellow, nodule-like and compact callus, showed the highest growth rate among these four types of calli, which was selected for further experiment (Table 1). After 2-month culture on solid medium, large amounts of Type 1 callus were accumulated (Fig. 1B), with the relative water contents less than 90% (Table 2) and a certain percentage of different size callus pieces (Fig. 2-4).

Characterization of suspension cultures

Suspension cultures were initiated by transferring approximately 0.5 g proliferated embryogenic callus into callus induction liquid medium containing 2.0 mg l^{-1} 2,4-D and 5.0 mg l^{-1} L-glutamic acid with continuous agitation at 100 rpm. After transferred callus into liquid medium, nearly all inoculated calli formed suspensions and began to cell divisions after the next following 1 week (Fig. 1C). These callus pieces were different from size, and according to different diameter sizes of callus pieces, very big (VB, diameter size > 3 mm), big (B, diameter size 3 mm > 2 mm), middle (M, diameter size 2 mm > 1 mm) and small (S, diameter size < 1 mm) callus, four types of calli were classified (Fig. 2). Unexpectedly, the fresh weights of different size callus pieces were also proportionately different (Fig. 3). After inoculation in liquid medium, these callus pieces began to produce single cells, and then these single cells began to aggregate and turned larger and larger. After 30 d suspension culture with continuous agitation at 100 rpm, the suspension cell amounts had as high as 22.3-fold increment (Fig. 5), with the average daily increment percentages of 65.1%-85.2% (Table 3) and relative water contents more than 90% (Table 2). The percentages of different size callus pieces were also evaluated after 30 d suspension culture (Fig. 4), but there was no remarkable

Table 1 Growth rates of different callus types during callus initiation and proliferation stage

Callus type[a]	Experimental callus number	Growth rate[b]
Type 1	90	3.34 ± 0.46 a
Type 2	90	2.23 ± 0.80 ab
Type 3	50	1.34 ± 0.38 b
Type 4	40	0.75 ± 0.29 b

[a] Callus type was distinguished based on the criterion described by Sun and Hong (2010)

[b] Growth rate evaluated by the fold value was calculated as the ratio of callus fresh weight after a 20 d culture on callus induction solid medium to the primary callus fresh weight. The growth rates are the mean of three repeated experiments. Means followed by the same letter in the same column are not significantly different at $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test

Table 2 Comparison of relative water contents between Type 1 callus and suspension cell

Experimental repeats	Callus line	Fresh callus weight (mg)	Dry callus weight (mg)	Relative water content[a]
1	Type 1 callus	2.64	0.27	89.77%
2		3.06	0.31	89.86%
3		12.17	1.24	89.81%
1	Suspension cell	1.14	0.08	92.60%
2		4.09	0.36	91.20%
3		2.24	0.22	90.18%

[a] Relative water content was calculated as the ratio of the difference of fresh callus weight and dry callus weight to fresh callus weight

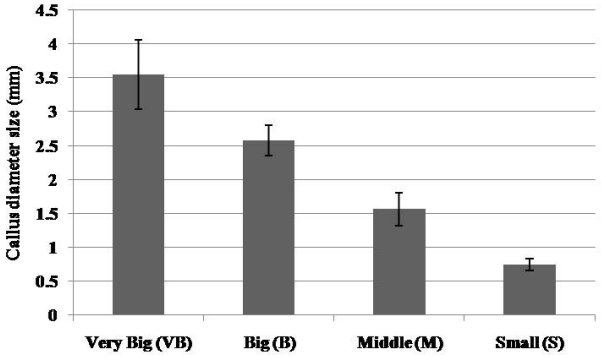

Fig. 2 Callus diameter size of various calli or cell aggregates. Very big (VB, diameter size > 3 mm), big (B, diameter size 3 mm > 2 mm), middle (M, diameter size 2 mm > 1 mm) and small (S, diameter size < 1 mm) callus, four types of calli or cell aggregates were classified. The values are the mean of three repeated experiments, and each experiment includes 6 synchronous repeats. *Vertical bars* represent standard deviation (SD) of the means

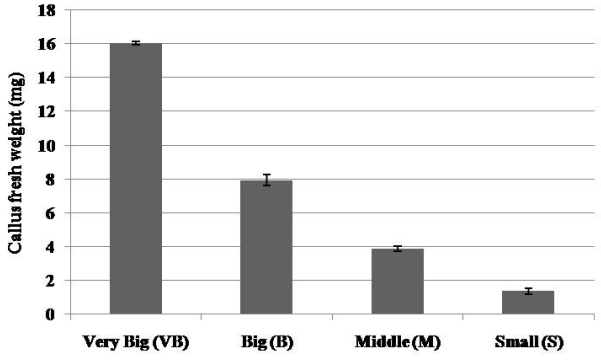

Fig. 3 Callus fresh weight of various calli or cell aggregates. The values are the mean of three repeated experiments, and each experiment includes 6 synchronous repeats. *Vertical bars* represent standard deviation (SD) of the means

change between before and after suspension culture. In addition, with suspension cultures, it made cell aggregates with higher rigidity and more somatic embryogenesis, that would enhance the potential of shoot formation (Fig. 6). Except Type 1 callus, Type 2-4 calli were also performed

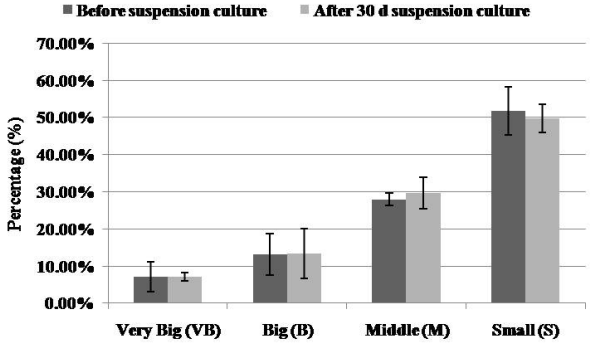

Fig. 4 Percentages of various calli or cell aggregates before suspension culture and after 30 d suspension culture. The values are the mean of three repeated experiments, and each experiment includes 6 synchronous repeats. *Vertical bars* represent standard deviation (SD) of the means

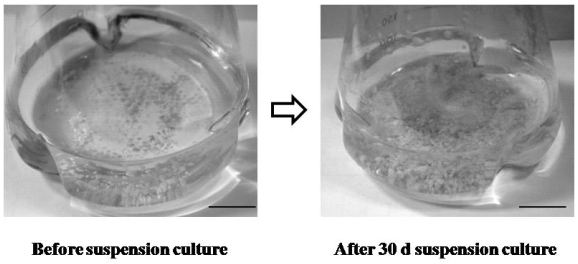

Fig. 5 Cell division and proliferation before and after 30 d suspension culture. Scale bar: 20 mm

suspension culture (data not shown). Rapid cell division and proliferation were though obtained from suspension cultures compared to that from routine tissue culture of Type 2-4 calli, the optimization of callus types was not obvious: the newly regenerated cell aggregates were pale-yellow or white, loose, soft and watery, which were not used for following shoot regeneration. After 30 d suspension culture, cell aggregates were suitable for further cell proliferation or shoot regeneration (Fig. 1D).

Regeneration and rooting

Suspension cell aggregates were plated on plant regeneration solid medium containing 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} Kn, and 2.0 g l^{-1} casamino acid for inducing shoots. Due to the sudden relatively dry conditions from liquid to solid medium, the necrosis often occurred (Fig. 1E). To improve the necrosis occurrence and quicken the adaption speed, suspension cell aggregates were firstly inoculated on plant regeneration solid medium under darkness for the 1st week, and then slow light intensity for the following 2 weeks and high light intensity for the following culture periods (Fig. 7). We also investigated the assuasive transition: suspension cell aggregates were firstly subcultured and proliferated for a subculture period on callus induction solid medium, to increase the capacity of adaption under the sudden relatively dry conditions, and then transferred onto plant regeneration solid medium directly under slow light intensity for 1 weeks and high light intensity for the following culture periods (Fig. 7). Even now, the suspension cell aggregates had lower potential of cell division on solid medium than Type 1 callus, with 50% decline compared to that of routine tissue culture

Table 3 Daily growth rates, total growth rates, and daily increment percentages of suspension cells in callus induction liquid medium during cell division and proliferation stage

Experimental repeats	Fresh weight 1 (mg)[a]	Fresh weight 2 (mg)[b]	Daily growth rate (mg d^{-1})[c]	Total growth rate[d]	Daily increment percentage[e]
1	479.7	12743.5	408.8	26.6	85.2%
2	480.4	12648.2	405.6	26.3	84.4%
3	601.9	12375.4	392.5	20.6	65.2%
4	602.4	12475.5	395.8	20.7	65.7%
5	568.3	11771.5	373.4	20.7	65.7%
6	568.4	11666.6	369.9	20.5	65.1%
Means	550.2	12280.1	391.0	22.3	71.1%

[a] Fresh weight 1 means the fresh weight of plant cells to be cultured in callus induction liquid medium
[b] Fresh weight 2 means the fresh weight of plant cells cultured for 30 d in callus induction liquid medium
[c] Daily growth rate was calculated as the ratio of the difference of fresh weight 2 and fresh weight 1 to the culture period, 30 d
[d] Total growth rate evaluated by the fold value was calculated as the ratio of fresh weight 2 to fresh weight 1
[e] Daily increment percentage (%) described how many percentage of fresh weight 1, the daily increment of fresh weight of plant cells occupied

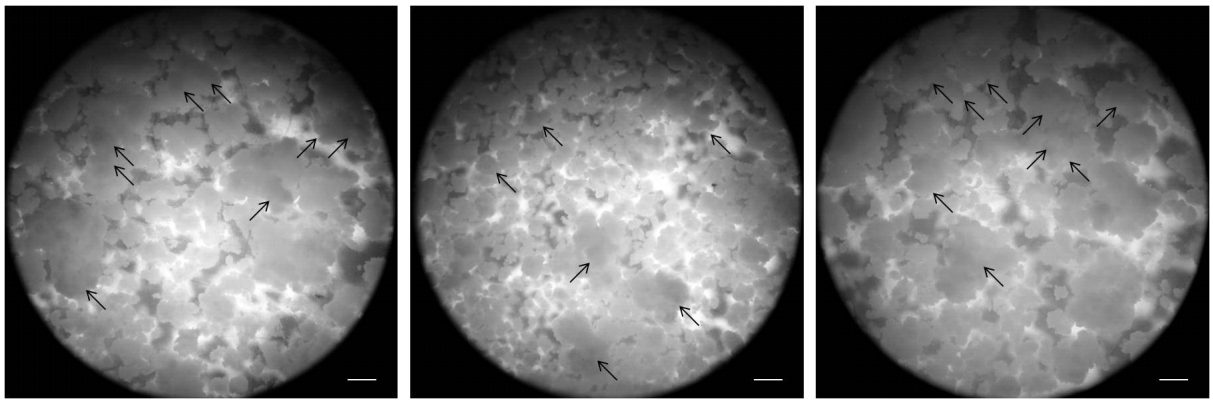

Fig. 6 Different size cell aggregates and somatic embryogenesis on the surface of cell aggregates. Arrows mean the obvious embryogenic calli which could produce shoots. These three pictures are from three synchronous repeats. Scale bar: 1 mm

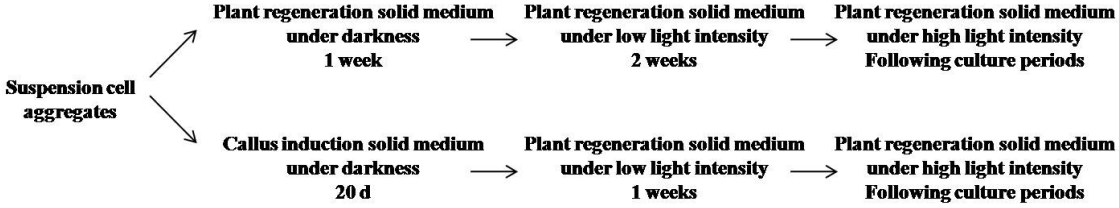

Fig. 7 The two pathways of plant regeneration from suspension cell aggregates

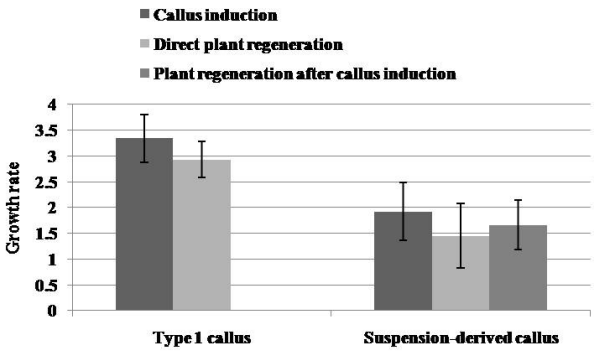

Fig. 8 Comparison of growth rates between Type 1 callus on routine tissue culture and suspension-derived callus on solid medium. The values are the mean of three repeated experiments, and each experiment includes 6 synchronous repeats. *Vertical bars* represent standard deviation (SD) of the means

(Fig. 8).

During 2-month culture with a 20 d subculture, cell aggregates began to form a few green spots and approximately 50% of the suspension-derived callus produced shoots (Fig. 1F, G). When shoots reached 3 cm in length, all of them were transferred to rooting solid medium containing half-strength MS basic salts. Nearly 100% regenerated shoots rooted within the next following week (Fig. 1H). The well-rooted plantlets were cultured in pots containing a mixture of sterilized soil and vermiculite (3:1) in greenhouse conditions. After 1 month in culture, all the plants appeared phenotypically normal (Fig. 1I).

Discussion

Somatic embryogenesis from callus was first reported in *L. chinensis* using immature inflorescences as explants (Liu et al. 2004). The callus induction frequencies of 72.11-82.19% are obtained on media with different hormones, however, immature inflorescence of this grass is seasonal, difficult to handle, and not available year round in bulk quantities. Later, we attempted mature seeds, leaf tissues, and root tissues which are easy to handle and obtain, as explants, and induced callus and obtained regenerated shoots and plantlets successfully (Sun and Hong 2009). Through regulating some growth factors affecting embryogenic callus induction and plant regeneration, Medium 6 is believed as the preferred callus induction medium, mature seeds were selected for a preferred explants, and plant regeneration frequency is enhanced to 70.8% in LcWT07. Recently, our lab improved the conditions of callus initiation further, by the addition of L-glutamic acid and casamino acid on callus induction and plant regeneration stage, respectively (Sun and Hong 2010). Until now, a highly efficient callus induction and plant regeneration of *L. chinensis* has been established. As the addition of 2.0 mg l^{-1} 2,4-D and 5.0 mg l^{-1} L-glutamic acid was believed to enhance significantly callus induction and

maintenance, and the formation of embryogenic callus in callus tissue culture (Sun and Hong 2010), liquid medium with the same agents was used in suspension culture in the present study.

Callus type used for suspension culture is very important for rapidly growing and providing a continuous supply of regeneration tissue. It has been reported that Type 1 callus, yellow, nodule-like, compact callus, rapidly and efficiently converts to embryogenic callus and has relatively high growth rate. Type 1 callus was selected as suspension explants in this study. In general, long period cultures increase the probability of gene mutation and lose the potential of regeneration. So it is proposed to use newly initiated callus as explants for suspension cultures and genetic transformation. In this study, continuous agitation at 100 rpm made the maintenance of a certain cell aggregate size and rigidity, and rapidly growing of cell aggregates made suspension cultures highly saving on time, implying that the probability of gene mutation could be cut down largely.

To obtain a fine suspension culture, it is of prime importance to resolve the problem of necrosis. One side is the dry condition, the other side is suddenly removal of the surrounding medium with abundant hormones. Similar evidence has suggested that removal of either hormone from the medium would normally result in culture death (Stafford 1996). Our assuasive transition, though, played determinate roles, the necrosis is still a problem to be resolved. Genotypic variation frequently is observed in tissue cultures (Doctrinal et al. 1989) and suspension cultures (Gürel et al. 2002), various suspension cultures of different lines of *L. chinensis* are also required to establish.

In conclusion, an efficient suspension cell culture using Type 1 callus as explants and plant regeneration from suspension-derived callus was firstly developed in halophytic *L. chinensis*, LcWT07 line. It provides a new and ideal way of the plant regeneration and genetic transformation to improve the traits in this grass.

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