INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0722/2018/20–2–375–381
DOI: 10.17957/IJAB/15.0501
http://www.fspublishers.org

Full Length Article

High-Efficiency Plant Regeneration from Immature Inflorescence Derived Callus Cultures of Two Phenotypically Distinct Accessions of Centipedegrass (*Eremochloa ophiuroides*)

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Abstract

Centipedegrass [*Eremochloa ophiuroides*(Munro) Hack] is an important warm-season (C_4) grass species widely used in lawn and forage industry. To achieve the purpose of improvement and innovation of centipedegrass germplasms, a high efficient plant regeneration system was established from immature inflorescence-derived callus cultures. Two centipedegrass accessions of E022 and E092-1 with different phenotypic characteristics were investigated for their performances in callus formation, development and as well plant regeneration. The optimum medium for initiation of callus induction was Murashige and Skoog (MS) medium supplied with 4.0 mg/L dichlorophenoxy acetic acid (2,4-D) and 0.2 mg/L N6-benzyl adenine (BAP), and the callus induction rate reached up to 53.4%and 80.0% in E092-1 and E022, respectively. Callus proliferation was best in the subculture medium consisting of a MS base medium supplemented with 2.0 mg/L2,4-D and 0.1 mg/L BAP, with enhanced osmotic pressure. Shoot organogenesis was brought about when the calli were shifted to MS media containing 2.0 mg/L Kinetin (KT) and 0.1 mg/L*a*-Naphthalene acetic acid (NAA), or 2.0 mg/L BAP and 0.1 mg/L NAA or 2.0 mg/L N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) and 0.1 mg/L NAA. Maximum organogenesis of E022 was obtained on medium containing BAP, but maximum organogenesis of E092-1 achieved on medium containing KT. Rooting of plantlets was achieved for both accessions under the rooting induction medium of MS plus 0.5 mg/L NAA. Plants with well-formed root system were hardened and transplanted to soil. Thus, a highly efficient, reproducible plant regeneration system was developed for two phenotypically distinct *E. ophiuroides* accessions by using immature inflorescence explants, which can effectively solve the problem of low percentage of embryogenic calli formation and meanwhile improve the regeneration frequency of plantlets in centipedegrass.

Keywords: Callus induction; Centipedegrass; *Eremochloa ophiuroides*; Inflorescence; Plant regeneration

Introduction

Centipedgrass [*Eremochloa ophiuroides*(Munro) Hack] is a significant warm-season (C_4) perennial grass species native to South and Central parts of China (Hanna and Burton, 1978) and widely distributed in South-east Asia. *E. ophiuroides* is famous for good adaptation to poor and ranges of changed soil-climatic conditions (Islam and Hirata, 2005; Liu *et al*., 2008). For its lower management requirements than the most of current warm-season turfgrasses, centipedegrass has a great advantage in the aspect of low-maintenance turf breeding and often is named 'poor man's grass' (Hanna, 1995). Centipedegrass is propagated by seed and stolons, is highly resistant to disease and insect infestation, and also has strong heat tolerance (Hanna, 1995). It is such low maintenance requirements, good adaptation to poor soil and excellent resistance to biotic or abiotic stresses that make centipedegrass a key important and popular turfgrass in tropical and subtropical regions. Besides, centipedegrass has also been used as a forage grass in some areas of Southeast Asia, such as Japan for its acceptable quality and palatability to animals (Islam and Hirata, 2005; Hirata *et al*., 2007). Despite the great advantageous of centipedegrass over other warm-season turfgrasses, there are some congenital defects, such as its sensitivity to salt, cold and drought stresses, which are hindering its mass application in the lawn industry. So it is of great urgency and significance to achieve germplasm improvement and innovation through transferring the useful genes in centipedegrass breeding.

To establish a highly efficient regeneration is a prerequisite for improvement of grass species via

To cite this paper: Ma, J., Y. Wang, J. Zong, B. Zhang, J. Chen, D. Li, L. Li, H. Guo, J. Liu, J. Li, J. She, 2018. High-efficiency plant regeneration from immature inflorescence derived callus cultures of two phenotypically distinct accessions of centipedegrass (*Eremochloa ophiuroides*). *Int. J. Agric. Biol*., 20: 375–381

biotechnological approach (Wang *et al*., 2001; Lu *et al*., 2006). So far, callus induction and plant regeneration have been reported in centipedegrass from several explant types including mature seeds (Ma *et al*., 2004; Shuto *et al*., 2004; Barampuram *et al*., 2009; Yuan *et al*., 2010), axillary buds or shoots (Yuan *et al*., 2008; Barampuram *et al*., 2009), young stems (Shu *at al*., 2011). However, all these embryogenesis and regeneration systems had low rates of callus induction and plant regeneration with long period required for establishing a regeneration system, and these "short boards" have been becoming a key limiting factor for achieving high frequency of genetic transformation in centipedegrass. Fortunately, it has been found in Kentucky bluegrass (Valk *et al*., 1989) and Sea Dallisgrass (Liang *et al*., 2008) that using immature inflorescences as explants was of great advantage because of higher frequencies of callus induction and plant regeneration. Up till now, there have been no systematic trial tests to develop a highly efficient plant regeneration from inflorescence-derived callus induction in *E. ophiuroides*. Therefore, the main purpose of the current study was to develop a highly efficient regeneration system from immature inflorescences of centipedegrass by testing the effects of different genotypes of inflorescences and medium composition on somatic embryogenesis and plantlet regeneration. The establishment of such efficient regeneration system will undoubtedly contribute to carrying out the further research on somaclonal variation and genetic transformation of centipedegrass, which serve as the basis for genetic improvement of this species.

Materials and Methods

Plant Materials and Culture Media

Two phenotypically distinct accessions of centipedegrass (*Eremochloa ophiuroides* [Munro] Hack.), E092-1 with green stolons and spikes and E022 with purple stolons and spikes were employed in the study. These two accessions are widely used in practical landscaping, water and soil conservation at present. The immature inflorescences were collected from centipedegrass accessions E092-1 and E022 during early heading stage. All plants were preserved and propagated in soils under natural conditions at the nursery garden of turfgrass in Nanjing Botanical Garden Mem. Sun Yat-Sen. and the Hushu experimental base in Jiangning District of Nanjing city, China.

The basal medium consisted of Murashige and Skoog (Murashige and Skoog, 1962) salts containing 3.0% sucrose (*w/v*) and different plant growth regulators (PGRs). The 0.8% (*w/v*) agar (Hirono Company, Japan) was used as curing agent in the medium for all cultures except for special instructions. The pH of the medium was adjusted to 5.8 before autoclaving. Then the medium sterilized at 121°C for 20 min.

Preparation of Explants

The immature inflorescences were soaked in 10.0% H_2O_2 solution for 5 min, followed by flushing with aseptic water 5 times. The inflorescences were then transferred to 70% (*v/v*) ethanol solution for sterilization about 30–45 s, and immediately rinsed by aseptic water for 3–5 times. The spikelets were finally excised and cut into ~1.0 cm length segments with scissors.

Induction of Callus

Inflorescence explants were inoculated on induction medium of MS salts supplemented with a range of 2,4-dichlorophenoxyacetic acid (2,4-D; 2, 4 and 6 mg/L) and 0.2 mg/L benzyladenine (BAP) for callus induction (Fig. 1A-I and 1B-I). The yellow granular calli were chosen from the initial primary calli and then transferred to subculture media, which consisted of MS medium supplemented with 2.0 mg/L 2,4-D and 0.1 mg/L BAP, and solidified by 1.4% (*w/v*) agar. The callus induction and subsequent subculture were implemented at 25±1°C in a culture chamberin darkness. The frequency of callus induction was calculated as the percentage of explants produced calli after a period of ~4 weeks calli culture. The callus subculture was carried out at a 4-week interval of on the subculture medium.

Shoot Organogenesis from Callus

Embryogenic calli of 0.5–1.0 cm in diameter were further subcultured to the fresh differentiation medium composed of MS basal medium supplemented with a combination of Kinetin (KT, 2.0 mg/L) or N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU, 2.0 mg/L) or BAP (2.0 mg/L) with Naphthalene acetic acid (NAA, 0.1 mg/L), and maintained in the culture chamber at 25±1°C under a photoperiod of 12 h dark and 12 h light (100 μmol/m·s illumination) for shoot organogenesis. After 3 weeks of culture on differentiation media, the numbers of shoots originated from calli were counted according to Liu *et al.* (2008). The rate of plantlet regeneration was calculated as a percentage of the number of regenerating calli divided by the total number of the inoculated calli. All the regenerating calli were subcultured for shoot proliferation and elongation. After attaining the height of ~5.0 cm, regenerated plantlets were transferred to media for root induction.

Rooting in Vitro and Hardening of Plantlet

Regenerated shoots were separated from calli and transferred onto MS media containing 0.5 mg/L NAA for rooting induction. Culture condition of plantlet rooting was the same as shoot organogenesis. The rooting percentage was documented after 2 to 3 weeks. Well-rooted plantlets were subjected for acclimatization in plastic pots (13.0 cm top diameter and 11.0 cm depth) filled with 1:3 (*v/v*)

perlite/peat and maintained in the culture chamber for 7 days. Plantlets were finally transferred to a greenhouse with automatic temperature control system at 28～35°C under natural light conditions. The plant survival rate was recorded after 4–5 weeks.

Experimental Design and Statistical Analysis

All experiments in tissue culture were performed in a completely randomized design. Each test comprised 100–105 explants and repeated thrice. Data pertaining to the study was recorded, statistically analyzed using Microsoft Office Excel 2010 software and the Statistical Package SPSS version 10.0 (Chicago, USA), and presented as mean± standard error (SE) of the three replicates.

Results

Influence of 2,4-D and BAP on Callus Induction of two Accessions of Centipedegrass

Using immature inflorescence segments of two distinct centipedegrass accessions as explants, callogenesis was studied on MS basal medium containing a combination of BAP and 2,4-D at different concentrations. Calluses formation was started after one week and similar effect of PGRs was observed on the induction of callus in two centipedegrass accessions. The induction rate achieved its highest level with 4.0 mg/L 2,4-D and 0.1 mg/L BAP, and the induction rate reached to 53.4% and 80.0% in E092-1 and E022, respectively (Table 1). Most of calli produced in both two accessions were embryogenic calli which showed a yellow and opaque colour, compact and granular texture (Fig. 1A-II and 1B-II). Rate of embryogenic calli induction ranged from 74.4 to 94.9% in E092-1 and from 87.9 to 95.7% in E022 (Table 1). It is obvious that there was significant difference in the frequency of callus induction between E092-1 and E022. The induction rate of total calli in E022 was much higher than that in E092-1; however, a considerable percentage of embryogenic calli induction was observed for the two accessions. After 4 weeks, the compact calli, consisting of yellow spherical cellular clumps were excised from the inflorescence explants and transferred to subculture media for further proliferation. All of the embryogenic calli continued to growth, but quite a few embryogenic calli gradually changed to white or light yellow in colour, watery and soft in nature and loose in structure with the extension of subculture time. This situation got even worse after successive subcultures, with a more easily occurrence in E022 than in E092-1. In view of this issue, an eminent subculture media was obtained through changing the composition of subculture media. The optimum subculture system for both E092-1 and E022 was MS media containing 2.0 mg/L 2,4-D and 0.1 mg/L BAP, and an agar content of 1.4% (*w/v*) as well (Fig. 1A-III and1B-III).

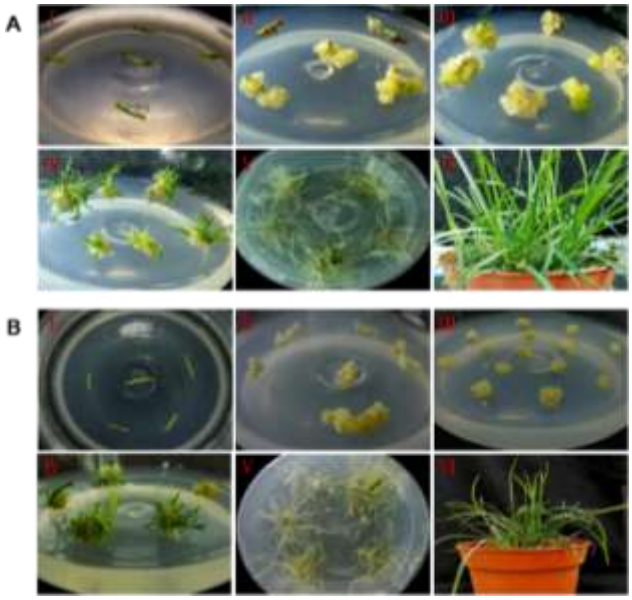

Fig. 1: Callus induction and plant regeneration from immature inflorescence of *E. ophiuroides* accessions E092-1 (A) and E022 (B). (Ⅰ) Inoculation of immature inflorescence explants; (Ⅱ) Calli induction and growth; (Ⅲ) Calli subculture; (Ⅳ) Shoot regeneration and growth from calli; (Ⅴ) Plantlets rooting; (Ⅵ) Transplanting survival plants

Effect of Different PGRs Combinations on Shoot Organogenesis from Calli

Shoots occurrence in callus were induced from both accessions of centipedegrass embryogenic calli after they were transferred to regeneration media supplemented with three different PGRs combinations of KT and NAA, CPPU and NAA, BAP and NAA (Table 2; Fig. 1A-IV and 1B-IV). Only after 3 to 5 days culture, buds firstly developed from both E092-1 and E022 calli induced by PGRs combination of KT and NAA. After 4-week culture on regeneration media, buds formed and proliferated for both accessions-derived calli cultured on all combined PGRs media. The number of shoot buds per callus ranged from 5.7 to 8.0 for E092-1 and from 5.0 to 8.0 for E022. Shoot length varied from 0.8 to 2.0 cm for E092-1 and from 1.3 to 1.9 cm for E022, while the rate of shoot differentiation was in the range of 93.7–100% for E092-1 and 63.3–83.3% for E022. The maximum shoot differentiation rate, as well as the highest number of shoot buds and the longest shoot length, was observed for E092-1 calli induced by combination of KT and NAA, while the highest differentiation rate and the greatest number of shoot buds occurred on E022 calli with the induction of BAP in combination with NAA, even if the longest shoot length was detected for E022 under induction of KT combined with NAA (Table 2 and Fig. 2A).

Table 1: Effects of combined 2,4-D and BAP on callus induction in two distinct accessions of centipedegrass

Accession number	PGRs		Rate of total calluses induction (%)*	Rate of embryogenic callus induction (%)**
	2,4-D (mg/L)	BAP (mg/L)		
E092-1	2.0	0.1	27.9 ± 1.08 c	83.8 ± 2.65 ab
	4.0	0.1	53.4 ± 0.72 a	94.9 ± 2.57 a
	6.0	0.1	36.2 ± 1.16 b	74.4 ± 6.78 b
E022	2.0	0.1	77.7 ± 0.90 a	88.8 ± 1.98 b
	4.0	0.1	80.0 ± 0.82 a	95.7 ± 1.45 a
	6.0	0.1	61.1 ± 2.04 b	87.9 ± 3.98 b

* Total number of calli produced/total number of explants inoculated; ** Total number of embryogenic calli/total number of calli produced; The mean values followed by different letters are significantly different at the 5% level

Table 2: Effects of PGRs combination on shoot differentiation from regenerated calli

Accession number	PGRs (mg/L)		Rate of shoot differentiation (%)*	Number of shoot buds formed	Shoot length of regenerated calli (cm)
E092-1	KT (2.0)	NAA (0.1)	100.0 a	8.0 ± 1.15 a	2.0 ± 0.09 a
	CPPU (2.0)	NAA (0.1)	93.7 ± 0.03 b	6.3 ± 1.33 b	1.4 ± 0.12 b
	BAP (2.0)	NAA (0.1)	96.6 ± 0.03 b	5.7 ± 0.33 b	0.8 ± 0.05 c
E022	KT (2.0)	NAA (0.1)	63.3 ± 0.09 b	5.0 ± 0.58 c	1.9 ± 0.12 a
	CPPU (2.0)	NAA (0.1)	60.0 ± 0.06 b	6.7 ± 1.20 b	1.4 ± 0.14 b
	BAP (2.0)	NAA (0.1)	83.3 ± 0.08 a	8.0 ± 1.53 a	1.3 ± 0.15 b

* Total number of calli with green shoot/total number of calli inoculated on regeneration medium; the mean values followed by different letters are significantly different at the 5% level

The differences of shoot differentiation responding to the PGRs combinations became more obvious with the prolongation of growth of subcultured shoots for both accessions (Fig. 2B). Further comparative test of cytokine in concentrations demonstrated that 2.0 mg/L KT plus 0.1 mg/LNAA was the optimum concentration for E092-1 shoot differentiation and development, while 2.0 mg/L BAP combined with 0.1 mg/LNAA was the optimum for E022 (Table 3 and Fig. 3).

Rooting *in Vitro* and Transplanting Plantlets of Two Distinct Accessions

Shoots derived from the calli of 4-week differentiation culture were transferred to MS basal media containing 0.5 mg/L NAA for rooting induction. All of the regenerated shoots could be induced to form roots on the rooting media, but there were marked differences in root development among shoots from different differentiation culture (Table 4). At the end of the 3-weekculture, the maximum number of 7.9 roots per shoot bunch was observed in E092-1 rooted shoots derived from KT induction, of 7.3 roots per shoot bunch in E022 from KT induction. The roots developed to 1.8–2.3 cm in length and 2.6–2.7 mm in diameter for E092-1, which is comparable to that of 1.4–2.0 cm in length and 2.6–2.8 mm in diameter for E022. Based on rooting number and morphological characteristics of roots, the optimum root development was observed in both E092-1 and E022 shoots derived from KT plus NAA induction (Table 4; Fig. 1A-V and 1B-V).

After 4 weeks culture in the rooting phase, plantlets with healthy shoots and well-developed roots were transplanted into plastic pots filled with 1:3 (*v/v*) perlite/peat and could be acclimated in a lighted culture chamber by 1 week. Then all hardened plants were finally transferred to a glasshouse and grew normally at 28～35°C temperature under natural light conditions (Fig. 1A-VI and 1B-VI). The survival rate of transplanting plants reached to 100% for both E092-1 and E022.

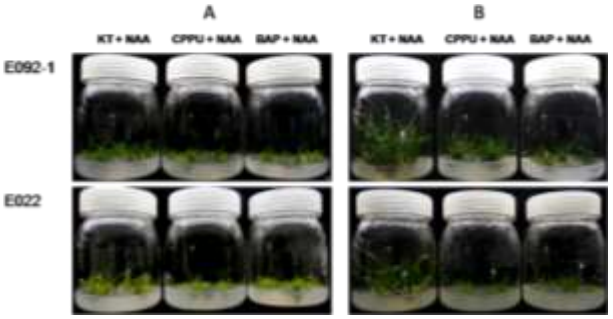

Fig. 2: Effect of three different combinations of PGRs on shoot organogenesis from calli of *E. ophiuroides* accessions E092-1 and E022. The left set (A) shows shoot organogenesis of E092-1 (upper) and E022 (lower) after 4-week treatments of three different PGRs combinations, and the right part (B) displays shoot organogenesis of E092-1 (upper) and E022 (lower) after 8-week same treatments of the PGRs combinations. BAP, N6-benzyl adenine; KT, Kinetin; CPPU, N-(2-chloro-4-pyridyl)-N'-phenylurea; NAA, *a*-Naphthalene acetic acid

Discussion

This study revealed the regeneration potential of *E. ophiuroides* genotypes using immature inflorescence as explants. A high efficient and reproducible plant regeneration system was established for centipedegrass in the present investigation, which was based on culturing callus induced from immature inflorescence.

Table 3: Screening of optimum cytokinin concentration for shoot differentiation from regenerated calli

Accession number	PGRs (mg/L)		Rate of shoot differentiation (%)	Number of shoot buds formed per callus	Shoot length of regenerated calli (cm)
E092-1	KT (1.0)	NAA (0.1)	94.7 ± 2.6 a	4.7 ± 0.89 b	1.61 ± 0.20 b
	KT (2.0)	NAA (0.1)	100.0 a	8.3 ± 0.67 a	2.18 ± 0.11 a
	KT (4.0)	NAA (0.1)	100.0 a	7.7 ± 1.20 ab	1.99 ± 0.12 ab
	KT (6.0)	NAA (0.1)	95.7 ± 1.9 a	6.0 ± 1.15 ab	1.56 ± 0.05 b
E022	BAP (1.0)	NAA (0.1)	81.1 ± 1.1 ab	5.6 ± 0.33 ab	1.09 ± 0.04 b
	BAP (2.0)	NAA (0.1)	87.8 ± 6.2 a	8.3 ± 0.89 a	1.40 ± 0.03 a
	BAP (4.0)	NAA (0.1)	82.2 ± 1.1 ab	6.7 ± 1.20 ab	1.18 ± 0.06 b
	BAP (6.0)	NAA (0.1)	72.7 ± 4.0 b	5.0 ± 0.58 b	0.90 ± 0.04 c

The mean values followed by different letters are significantly different at the 5% level

Table 4: Comparison of plantlet rooting among the shoots derived from different PGRs induction in two distinct centipedegrass accessions

Accession number	Shoots derived from	No. of root	Root length (cm)	Root diameter (mm)	Rate of rooting (100%)
E092-1	KT	7.9 ± 0.17 a	2.3 ± 0.2 a	2.7 ± 0.09 a	100 a
	CPPU	6.7 ± 0.19 b	2.0 ± 0.12 ab	2.7 ± 0.06 a	100 a
	BAP	5.6 ± 0.15 c	1.8 ± 0.11 b	2.6 ± 0.07 a	100 a
E022	KT	7.3 ± 0.14 a	2.0 ± 0.08 a	2.8 ± 0.12 a	100 a
	CPPU	7.0 ± 0.15 ab	1.9 ± 0.03 a	2.7 ± 0.06 a	100 a
	BAP	6.6 ± 0.19 b	1.4 ± 0.05 b	2.6 ± 0.09 a	100 a

The mean values followed by different letters are significantly different at the 5% level

The genotypes of explants and culture conditions showed significant effect on the induction of calli induction and the regeneration of plantlets. The ultimate objective of this study was to establish the optimum culture conditions for the formation and development of embryogenic callus induced from immature inflorescence of *E. ophiuroides*, subsequently plantlet regeneration, *in vitro* rooting and *ex vitro* acclimatization of plantlets.

During the *in vitro* culture of immature embryos which often showed higher efficiency of callus induction and plant regeneration than that by mature embryos, immature inflorescences are frequently used as explants. In this study, the immature inflorescences of both E092-1 and E022 accessions could successfully produce embryogenic calli with a high frequency, which was much higher than previous reports in centipedegrass using other types of explants (Ma *et al*., 2004; Liu *et al*., 2008; Barampuram *et al*., 2009; Yuan *et al*., 2010), and a significant difference was found on callus induction rate between genotypes. This demonstrated that callus induction from the immature inflorescence in centipedegrass might be genotype dependent, which was also found in other turfgrass species (Salehi and Khoshkhui, 2005) and other grass plants (Şener *et al*., 2008; Vendruscolo *et al*., 2008; Głowacka *et al*., 2010; Zhao *et al*., 2016).

Frequency of callus induction often varied considerably depending on PGRs (Praveena and Giri, 2012). The PGRs plays a decisive role to the induction and development of callus. 2,4-D was frequently used as a PGRs during the progress of callus induction and subculture in grass species (Li *et al*., 2006). The combination of 2,4-D with cytokinin often performed better for higher degree of callus induction, and 2,4-D in combination with BAP was reported to promote somatic embryo formation in many

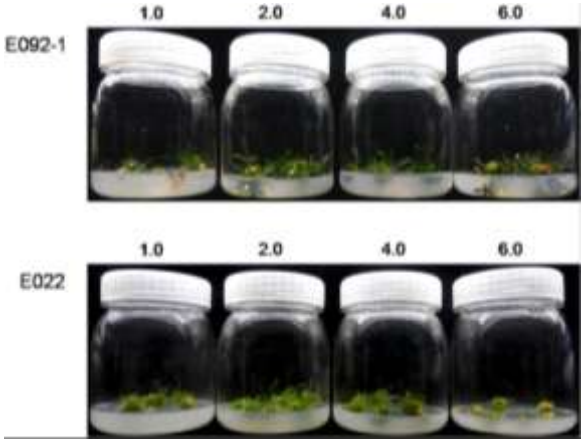

Fig. 3: Effect of different concentrations of cytokinin on shoot organogenesis from calli of *E. ophiuroides* accessions E092-1 and E022. The upper set shows the treatment of different KT concentrations in E092-1, and the lower part displays the treatment of different BAP concentrations in E022. The numerical value on each picture represents the concentrations (mg/L) of the cytokinin used

grass plants (Griffin and Dibble, 1995; Chaudhury and Qu, 2000; Wang *et al*., 2003; Li *et al*., 2006; Liu *et al*., 2008). Inclusion of lower concentration of BAP could increase the induction percentage of calli derived from the matured seed or shoot base explants of centipedegrass (Liu *et al*., 2008; Barampuram *et al*., 2009). So under the premise of low content of BAP (0.2 mg/L), the high rate of callus induction, as well as the high proportion of embryogenic callus formation, was obtained in this study by using the media containing 4.0 mg/L 2,4-D. This optimal level of

2,4-D is basically in accord with the results of callus induction using seeds as explants in centipedegrass (Liu *et al*., 2008; Barampuram *et al*., 2009), and is also consistent with the reports for callus induction with immature inflorescence explants in other grass species (Dahleen and Bregitzer, 2002; Głowacka *et al*., 2010; Yu *et al*., 2015).

Selection of optimum culture media is another important aspect for callus induction and plantlet regeneration. Many studies have indicated that the MS basal media supplemented with auxin and cytokinin was one of the best medium for callus induction and plant regeneration in main warm-season turf grasses, such as bermuda grass (Chaudhury and Qu, 2000; Jain *et al*., 2005; Zhang *et al*., 2007), zoysia grass (Lee *et al*., 2004; Wang *et al*., 2010; Chai *et al*., 2011), and centipedegrass (Ma *et al*., 2004; Liu *et al*., 2008; Barampuram *et al*., 2009; Yuan *et al*., 2010) etc. So the present study also adopted the MS-based media and could achieve high efficiency of callus induction rate and plant regeneration.

In this study, all of the embryogenic calli produced in the induction phase could keep proliferating when they were transferred onto the subculture media or proliferation medium with the same medium composition as callus induction media. However, a considerable part of embryogenic calli became non-embryogenic calli in shape and appearance during subculture phase, and this issue was more prominent, while performing subcultures of multiple generations, with E022 having more high incidence rate than E092-1, which demonstrated that the maintenance of embryogenic competence is genotype dependent, too. This problem was easy to occur during proliferation, long-term subculture or maintenance of embryogenic calli, and was also met in the previous investigations of centipedegrass (Liu *et al*., 2008; Barampuram *et al*., 2009; Yuan *et al*., 2010) and other turfgrasses (Chai *et al*., 1998; Zhang *et al*., 2007). Some studies have suggested that embryogenic callus formation of grass species can be regulated by altering the concentration of hormone and osmotic pressure of the media (Yamakata *et al*., 1993; Lu *et al*., 2011). Through reducing the concentration level of PGRs and enhancing osmotic pressure of subculture media by increasing agar content [1.4% (*w/v*)], the embryogenic calli of both E092-1 and E022 could keep normal proliferation capacity and constant embryogenic competence even after many times of subculture in this study.

In general, plant regeneration is more challenging than callus induction in grass plants. In the present study, high frequencies of shoot differentiation and development were achieved for both centipedegrass accessions of E092-1 and E022 embryogenic calli cultured on the differentiation media supplemented with three different PGRs combination. The shoot differentiation and development of different genotypes showed different preference to cytokinin, with highest differentiation performance of shoot in E092-1 induced by KT and in E022 induced by BAP. The optimum PGRs concentration for shoot differentiation was 2.0 mg/L KT or BAP in combination with 0.1 mg/L NAA in this trial, which was completely consistent with the results using seeds as explants in centipedegrass (Liu *et al*., 2008; Yuan *et al*., 2010). Although all regenerated plantlets rooted successfully after transferring onto the media containing 0.5 mg/L NAA, the number, the length and diameter of roots generated from each shoots were found to vary depending on PGRs combinations in the previous differentiation medium on which the shoots were differentiated from the inoculated calli, and were also found to be related to genotypes. This situation can be explained by the carryover effect of cytokinin into the rooting phase, which occurs universally during establishment of plant regeneration system, and the intensity of the carryover effect is different between genotypes.

Conclusion

In this research, a highly efficient, reproducible plant regeneration system was developed based on immature inflorescence-derived callus induction of two phenotypically distinct accessions of *E. ophiuroides*. For this system, the rate of callus induction and the occurrence of embryogenic calli, the rates of shoot regeneration from callus, rooting and transplanting survival were higher than ever reported using seed and young shoot base explants. This work effectively solved the problem of low percentage of embryogenic calli formed in callus induction phase, and obtained the optimum media composition for the proliferation and maintenance of embryogenic calli during calli subculture, and also improved the regeneration frequency of plantlets in centipedegrass. The newly established regeneration system will be helpful for the further study of somaclonal variation and genetic transformation of centipedegrass.

Acknowledgements

This work was funded by Scientific Facility Project of Jiangsu Province (Grant No. BM2015019-1) and was partly supported by Natural Science Foundation of Jiangsu Province (Grant No. BK20171327).

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(Received 29 June 2017 Accepted 14 October2017)