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EFFECT OF GENOTYPE AND INFLORESCENCE DEVELOPMENTAL STAGE ON CALLUS INDUCTION AND PLANT REGENERATION OF *MISCANTHUS LUTARIORIPARIU*, A NON-FOOD BIOMASS CROP

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Abstract

Miscanthus lutarioriparius, is a perennial C4 weeds. In this study, tissue culture responses were evaluated in four genotyoes of *M. lutarioriparius* which collected from the Yangtze River basin. A total of four types of callus with different characteristics and induction frequencies were induced from four genotypes immature inflorescences on medium Murashige and Skoog (MS) containing 4.0 mg/L 2,4-dichlorophenoxyacetic acid (2, 4-D). Only ML04 generated the embryogenic callus. According to the length of immature inflorescences, they divided into three inflorescences developmental stages which generated different induction percentages of embryogenic callus. Developmental stage B with 50-100 mm length inflorescence generated the highest induction percentages of embryogenic callus. The regeneration rate was 65.56% (4.0 mg/L kinetin), 91.50% (5.0 mg/L KT) and 26.67% (6.0 mg/L KT) from embryogenic callus, respectively. The shoots were transferred onto the Murashige and Skoog (MS) basal medium for rooting, the rooting rate was 100%.

Key words: *Micanthus lutarioriparius*, Inflorescences developmental stage, Embryogenic callus, Plant regeneration.

Introduction

Miscanthus lutarioriparius is a perennial C4 weeds. *M. lutarioriparius* was also used as feed and papermaking in the past. *M. lutarioriparius* was first discovered by Dr Liu Liang and originated in the Yangtze River basin of China (Liu, 1990). Liu & Yu (2009) estimate an average yields for *M. lutarioriparius* of 10-22.5 t ha^{-1}. *M. lutarioriparius* grows in the flood areas, this makes it prevent flooding and protect environment. *Miscanthus* is also considered to be a promising candidate for second-generation energy crops (Clifton-Brownet *et al*., 2007; Karp & Shield, 2008; Oliver *et al*., 2009). *M. lutarioriparius* also had been identified as having strong potential as a biomass crop, adapted to a wide range of growing conditions and resulted in more efficient biomass conversion, will further improved its suitability as a biomass crop (Jakob, 2009). *M. lutarioriparius* is especially suitable for growing in the temperate regions of the world, with low-nutrient requirements, rich-cellulosic production and exceptionally cold tolerant (Yi *et al*., 2001; Naidu *et al*., 2003; Heaton *et al*., 2004; Wang *et al*., 2008; Yan *et al*., 2012). These excellent characteristics make *M. lutarioriparius* a useful landscaping and bio-energy feedstock. New agronomic techniques and new genotypes with improved characteristics are being developed and screened over the wide range of ecological conditions of *Miscanthus*× *giganteus* for large-scale production in Europe (Chou, 2009; Lewandowski *et al*., 2000). To fulfill the requirement of large-scale production, and establishment in the field, the researchers utilized rhizome propagation or *In vitro* propagation by axillary shoots. However, these two propagation methods can't meet the large demands in production. Development of an efficient tissue culture system could provide disease-free plants and be useful for breeding purpose.

The plant regeneration system had been developed in *M.* × *giganteus* (Nielsen *et al*., 1993; Nielsen *et al*., 1995; Holme & Petersen 1996; Zhao *et al*., 2013). Shoot apices, leaf, immature inflorescence and root sections have been used as explants in *M.* × *giganteus* (Holme & Petersen 1996) for callus induction, embryogenic callus derived from immature inflorescence had the highest regeneration capacity. In *Miscanthus*, calli have been induced from shoot apices, leaf, immature inflorescence (Petersen *et al*., 2002), also mature seeds as explants have been induced callus (Zhang *et al*., 2011) and achieved in bombardment-mediated transformation by Wang *et al*. (2011). In many species of *Poaceae*, immature inflorescence produced embryogenic callus which had a higher regeneration frequency and grown better in maintenance medium (Tabaeizadeh *et al*., 1990).

The main aim of the present investigation was to find the influence of the genotypes and inflorescences developmental stage on embryogenic callus induction, and the effect of KT concentration on shoot regeneration, in order to develop an efficient technique for plant regeneration of *M. lutarioripariu* to fulfill a lot of demand for landscaping.

Methods and Materials

Plant materials: Four typical genotypes of *M. lutarioripariu* were collected from four provinces along Yangtze River flood area, including Jiangsu (ML01), Anhui (ML02), Hubei (ML03) and Hunan (ML04) Province. Their rhizome were collected and transplanted in a germ-plasm nursery at Wuhan University, China. The locations and characters of four genotypes of plants were shown in Table 1.

Table 1. Callus induction frequency and character from 50-100 mm length inflorescence of four genotypes on 4.0 mg/L 2, 4-D medium.

Genotypes	Latitude (N)	Longitude (E)	The characteristic of callus	The induction frequency (%)
ML01	32°15.903'	119°56.443'	Yellow/Water stain/soft	60 b
ML02	31°15.863'	118°19.341'	Yellow/Granular/Compact	15 c
ML03	30°23.913'	113°01.310'	Canary yellow/Granular/Friable	95 a
ML04	29°04.486'	112°19.117'	White/Granular/Compact and Yellow/Water stain/soft	95 a

Mean values marked with the same letter are not significantly different at $p<0.05$

Callus induction: In the first experiment, immature inflorescence tissues, approximately 50–100 mm in length, were surface-sterilized by washing with 75% ethyl alcohol for 30 s, 0.1% (w/v) mercuric chloride ($HgCl_2$) for 2 min, and then rinsed thoroughly (at least three times) with sterile distilled water. The sterilized explants were then placed on callus induction medium containing Murashige and Skoog (MS) basal salts (Murashige and Skoog 1962) supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D; 4.0 mg/L), 750 mg/L $MgCl_2 \cdot 6H_2O$, 2.88 g/L proline, 30 g/L sucrose. All media were supplemented with 3 g/L Phytagel™ agar (sigma), and adjusted to pH 5.6 prior to autoclaving. Two immature inflorescences of every genotype were cut off about one centimeter, ten subsections immature inflorescences were placed on each bottle for induction. Cultures were incubated in the dark at 27±2°C and sub-cultured at 3-week intervals with the same medium as used for callus induction. Six weeks after culture initiation, the total number of callus induced was recorded to calculate callus induction percentage. The different callus types were separated by their visual appearance , characteristics and number of each callus from different locations were recorded.

In the second experiment, according to the length of immature inflorescence tissues, the inflorescences of ML04 were divided into three stages, including A stage (<50 mm), B stage (50-100 mm) and C stage (100-200mm). The explants of three stages were all shown in Fig. 2a. The first two inflorescences on the left were at the A developmental stage, the middle two inflorescences were at the B developmental stage and the last one on the right was at C developmental stage (Fig. 2a). The surface-sterilized method, culture method and the calculation method of callus induction percentage were same to the first experiment. But the two types calli induced from the inflorescences of ML04 were calculated respectively.

Plant regeneration and rooting: Four weeks later, the four major types callus including yellow water stain soft callus, yellow friable callus, yellow compact callus, and white compact callus (embryogenic callus) induced from immature inflorescence tissues were transferred onto regeneration media containing Murashige and Skoog (MS) basal salts supplemented with kinetin ((KT); 4.0, 5.0 and 6.0 mg/L) (Sigma), 750 mg/L $MgCl_2 \cdot 6H_2O$, 2.88 g/L proline, 30 g/L sucrose. All media were supplemented with 3 g/L Phytagel™ agar (Sigma), and adjusted to pH 5.6 prior to autoclaving. Thirty pieces callus were placed on each callus regeneration media, with three replicates per treatment. Six weeks later, in vitro shoots regenerated from calli were transferred to MS basal medium without supplementation to induce roots. Cultures were incubated in the light at 27±2°C. Growth conditions were 16 h of cool-white fluorescent light (40 $\mu molm^{-2}s^{-1}$ of photosynthetically active radiation) and 8 h dark growth at 27±2°C. After four week, the shoots were transferred to rooting medium. The regeneration frequency was calculated from the number of regenerated shoots / the total number of callus pieces plated for differentiation. The plantlets were generated one week later, then transferred to outside according to the method of Zhao (Zhao *et al*., 2013).

The calculation of the least square means and analysis of variance (ANOVA) as well as general linear model procedures were performed by SPSS (Statistical Product and Service Solutions) software.

Results and Discussion

Callus induction: The results showed that the genotypes of immature inflorescences were the critical factors which determining the efficiency and the characteristics of callus. The calli were effectively induced in all of the tested genotypes on callus induction medium containing 4.0mg/L 2, 4-D. Callus appeared two weeks after culture initiation and during the following weeks different callus types were formed, and tested 4 genotypes generated calli with different frequencies and characteristic (Table 1). Four weeks after culture initiation four types of callus could be identified, three types callus classification were same as described by Petersen (1997), Holme & Petersen (1996) and Kim (2010) for *M.* × *giganteus* callus evaluation. The calli induced by ML01 was yellow, water stain, friable and soft characteristics (Fig. 1a), and the induction frequency was 60%. The yellow, granular and compact type calli was induced by ML02 and the induction frequency was 15% (Fig. 1b). The calli induced by accessions ML03 showed canary yellow, granular and friable characteristics, the induction frequency was 95%, (Fig. 1c). Only ML04 generated two types of calli, one is the embryogenic callus with white, granular and compact characteristic and the other callus with yellow water stain characteristic, the total induction frequency was 95% (Fig. 1d). The highest 95% of callus induction rate was from inflorescence B stage and the embryogenic callus ratio of total callus that induced from three inflorescence developmental stage including A stage(<50 mm), B stage (50-100 mm) and C stage (100-200mm) were 45.5%, 74.8% and 49.7% respectively (Table 2). The calli induced from three inflorescence developmental stage were shown in Fig 2b, 2c and 2d. Therefore B stage with 50-100 mm inflorescence length was the best materials for the

embryogenic callus induction because of their high callus induction rate and high percentage of embryogenic callus, which had a high level regeneration rate in the next step experiment. *In vitro* tissue culture is recognized as a globally profitable option for increasing the production capacity and saving germ-plasm resources of nonfood plant materials for human use. Immature inflorescences are the optimal explants tissues to induce callus, with 2, 4-D as the only plant growth regulator required for callus induction (Holme & Petersen 1996; Petersen 1997). Accessions collected in warm and temperate regions displayed high callus formation under the callus culture conditions of this study (Wang *et al*., 2011). The incubating temperature at 25-29°C is suitable for callus culture of warm season grass species (Smith *et al*., 2002). The developmental stages of inflorescences played an important role on embryogenic callus induction and plant regeneration in *M. sinensis* and *M.* × *giganteus* (Glowacka *et al*., 2010), this result was also proved and the best developmental stage of inflorescences was also found in our report. A, B and C developmental stage of inflorescences had different percentages of embryogenic callus (Fig. 2a, 2b, 2c and 2d). Genotype was the fundamental factors for the characteristics of callus, and then affected the plant regeneration efficiency of the fifteen accessions (Głowacka *et al*., 2010). Some of yellow water stain calli (Fig. 1a) transformed into root callus after subculturing three times. Our experimental data demonstrated that the genotypes of the tested materials are the critical factor.

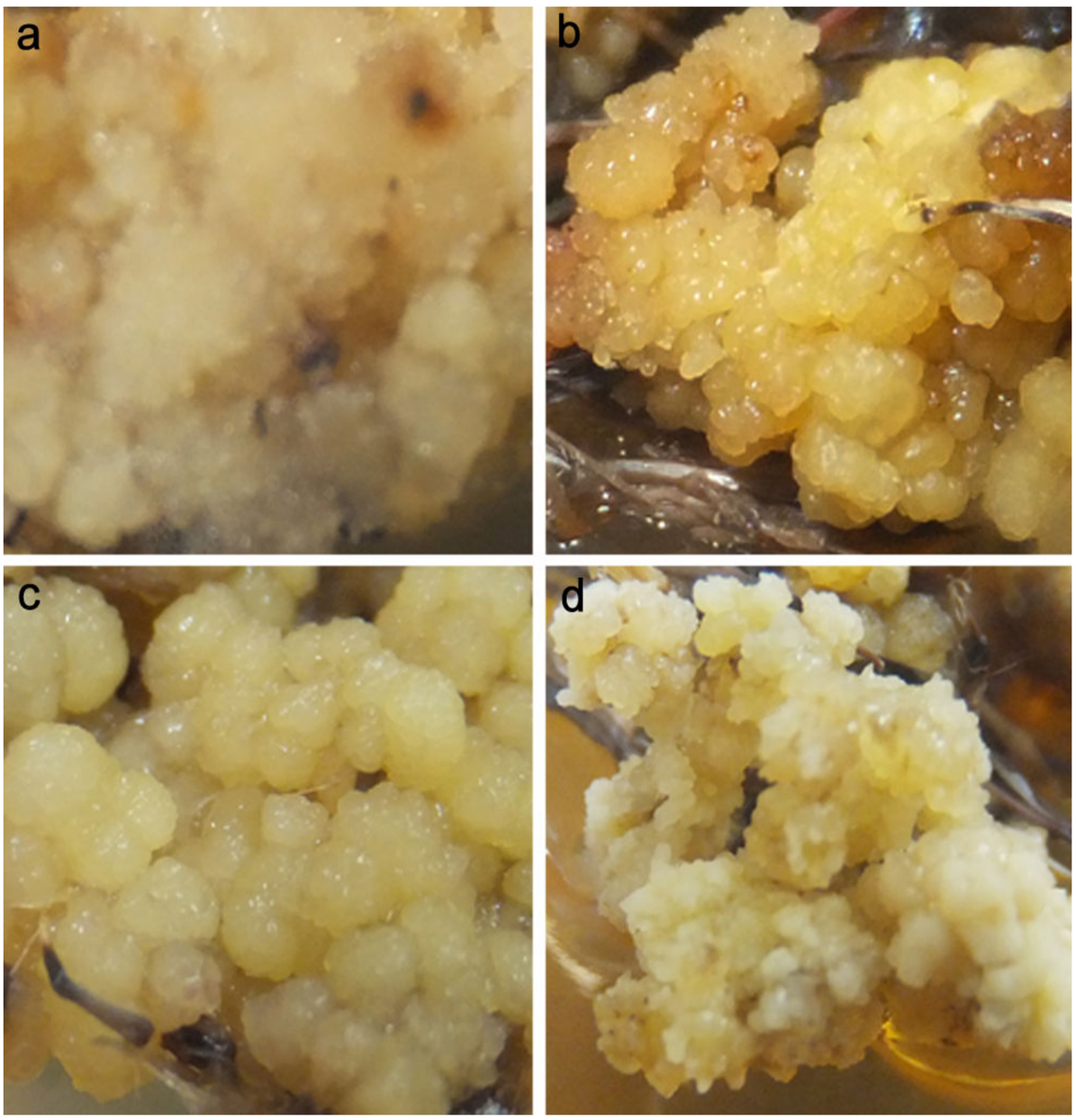

Fig 1 Four types of calli induced from immature inflorescences of 4 accessions. (a) Yellow water stain callus; (b) yellow compact callus; (c) yellow friable callus; (d) embryogenic callus with white compact characteristic.

Plant regeneration and rooting: According to their characteristic of calli induced from the immature inflorescences, four major types of calli were used to regenerate. Only embryogenic callus generated the plantlets on plant regeneration medium among four types callus. The yellow friable soft calli, yellow compact callus and yellow friable callus began to brown slowly when transferred onto regeneration medium, coarse roots started to grow out after one month. The rates for shoot regeneration of embryogenic callus were shown in Table 3. On the medium supplemented with 4.0 mg/L KT, the regeneration rate was 65.56%. When the KT concentration was increased to 5.0 mg/L, the regeneration rate decreased to 91.50%. The lowest regeneration rate was 26.67% when the KT concentration was increased to 6.0 mg/L in our study. The shoots could be divided into two types, the single and the cluster. The percentage of the two types of shoot showed in Table 3. We also tested the methods that combining NAA and BA in regeneration medium stimulates the rapid production of shoots for *M.*×*giganteus* and *M. sinensis* (Kim *et al*., 2010; Wang *et al*., 2011), but only roots initiated among all the types of calli. So we replaced with KT for differentiation. The yellow compact and friable callus was described in (Fig. 1b and 1c), although most of these calli initiated differentiation through formation of green spots on the surface, but no shoots generated at last. As a comparison, embryogenic calli (Fig. 1d) initiated differentiation through formation of green spots on the surface, a few short multiple shoots appeared later. The generation process of shoots was same as *M.* × *giganteus* (Kim *et al*., 2010). The embryogenic callus may be similar with the shoot forming calli (Holme & Petersen 1996; Petersen 1997; Glowacha *et al*., 2010; Kim *et al*., 2010). This type callus showed the greatest regeneration frequencies on the medium containing cytokinin of KT in our experiments. From the perspective of differentiation rate, 4.0 mg/L of KT concentration was the best for differentiation (Fig. 2e). Root induction achieved success on MS medium, and rooting rate was 100% for all shoots. The roots generated completely and the entire plantlets were successfully obtained after two weeks of transferring to rooting medium , two weeks later, the plantlets could be transferred to water in the test tube when they were much taller and stronger (Fig. 2f). The plantlets should be transferred into soil after three days domestication in the test tube (Fig. 2g). The whole plantlets could be transferred to soil when they were much taller and stronger and three days domestication. Two months later, the whole plants were obtained (Fig. 2h). So, *In vitro* regeneration system was established of *M. lutarioripariu*.

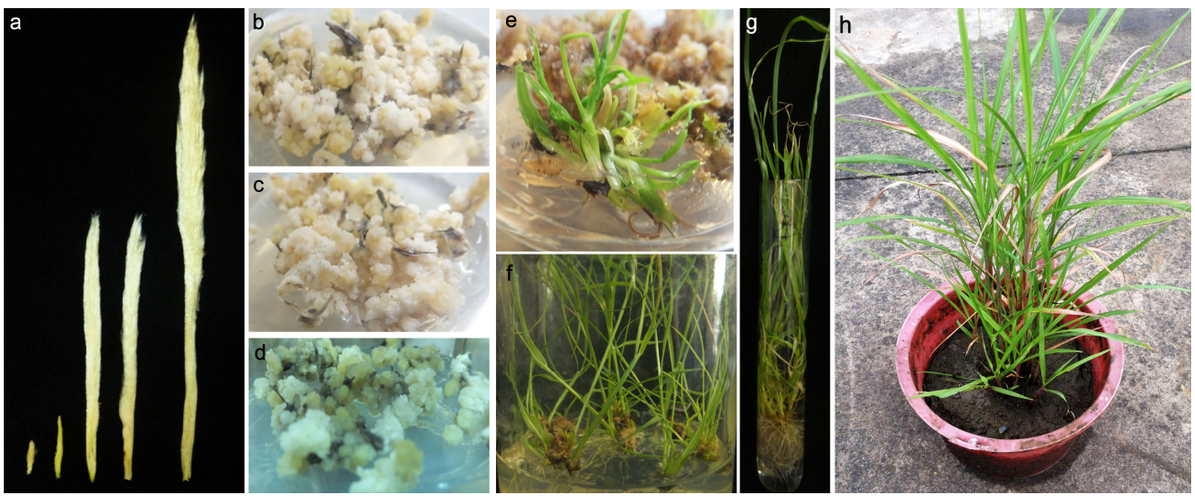

Fig 2 Embryogenic callus induction and regeneration from A, B and C developmental stages of inflorescences. (a) the first two inflorescences on the left were at the A developmental stage, the middle two inflorescences were at the B developmental stage and the last one on the right was at C developmental stage; (b) two types callus induced from inflorescences at A developmental stage; (c) two types callus induced from inflorescences at B developmental stage; (d) two types callus induced from inflorescences at C developmental stage; (e) the plantlets on the regeneration medium after two weeks; (f) the plantlets on the rooting medium after four weeks; (g) the domestication of the plantlets in the test tube; (h) the plantlets transplanting outside after two month.

Table 2. Influence of development stage of immature inflorescence on embryogenic callus induction and each type percentage of total callus.

Inflorescence length	Callus induction percentage (%)	Percentage of callus type (%)	
		Embryonic callus	Water stain soft callus
A (<50 mm)	70 b	45.5 b	54.5 a
B (50-100 mm)	95 a	74.8 a	25.2 b
C (100-200 mm)	35 c	49.7 b	50.3 a

Mean values marked with the same letter are not significantly different at $p<0.05$.

Table 3. Effect of KT concentration on embryogenic callus regeneration rate and percentage of single shoot and cluster shoots.

Test	The concentration of KT (mg/L)					
	4.0		5.0		6.0	
	Callus for regeneration	Generated shoots	Callus for regeneration	Generated shoots	Callus for regeneration	Generated shoots
1	30	21	30	19	30	6
2	30	20	30	17	30	12
3	30	18	30	18	30	7
% Shoot regeneration	65.56 b		91.50 a		27.78 c	
% Single of shoot	25.4 a		5.5 b		4.0 b	
% Cluster of shoots	74.6 b		94.5 a		96.0 a	

Mean values marked with the same letter are not significantly different $p<0.05$

Conclusion

In our study, the induction rates, the callus characteristics and plant regeneration efficiencies of the tested materials with different genotypes were displayed. Among the tested genotypes, ML04 collected from Hunan province were the optimal genotype for embryogenic callus induction and regeneration. The suitable genotype for embryogenic callus induction and the optimal type of calli for plant generation and transformation as well as the appropriate KT concentration for plant regeneration were found. The genotype was the critical factor for callus induction and the callus types affected the plant regeneration in *M. lutarioripariu*. A system for *in vitro* propagation from embryogenic callus in *M. lutarioripariu* had already been established basically, including the explants selection, induction medium and regeneration medium concentrations. Furthermore the special genotypes and callus type were found out for test. It has potential to generate starting material for landscaping and biomass production in a short period of time. The high efficiency plant regeneration system was established. Furthermore, agrobacterium-mediated transformation based on embryogenic callus was going on in our next experiments for large-scale production.

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