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Full Length Research Paper

Effects of genotypes and medium on callus induction of proso millet derived from anther culture

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Proso millet is a promising species for rich nutrition and drought tolerance. Factors influencing anther-derived callus induction efficiency were investigated when microspores of anthers taken at the late-uninucleate to early- binucleate stage were used as explants. The results indicate that callus induction is strongly affected by genotype. Several highly responsive cultivars of proso millet with desired traits have been identified that could be directly used in crop improvement by breeding. Cold pre-treatment of spikes for three weeks, followed by 32.5°C heat treatment for a short time significantly improved callus yield. Moreover, W14 basal medium and a combination of 60 g/L sucrose with 60 g/L maltose as carbon source were optimal for anther culture of proso millet in this study. To our knowledge, this is the first report on successful regeneration of plants from pollen-derived callus in proso millet for use as a source to accelerate breeding programs.

Key words: Proso millet, temperature, medium, genotype, carbohydrate.

INTRODUCTION

Proso millet (*Panicum miliaceum* L.) is a warm-season annual cereal crop that shows tolerance of drought and infertile soils (Rachie, 1975). It is usually used as a staple grain in Asia and Africa, especially when sown as an emergency grain after natural disaster because of short growing season. It has the lowest water and nutrient requirements (http://en.wikipedia.org/wiki/Proso millet) of any major grain. Moreover, the millet grains are rich in protein, minerals, vitamins, calcium, phosphorus, iron, and essential amino acids (Kalinova and Moudry, 2006). Having higher nutritional quality and digestibility but less fat, the ground millet grain is commonly used as a nourishing food for infants and as a side dish for meat, vegetable soup, dumpling, cake, pudding or porridge (Ang et al., 1999). The increasing demands for healthy nutrition have supported and increased interest in the planting and consumption of proso millet (McDonough et al., 2000). However, conventional breeding is cumbersome, laborious, time-consuming and sometimes rather inefficient in proso millet due to complicated pollination. This bottleneck may be overcome through employing a strategy which would speed up proso millet breeding process and shorten the duration to release cultivars to meet the new market demands through tissue culture generated haploid plants. The most frequently-used methods to produce haploids are embryogenic callus induction from anther culture or isolated microspore culture. Although, improved protocols of anther culture in other cereals, including barley, wheat, triticale, maize, rice, and rapeseed are available (Wędzony et al., 2009), there is still no method that can be universally recommended with proso millet (Dunwell, 2010). Lack of success is due not only to the recalcitrance of proso millet, but also to low international interest and consequently, less financial input for its

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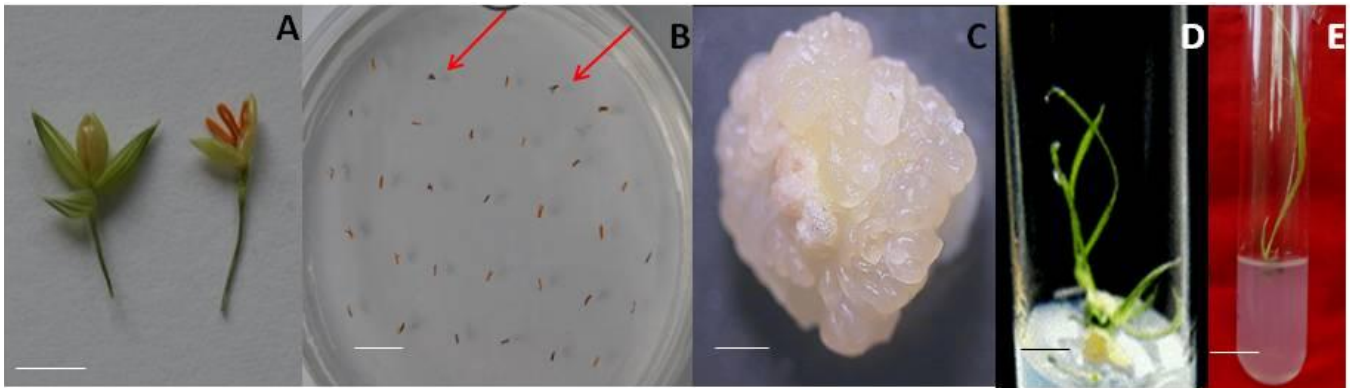

Figure 1. Callus induction from anther culture of proso millet: (A) morphological characteristics of anther (Bar: 5 mm); (B, C) calli emerging from anthers inoculated on culture medium (Bars: 10 and 1.5 mm, respectively) (D, E) anther-derived regenerated plantlets (Bar: 10 mm).

research. Development of genotype-independent *in vitro* culture methods for proso millet through the exploration or improvement of existing protocols will provide a useful breeding tool. As far as we know, this is the first report on optimization of anther culture in proso millet. The main objectives of this study were to investigate the effects of genotype, basal culture medium, carbon source and temperature stress treatment on induction efficiency in proso millet anther culture.

MATERIALS AND METHODS

Eleven commercially important cultivars were chosen to carry out diverse experiments on the basis of their grain quality and agronomic performance. Donor plants were grown in an experimental field of Hangzhou Normal University under general management practices. Spikes were collected from the donor plants in mid July to the end of August, depending on the sowing time. Determination of the optimal stage of pollen development is an essential step to increase the efficiency of anther culture. In general, anthers at the early-uninucleate to early-bicellular microspore stage are suitable as explants for callus induction of cereals (Piccirilli and Arcioni, 1991). However, routine identification of the developmental stages of pollen within the spike is very difficult. On the basis of cytological observation of squashed samples stained with aceto-carmine acid viewed under an optical microspores, we found the majority of samples for the tested genotypes in this study were at mid-late uninucleate or early-binucleate stage when the anther length was about 1.5 to 2 mm. Morphologically, this stage is characterized by the sheath of the flag leaf emerging 2 to 5 cm. However, the microspores were beyond the optimally responsive period if the anther was much over 2 mm in length and dark yellow in color (Figure 1A). Therefore, a few anthers at the upper tip of the inflorescence of donor plants were measured and then appropriate spikes were collected, wrapped with clean cotton gauze and stored at 4°C.

Sterilization and inoculation

Spikes were surface sterilized by immersion in 70% (v/v) ethanol for 1 min, followed by 8 to 10 min in 0.1% $HgCl_2$ with the addition of a few drops of Tween20, and finally rinsed three times for 5 min each with sterile distilled water. Intact anthers were carefully isolated and placed under aseptic conditions onto callus induction medium at a density of approximately 100 anthers per 90 mm diameter Petri dish with 25 to 30 ml of medium. Care was taken to avoid inoculation of anther wall or connective tissues, and if that occurred, the explants were marked and were later discarded.

Culture medium and condition

If not stated otherwise, the standard medium (W14 supplemented with 300 mg/L casein hydrolosate, 100 mg/L Myo-Inositol, 60 g/L sucrose + 60 g/L maltose, 2 mg/L 2, 4-D and 0.5 mg/L kinetin and solidified by 0.8% agar) was used. The medium was adjusted to pH 5.8 with 1 N KOH and autoclaved at 121°C for 15 min. Filter-sterilized L-glutamine (500 mg/L) was added when autoclaved medium cooled below 55°C. Each Petri dish containing approximately 100 anthers was incubated at 25 ± 1°C in darkness for 4 to 8 weeks. The calli or embryoids produced from induction experiments were transferred to a regeneration medium (N6+ 1 mg/L 6-BA + 1 mg/L KT) and incubated at a 16 h photoperiod and at 25°C.

Experimental design and data analysis

In experiment 1, six genotypes including Neimi5, Longmi7, Longmi8, Yixuanhuangmi, Yumi2, and Ningmi14 were used to study the effect of basal media on callus induction. Anthers were excised and inoculated on N6 (Chu, 1978), FHG (Hunter, 1988), W14 (Ouyang et al., 1989), MS (Murashige and Skoog, 1962) basal media supplemented with the same additions as standard medium except for using 90 g/L sucrose as carbon source. In experiment 2, W14 standard medium containing different carbon combinations (Table 3) was used to investigate the effect of carbon concentration on callus induction of 3 cultivars (Jinshu8, Longmi5 and Ningmi14). In experiments 3 and 4, the effects of genotype and temperature stress treatment on callus production were carried out, respectively. For experiment 4, freshly isolated anthers (Jinshu8, Yixuanhuangmi, Longmi8 and Ningmi14) from different cold pre-treatment durations were incubated at 25°C in the dark, and half of the Petri dishes for each cold pre-treatment were subsequently incubated at 32.5°C for 3 days (heat shock treatment) before being returned to 25°C. All experiments were completely randomized designs with three replicates for each genotype and cold pre-treatment. The number of calli was recorded after 4 to 8 weeks and

values presented are means (genotypes with contamination not analyzed). Statistical analysis was performed by analysis of variance (ANOVA) using SPSS version 18 software (SPSS Inc., Chicago, IL, USA). Difference between treatments was separated by the least significant difference (LSD) test at a probability level of 0.05 or 0.01 as indicated in individual experiments.

RESULTS AND DISCUSSION

Effect of basal medium and genotype on callus induction

The anthers inoculated on 4 basal media became brown and swollen then callus emerging from the surface of some anthers was observed after 2 to 3 weeks (Figure 1B). In all genotypes studied, more calli were produced on W14 medium than on MS medium (Table 1). The induction response on FHG and N6 media was intermediate. The highest rate of callus induction was found in the culture of Yumi2 on W14 medium (10.6 calli per 100 anthers). Statistical analysis showed that difference in the callus induction efficiency was significantly affected by medium ($P<0.01$). These data also indicated that MS medium was not ideal for anther culture of the proso millet cultivars studied. A possible basis is that only nitrate nitrogen and no ammonia nitrogen is present in W14 medium, while a large ratio of nitrate nitrogen to ammonia nitrogen is present in FHG and N6 media. This result suggests that ammonia nitrogen has an adverse affect on callus-derived anther production of proso millet. Callus induction in anther culture of barley was also higher on FHG and N6 media than MS (Castillo et al., 2000; Lazaridou et al., 2005).

Anther culture studies in other crops also commonly used basal media N6, (modified) MS, Nitsch (Nitsch J and Nitsch C, 1969) and B5 (Gamborg et al., 1968) but there are many others (Wedzony et al., 2009). The results demonstrated that specific basal medium for callus anther-derived induction should be designed for each species.

Among the factors previously reported to affect callus induction in anther culture, genotype is a major one (Chen and Dribnenki, 2002; Perrin et al., 2004; Lazaridou et al., 2005; Fu et al., 2008). Genotypic differences in anther culture response were observed among eight cultivars in this paper excluding two genotypes (Longmi8 and Niming14) because of contamination on W14 medium (Table 2). The percentage of anthers producing callus ranged from 5.1 for Yixuanhuangmi to 11.0 for Longmi5. Seven non-glutinous genetic background cultivars had significantly higher callus induction efficiency than glutinous Yixuanhuangmi. The strong genotypic effect in anther culture of proso millet was also confirmed in experiment 1 (Table 1). The highly responsive cultivars or advanced breeding lines (such as Longmi5, Longmi7) identified in this study could be used as parent to cross with agronomically desirable genotypes to produce F_1 hybrids for routine doubled haploid production. Embryogenic calli (Figure 1C) were successfully transferred to fresh differentiation medium to regenerate plantlets (Figure 1D and E).

Effect of carbohydrate on callus induction of proso millet

Anthers incubated on W14 basal medium containing 60 g/L sucrose in combination with 60 g/L maltose had the highest callus induction (9.738 calli/100 anthers). As shown in Table 3, culturing anthers on 30 g/L sucrose ± 60 g/L maltose medium dramatically reduced the percentage of anther-derived callus as compared with 60 g/L sucrose in combination with 60 g/L maltose. Callus production from the three genotypes tested (Jinshu8, Longmi5 and Ningmi14) also decreased when 90 g/L sucrose or 90 g/L maltose were separately used. The results in this paper indicate that relatively high sucrose concentration in the medium are beneficial for initiation of callus, whereas too high sucrose level is deleterious. The beneficial and adverse effects of high sucrose on callus induction have been reported previously in anther culture of some Gramineae and Cruciferae species, respectively (Keller et al., 1975; Keller and Armstrong, 1977; Dunwell and Thurling, 1985; Dunwell, 2010).

Sucrose as the osmotic and nutritional source appears to inhibit division of anther wall and promote division of pollen cells during the callus induction phase. The optimal sucrose level seen here is very different than for anther culture of previously examined species. Callus induction was greater on 90 g/L maltose medium than 90 g/L sucrose medium (Table 3). Maltose has successfully been used in anther culture of barley, wheat, triticale, rye and rice at concentrations ranging from 60 to 90 g/L (Wędzony et al., 2009). Sucrose is heat labile, so that autoclaved media contain a mixture of D-glucose and D-fructose (Powell, 1990). Fructose has been shown to be inhibitory to pollen embryogenesis in Petunia anther culture (Raquin, 1983). The use of maltose as the osmotic regulator may effectively stabilize the callus in anther culture because of slower decomposition than sucrose. Thus, the appropriate carbon source combination depends on species and genotypes, and means that additional specific protocols should be explored for anther culture of proso millet.

Effect of duration of cold pre-treatment on callus induction

The spikes of four genotypes (Jinshu8, Yixuanhuangmi, Longmi8, Ningmi14, and Figure 2) were collected from the field and stored in the dark at 4°C as described previously. The effect of cold pre-treatment of various durations (1 to 4 weeks and control with no cold) was

Table 1. The effect of basal medium on callus induction in proso millet anther culture.

Media	Longmi5	Longmi7	Longmi8	Yixuan-huangmi	Yumi2	Ningmi14	Mean
N6	8.0708	6.6166	8.0008	4.7937	8.2763	7.3783	7.4265^{B}
W14	9.1501	10.0674	10.5890	6.2552	10.5903	9.5092	9.1291^{A}
FHG	7.6555	7.3713	8.0582	4.2347	8.4907	7.0350^{A}	7.1688^{B}
MS	3.9261	3.8128	4.3832	1.9035	3.0090	4.2254	3.5432^{C}
Mean	7.200	6.9670	7.7578	4.2968	7.5916	7.0370	

Means followed by the same letter are not significantly different from each other for the same genotype at 1% level after analysis by transforming percentage data into arcsin.

Table 2. The Effect of genotype on callus induction in proso millet anther culture using W14 medium and a combination of 60 g/L sucrose with 60 g/L maltose.

Genotype	Glutinous	Calli induced frequency ±SE	1% level
Longmi7	N	10.3499±0.8211	A
Longmi5	N	11.0313±0.5321	A
Neimi5	N	9.7537±0.4979	AB
Yixuanhuangmi	Y	5.1152±0.3674	C
Yumi2	N	9.7656±1.0062	AB
Yimi5	N	9.5274±0.8104	AB
Huiningda huangmi	N	7.4252±0.6705	BC
Ningmi13	N	6.7219±0.5638	C

Means followed by the same letter are not significantly different from each other for the same genotype at the 1% level. SE: standard error. Percentage data have been transformed into arcsin for analysis.

Table 3. The effect of carbohydrate on callus induction in proso millet anther culture using W14 basal medium**.**

Carbohydrate (g L^{-1})	Longmi5	Jinshu8	Ningmi14	Mean
90 sucrose	8.6003	6.0031	6.4132	6.9964^{AB}
90 maltose	9.1826	6.5821	7.1899	7.6428^{AB}
30 sucrose / 60 maltose	5.3783	3.9962	4.6138	4.6608^{C}
60 sucrose / 30 maltose	7.0136	6.3949	5.9945	6.463B^{C}
60 sucrose / 60 maltose	11.4519	9.2286	8.6222	9.7535^{A}

Means followed by the same letter are not significantly different from each other for the same genotype.

investigated. The results showed callus induction increased over control by cold pre-treatment and significantly increased ($P<0.05$) by 3 weeks of cold pre-treatment in all tested genotypes except Yixuanhuangmi at 25°C post-incubation culture (SPSS procedure, results not shown). Tenhola-Roininen et al. (2005) reported that a cold pre-treatment (4°C) was beneficial for the induction of the anther culture response in rye, as is also well documented in other cereals (Devaux et al., 1993; Głowacka and Jeżowski, 2009; Motallebi-Azar, 2010). Cold pre-treatment for 3 to 7 weeks was most effective in anther culture of barley (Huang and Sunderland, 1982; Hou et al., 1993). Here, however, callus induction from the genotypes investigated was reduced after cold pre-treatment for 4 weeks (Figure 2). It has also been reported that a cold pre-treatment reduced the anther response in wheat (Ghaemi et al., 1995; Massiah et al., 2001; Xynias et al., 2001). These differences may be due to the species, genotypes, and various micro-conditions during culturing.

Furthermore, interactions between genotypes and cold pre-treatment were also observed for callus induction in this experiment. This was in line with previous studies (Powell, 1988; Osolnik et al., 1993). To elucidate the effect of heat to callus induction, we pre-incubated all of 4 genotypes materials at 32.5°C for 3 days (as earlier described) to observe the callus induction rate. It was found that the effect of heat to callus induction seems not to be obvious in Jinshu8, Longmi8 and Ningmi14 cultivars (Figure 2a, c and d), although, a difference of callus induction can be found in fresh Ningmi14 followed by heat treatment. But heat effect in Yixuanhuangmi cultivar

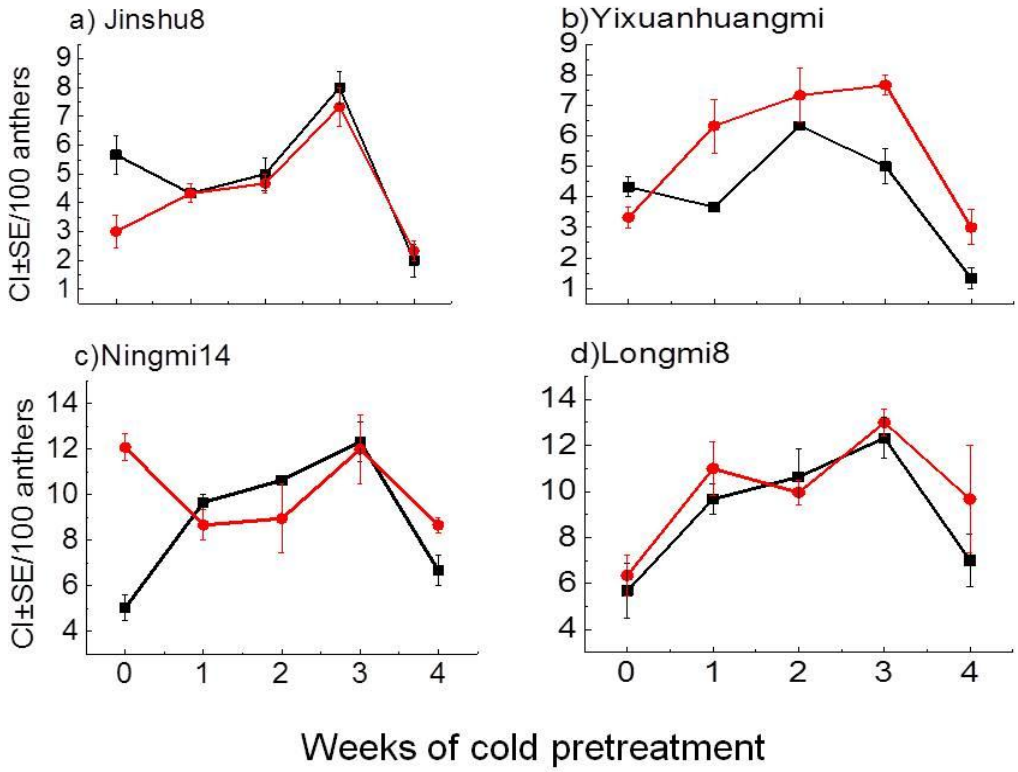

Figure 2. The effect of different durations of cold pre-treatment of spikes and heat stress post-treatment of plated anthers on callus induction. (a) Jinshu8 (b) Yixuanhuangmi (c) Ningmi14 (d) Longmi8; the symbol-■(black)-indicates general treatment (25°C dark culture) and -●(red)-indicates heat post-treatment(32.5°C for 3 days) before plating anthers. Standard error (SE) is shown as segments of lines.

to increase callus induction seemed to be significant (Figure 2b, p=0.013, ANOVA analysis). Especially in the application of 3 days heat following 3 weeks cold treatment, the difference of callus induction frequency reached a maximum (Figure 2b). Dunwell et al. (1983) reported that high temperature shock before further culture at 25°C can efficiently switch the developmental pathway but the highest callus yield was obtained at 35°C heat shock treatment for some rape cultivars (Dunwell et al., 1983). Heat post-treatment did not obviously increase callus production of Jinshu8 or Longmi8, but heat without any cold treatment greatly increased the response on Ningmi14 to a level (12%) nearly as high as any response seen. These results show the optimum temperature and duration of heat post-treatment in the anther culture of proso millet is also genotype-dependent.

Conclusion

Several factors investigated in the present study (namely, genotype of the donor material, cold pre-treatments and heat shock post-treatment, carbohydrate and media composition) had significant effects on callus induction of anther culture in proso millet. In accordance with the existing literature, our results showed that genetic background played a key role during callus induction. Genotypes without glutinous was a better response than glutinous Yixuanhuangmi in anther culture. W14 basal medium supplemented with 60 g/L maltose and 60 g/L sucrose as carbon source was optimal among the media tested for genotypes selected in this paper. Furthermore, anthers of donor plants subjected to cold pre-treatment at 4°C for 3 weeks and in some cases followed by a short heat post-treatment at 32.5°C before culturing at 25°C in darkness increased the frequency of callus induction. Evaluation of eleven genotypes in this study resulted in the identification of several highly responsive genotypes. Tests for suitability of routine production of double haploid plants from crosses involving these genotypes will be initiated. However, it should be noticed that this investigation could provide only a preliminary strategy for anther culture of proso millet. More effort is needed to improve induction efficiency and to overcome genotypic limitations. In order to use anther culture as a viable breeding tool, it will be essential to investigate more additional factors such as plant growth regulators and other genotypes with desirable traits to establish reasonable rates of callus formation and regeneration of plantlets.

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