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Study on Callus Induction from Anther and Inflorescense Culture of Sorghum

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Five genotypes of *Sorghum bicolor* Moench and five MS basal media supplemented with two kinds of hormone at various concentration were tested for their response to anther culture. TX403 produced calli with the highest frequency. Anthers cultured on medium containing 2.0mgL^{-1} kinetin, 3.0mgL^{-1} 2,4-D and 1,500mgL^{-1} gellan gum gave higher callus inducing rate of 1.25% than those cultured on other four medium. Frequency of callus producing decreased as anther aged. Rate of callus forming was high as panicle length ranged from 4.0 to 8.0 cm, corresponding with anther size ranged from 1,200 to 1,750 μm. The average number of plantlets regenerated derived from anthers with calli was 14.3%. Chromosome number of 20 from root-tip cells was found for most regenerated plantlets. For inflorescence culture, medium containing 2.5mgL^{-1} kinetin, 3.0mgL^{-1} 2,4-D and 5,000mgL^{-1} agar powder gave callus forming rate of 39.4%. The young inflorescence was an excellent source for inflorescence culture.

INTRODUCTION

Sorghum (*Sorghum bicolor* Moench) is considered as one of the top five most important cereal crops in the world behind wheat, rice, maize and barley (Kumaravadivel and Regasamy 1994). Improving the four major crops have been successfully undertaken through various appropriate methods but studies on sorghum have been still a big challenge. Since tissue culture method has been developed, anther culture is considered as an effective tool for the production of haploids for breeding programs. In 1964, Guha and Maheshwari reported the direct development of embryos from microspores of *Datura innoxia* through anther culture. Since then this technique has been improved and developed, specially in studies on cereal crops. Studies of anther culture have been successful for wheat (Liang *et al.* 1987; Hassawi *et al.* 1990). Regarding to inflorescence culture of rice, Mandal *et al.* (1993) reported that young inflorescences were a source for rice tissue culture. There are many studies on maize with respect of applying anther culture method (Ting *et al.* 1981; Genovesi and Collins 1982; Ma *et al.* 1987).

In sorghum, study on anther culture has been limited. Rose *et al.* (1986) first reported callus induction from anther culture of sorghum, but results from this study were limited because only four albino plants were obtained from more than 1,000 anther-calli. Wen *et al.* (1991) cultured over 21,000 anthers from 29 strains and found out that only six genotypes formed calli with various frequencies: Xin While and TX403-TSB produced more calli than four other genotypes. In addition, the production of calli was also dependent on medium used, which required to find the optimum hormone combinations for anther culture. Kumaravadivel and Rangasamy (1994) reported the induction of callus depended on temperatures and found that 30 °C was optimum. When

different levels of 2,4-D were used, the highest number of callus forming in medium containing 2.0mgL^{-1} 2,4-D was also reported. Therefore, several factors could affect haploids production through anther culture of sorghum: culture conditions, genotypes, anther stage, quantity and quality of hormones in the medium, etc. But previous studies have not reported about genotype × media interaction and details of anther stage which are considered as one of the important factors affecting callus induction.

In this study, we attempt to characterize both anther stage and medium components affecting anther culture in order to describe the optimum procedure of these techniques in a given condition. Simultaniously, a sub-experiment on inflorescence culture was also conducted to test for their response to callus production and to assess the potentiality of using young inflorescence as a good souce of explants and haploid production.

MATERIALS AND METHODS

1. Effect of genotype and anther stage on callus induction

The study was conducted from June 1995 to December 1996. Three inbred lines from USA (Xin White, TX403 and PP290) and two lines obtained from crossing of D_4 × Xin White and D_5 × Xin White (D_4 and D_5 origin from ICRISAT) were used as a major source of materials for anther culture. One plant was grown in a pot (200 mm in diameter) in a greenhouse and in a phytotron at 20 °C during winter season (November to March). Lighting was natural sunlight and no supplementary light was provided. A plant was fed as basal dressing of N, P_2O_5 and K_2O at the rate of 1.44 g per each pot.

To characterize anther stage, a tiller was collected when the panicle just caused the leaf sheath to split open. Distance between collars of penultimate leaf and flag leaf, the length of panicle, distance between panicle top and flag leaf collar, the length of anther and color of anther were measured and recorded. Immature panicle branches at one-third upper part from the main stem were collected, sterilized with 60% EtOH for 3 minutes then washed three times with sterilized water. Thirty-five anthers were placed on each medium in each petri dish (90×15 mm). Petri dishes were sealed with parafilm, kept in an incubation chamber in darkness at 28±1 °C until calli were formed and appropriated for transferring to regeneration medium. Over 26,000 anthers were used over a period of 20 months.

2. Effect of basal medium and medium supplement on callus induction

The five MS basal media (Murashige and Skoog 1962) supplemented with 2,4-D at 3.0mgL^{-1} and the two kinds of hormone, kinetin and zeatin, at various concentration were tested for their effect on the response to anther culture. The five MS basal media suplements included: (1) MS-na-1, (2) MS-na-2, (3) MS-na-3, (4) MS-na-4 and (5) MS-na-5 (Table 1). The medium was adjusted to pH5.8 with 0.1N Sodium Hydroxide Solution (NaOH) or HCl before autoclaving. To determine the effect of agar concentration for anther culture, MS-na-1 supplemented with different agar levels: [5,000mgL^{-1} agar powdwe; 7,000mgL^{-1} agar powder; 1,000mgL^{-1} gellan gum and 1,500mgL^{-1} gellan gum] was used.

3. Regeneration plants and chromosome counts

Table 1. Composition of the five callus-induction medium used in the study.

Components	Media[a] [mgL^{-1}]				
supplemented[b]	MS-na-1	MS-na-2	MS-na-3	MS-na-4	MS-na-5
2,4-D	3.0	3.0	3.0	3.0	3.0
Kinetin	2.5	2.0	0.5	-	0.3
Zeatin	-	-	2.0	2.0	2.2
Agar powder	5,000	-	-	-	5,.000
Gellan gum	-	1,500	1,000	1,500	-
pH	5.8	5.8	5.8	5.8	5.8

[a] MS (Murashige and Skoog) basal medium

[b] Alanine, proline, sucrose supplemented at the same level for each medium (50mg L^{-1}, 50mg L^{-1} and 20,000mg L^{-1}, respectively.)

For plant regeneration, we used MS basal medium plus IAA, 2.0mgL^{-1}; kinetin, 2.5mgL^{-1}; sucrose, 2.0% and gellan gum, 1,500mgL^{-1}. The medium was also adjusted to pH5.8 with 0.1N Sodium Hydroxide Solution (NaOH) or HCl before autoclaving. Anthers with calli at about 5 mm in diameter were tranferred into Pyrex glass containing regeneration medium and kept in a growth chamber at 25 °C and the light was at 400 μ molm^{-2}s^{-1}.

Chromosome number of root tips derived from anther calli was examined following the method by Hyuga (1990). Root tips were collected and pretreated in colchicine solution for 2 to 3hours in darkness at room temperature (24–25 °C). After pretreating, root tips were fixed in a freshly prepared solution containing 1 part of acetic acid: 3 part of ethanol at 4–5 °C for 40 to 50 minutes. After root tips were removed from the fixtative, they were rinsed in distilled water and treated with an enzyme mixture containing 4% cellulase and 1% pectolyase solution at 37 °C for 30minutes. Samplings with root tips cells were stained in 5% Giemsa's stain solution at room temperature for 30 minutes. For comparison, chromosome number of root tips derived from germinated seeds of Xin White was also examined following the procedures as described above.

4. Inflorescence culture

Inflorescence culture was also conducted during period of July to September 1995. Xin White was used to test for its response to inflorescence culture. Distance between penultimate leaf and flag leaf collars, and the length of inflorescence were measured. Inflorescences were rinsed in water, sterilized with 60% EtOH for 3 minutes and washed three times with sterilized water. Immature spikelets from the portion of the inflorescences were cultured on petri dishes containing MS-na-1 and MS-na-5 medium. Cultures were kept in total darkness at 28 ± 1 °C in an incubation for callus induction.

RESULTS AND DISCUSSION

1. Effect of genotype and anther stage on callus induction

Thirty days after inoculation on the callus-induction medium, calli were initiated to form. Peak callus forming was about 40days after inoculation. Effect of genotype on

Table 2. Effect of genotype and medium composition on callus producing from *Sorghum bicolor* anthers.

Genotype	Medium				Average
	MS-na-1	MS-na-2	MS-na-3	MS-na-4	
Xin White	0.72±0.21*	1.00±0.74	0.14±0.09	0.86±0.47	0.68
TX403	0.18±0.14	4.29±1.62	0.11±0.22	3.38±1.29	1.99
PP290	0	0.95±0.83	2.86±5.60	0.63±0.88	1.11
D4×Xin White	0	0	0	0	0
D5×Xin White	0	0	0	0	0
Average	0.18	1.25	0.62	0.97	0.76

* Mean value of number of anthers with calli induced from 100 anthers inoculated ±S.E., Standard error of the mean value.

producing callus on various medium was presented in Table 2. On average, TX403 genotype produced calli with higher frequency as compared to four other genotypes. There was no callus forming for anthers from lines of D_4 × Xin White and D_5 × Xin White. This showed that calluses forming was dependent on genotype. A similar result was pointed out for *Sorghum bicolor* in USA, in which only six genotypes responsed to anther culture from 29 strains (Wen *et al.* 1991).

Results from Table 2 indicated that there was an interaction between Genotype × Media. TX403 anthers cultured in MS-na-2 and MS-na-4 medium produced more calli than those cultured in the two other media Ms-na-1 and MS-na-3 (4.29% and 3.38% compared to 0.18 and 0.11%, respectively). On the other hand, PP290 anthers cultured in MS-na-3 gave the highest callus producing rate of 2.86% as compared to those cultured in the three other media.

A series of data from anthers cultured in MS basal medium supplemented 2.0mgL^{-1} kinetin [MS-na-2] and in MS basal medium supplemented 2.0mgL^{-1} zeatin [MS-na-4] was used for analyzing the effect of anther stage. In general, number of callus forming per 100 anthers decreased as panicle aged. There was a significant association between callus yield and panicle length (r =–0.33**). Yield of callus inducing was quite high with panicle

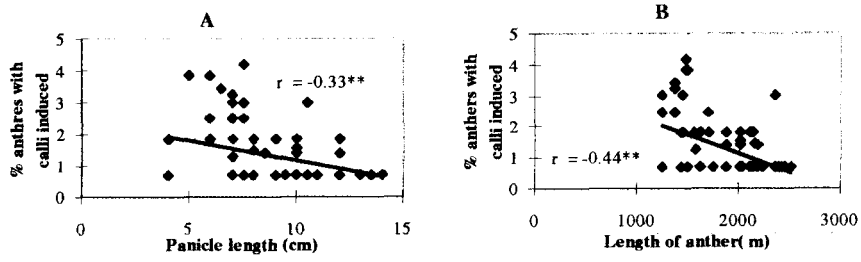

Fig. 1. Relationship between callus yield and the age of Sorghum anther.
A) Callus yield and panicle length. B) Callus yield and anther length.

length ranged from 4.0 to 8.0 cm then declined. Simultaniously, relationship between callus yield and the length of anthers was found ($r = -0.44^{**}$). The length of anthers ranged from 1,200 to 1,750 μm gave high frequency of calli and then decreased as anthers aged (Fig. 1).

2. Effect of basal medium and medium supplement on callus induction

Among the 5 MS basal media suplements tested, MS-na-2 [basal medium plus kinetin ($2.0mgL^{-1}$), 2,4-D ($3.0mgL^{-1}$), gellan gum ($1,500mgL^{-1}$)] produced the highest calli while other mediums produced less than 1% of calli. In our experiments, the yield of callus induction from anther culture was low, which might be due to the effects of media composition and others unknown or uncontrollable factors.

Comparing callus-induction medium containing different gelling agents showed that callus producing was the highest with medium containing $1,500mgL^{-1}$ gellan gum, and no callus forming was observed in medium containing $7,000mgL^{-1}$ agar powder (Table 3). This indicated that quality and levels of gelling agent played an important role in callus-induction medium. Number of callus declined with medium containing $5,000mgL^{-1}$ of agar powder and $1,000mgL^{-1}$ of gellan gum.

Table 3. Effect of gelling agents on callus induction from Xin White anther culture.

Gelling agents and contents[a] (mgL^{-1})	Mean value of number of anthers with calli induced from 100 anthers inoculated
Agar powder (5,000)	0.55 ± 0.34[b]
Agar powder (7,000)	0
Gellan gum (1,000)	0.30 ± 0.21
Gellan gum (1,500)	1.25 ± 0.41

[a] MS basal medium plus $2.5mgL^{-1}$ kinetin and $3.0mgL^{-1}$ 2,4-D was used for this experiment.
[b] $\pm$ S.E., standard error of the mean value.

3. Regeneration plants and chromosome counts

From 25 to 30days after calli were transferred to the regeneration medium, they initiated roots then formed shoots (Fig. 2). In our experiment calli developed regenerated plants poorly with an average of 14.3%. PP290 anthers with calli developed the highest regeneration plants as compared to Xin White and TX403 (Table 4). Low-

Table 4. Regeneration ability from anther derived calli of three genotypes.

Genotype	Anthers	Anthers with calli	Regeneration
Xin White	18,025	73	7 (9.6)*
TX403	5,635	60	5 (8.3)
PP290	2,205	8	2 (25.0)
Total	25,865	141	14 (14.3)

* Values in parentheses are percent regenerated plantlets derived from anthers with calli.

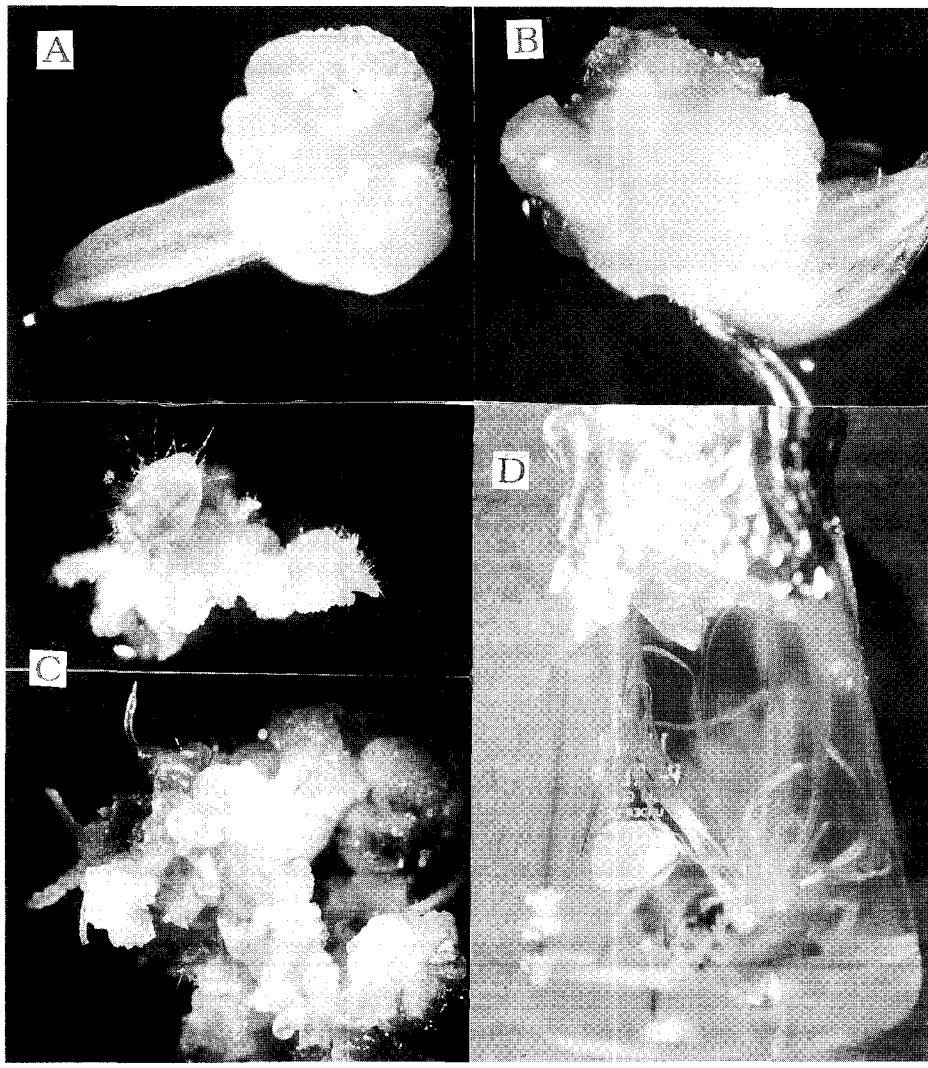

Fig. 2. Callus and inflorescence culture of Sorghum. A) Callus forming from anther culture. B) Callus forming from inflorescence culture. C) Shoots regenerated from anther with calli (25days after transferring to the regeneration medium). D) Shoots regenerated from anther with calli (40days after transferring to the regeneration medium).

Table 5. Chromosome number of root tip cells of regenerated plantlets from anther culture of sorghum.

	Chromosome number of		
	10	20	40
No. of cells with specific characteristic/ Total cells observed	11/170	150/170	10/170

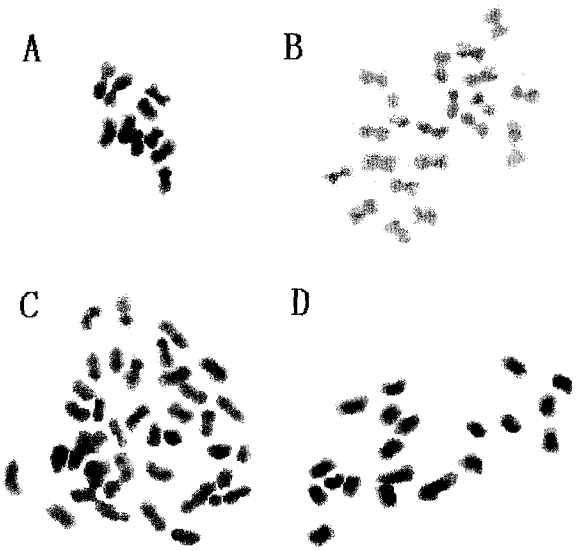

Fig. 3. Chromosomes of root tip cells of plants regenerated from anther culture and from germinated seeds of sorghum. A) A root tip from plants regenerated showing 10 somatic chromosomes. B) A root tip from plants regenerated showing 20 somatic chromosomes. C) A root tip from plants regenerated showing 40 somatic chromosomes. D) A root tip from germinated seeds showing 20 somatic chromosomes.

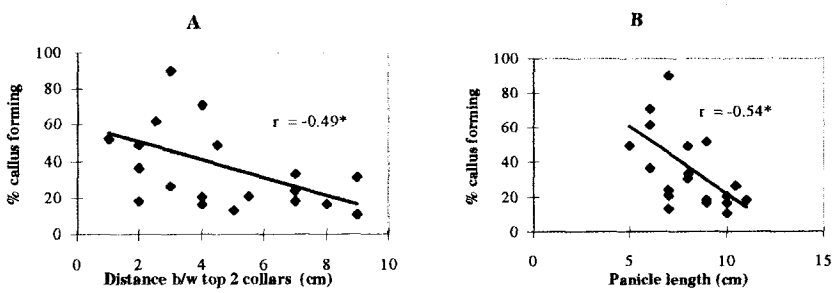

Fig. 4. Relationship between callus yield and the age of Sorghum. A) Callus yield and distance b/w penultimate leaf and flag leaf collars through inflorescence culture. B) Callus yield and the length of panicle through inflorescence culture.

frequency and unstability of regenerated plantlets were also reported in some previous studies for Sorghum (Rose *et al.* 1986; Wen *et al.* 1991).

For most cells from root-tip of regenerated plants of Xin White, the chromosome number observed was 20. Cells with chromosome numbers of 10 or 40 were also observed (Fig. 3). In an observation, 150 out of 171 cells had chromosome number of 20,

while only 10 and 11 cells had chromosome number of 40 and 10, respectively (Table 5). A similar finding of 20 chromosomes was reported for regenerated plants of *Sorghum bicolor* (Wen *et al.* 1991). Chromosome number of 20 were observed for root tip cells derived from germinated seeds of Xin White. In root tips from the major of mitotic cells, chromosome number of 20 often were observed for *Sorghum bicolor* (Tang and Liang 1987). According to this result, chromosome number of 20 from anther culture was suggested for most regenerated plants. The source of chromosome numbers of 10 and 40 were unknown. Basically, the origin of chromosome numbers of 10 was considered as from haploid (pollen) cells, 20 chromosomes from doubled haploid during regeneration or somatic cells as of filament and 40 chromosomes from doubled diploid during regeneration. However, the exact chromosome number and its source were proposed for further study.

4. Inflorescence culture

Over 1600 immature spikelets from the portion of Xin White inflorescences were placed on 55 petri dishes containing MS-na-1 and MS-na-5 media. Ten days after inoculation, calli were initiated to form mostly from rachis. Peak callus inducing was 15 to 20 days. Frequency of callus producing was low as 39.4 ± 3.1 on MS-na-5 medium and only 5.0 ± 4.0 on MS-na-1 medium per 100 spikelets. Wen *et al.* (1991) obtained 95% on the average of callus induction for Xin White. This suggested the effect of medium used for inflorescence culture. There was a marked that both MS-na-1 and MS-na-5 medium also produced calli with low frequency from anther culture. In addition, callus producing depended on inflorescence stage. It decreased as inflorescence aged (Fig. 4). This result was coincided with investigations on *Sorghum bicolor* and on rice that young inflorescences were a good source for tissue culture (Wen *et al.* 1991; Mandal *et al.* 1993).

In conclusion, results from our experiment indicated that the induction of calli derived from anther culture and regeneration of plantlets from calli were always unstable and depended on genotype, anther stage, medium and culture conditions. Among the five genotypes tested, TX403 showed the highest frequency of callus inducing. A significant association between yield of callus inducing and anther stage was occurred. Yield of callus inducing declined as anther aged. Medium containing $2.0 mgL^{-1}$ kinetin and $3.0 mgL^{-1}$ 2,4-D was effective in inducing anther calli. Young infloscence produced calli with high frequency. Therefore, the possibility of using young infloscence as a benefit souce for explants was high. For further studies, the effect of environment conditions such as the season, how the parent plants are grown, and from which anthers used for culture was proposed.

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