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CONSTRUCTION OF AN EFFICIENT TISSUE CULTURE SYSTEM FOR SORGHUM USING MATURE EMBRYOS

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Abstract

Mature embryos as explants are more convenient to operate and not subjected to time constraints in tissue culture systems. In this research, 120 sorghum varieties were screened to select an appropriate material for establishing a high efficient tissue culture system using mature embryos as explants. Loose, soft, and yellowish primary calluses were induced from the mature embryos of only one variety (No. 32) out of the 120. Therefore, No. 32 was selected to establish a culture system. The highest callus induction frequency was 58.58% when mature embryos werecultured on induction medium supplemented with 2.375 mg/L $CuSO_4 \cdot 5H_2O$ and 5 mg/L 2,4-D. Higher frequency of embryogenic calluses induction was achieved on subculture medium with 2 mg/L 2,4-D. The best combination of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) in regeneration medium was 1 mg/L of each. The results showed that the optimal subculture time for callus regeneration was no more than one week. Our research provides a solid basis for genetic transformation of sorghum using mature embryos as explants.

Key words: Sorghum, Mature embryo, Tissue culture system, Regeneration.

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the main forage crops and food crops in the world (Singh *et al.*, 2016). It is mainly planted in arid and semi-arid regions. Sorghum can be processed into a variety of products including food, feed, sugar, and biofuel using sorghum straw and other parts (Yong *et al.*, 2015). Demand for food and energy is soaring with global population increase. Therefore, sorghum production is of great significance (Visarada *et al.*, 2014).

In the past 20 years, research of sorghum breeding has mainly focused on yield in China (Mekbib, 2006). Extensive and in-depth studies with different purposes have been conducted and great progresses have been made (Nabukalu *et al.*, 2016). However, sorghum breeding is still faced with many problems such as small genetic variation, low heritability, and slow variety improvement. Transgenic technique provides an efficient way to solve these problems, and an efficient tissue culture system is the basis for the establishment of a sorghum genetic transformation system (Girijashankar & Swathisree, 2009). Immature embryos are mainly used in sorghum tissue culture. Although immature embryos as explants have higher efficiency in callus induction, mature embryos as explants are more convenient to operate and not subjected to season limit. It means that mature embryos can be induced in spring or winter (Wang *et al.*, 2006). Callus induction and regeneration of sorghum are affected by many factors, such as variety as well as the genotype, aspartic acid, proline, copper, 2,4-dichlorophenoxyacetic acid (2,4-D) (Pasternak *et al.*, 2002), indole-3-acetic acid (IAA) (Kopertekh *et al.*, 1995), kinetin, α-naphthaleneacetic acid (NAA) (Jiménez, 2005), and 6-benzylaminopurine (BAP) (Agarwal *et al.*, 2004), and variety and genotype has strong influence. Therefore, screening an appropriate variety is the basis for the establishment of a high efficient tissue culture system using mature embryos as explants.

In the present research, one out of 120 sorghum varieties was selected to establish a high efficient tissue culture system where conditions were optimized for callus induction and sorghum plantlet regeneration.

Materials and Methods

Plant materials and medium maintenance: Mature dry seeds of 120 sorghum varieties were used in the present research. Variety names and sources are listed in Appendix 1. The seeds were first surface-sterilized with 75% ethanol and shaken at 200 rpm for 5 min, and then soaked in sterilized water under a laminar air flow hood. After that, the seeds were soaked in 0.1% mercuric chloride solution with two drops of Tween 20 added and shaken at 200 rpm for 15 min. Then, the seeds were rinsed with sterilized water for at least five times and placed into aseptic Petri dishes to be desiccated under a laminar air flow hood. Callus induction medium and subculture medium were prepared and maintained at 28°C in darkness. Regeneration medium and rooting medium were prepared and maintained at 28°C under cool white fluorescent lights with a 16/8 h (light/dark) photoperiod. Greenhouse condition is28°C under cool white fluorescent lights with a 16/8 h (light/dark) photoperiod and humidity is 70%.

Medium formula: MS medium: MS (Murashige & Skoog, 1962) powder with Gamborg vitamins, 30g/L sucrose, and 3.5g/L Phytagel.

Callus induction medium:MS medium with 1 g/L L-proline, 1 g/L L-asparagine, 1 g/L potassium dihydrogenphosphate (KH_2PO_4), 0.5 mg/L nicotinic acid, 0.1 mg/L thiamineHCl, 0.5 mg/L pyridoxineHCl, 10 mg/L ascorbic acid, 0.1 g/L myo-inositol, 0.4 mg/L glycine, 5 mg/L 2,4-D and different $CuSO_4 \cdot 5H_2O$ (Table 1).

Appendix 1. Variety names and sources of different sorghum genotypes studied for *In vitro* regeneration.

S.NO	Source country	S.NO	Source country	S.NO	Source country
1	USA	41	India	81	South Africa
2	USA	42	India	82	South Africa
3	USA	43	Uganda	83	South Africa
4	USA	44	Nigeria	84	Argentins
5	USA	45	Nigeria	85	Botswana
6	India	46	Nigeria	86	Cameroon
7	India	47	Nigeria	87	Cameroon
8	China	48	Nigeria	88	Cameroon
9	China	49	Nigeria	89	Cameroon
10	China	50	Japan	90	Cameroon
11	India	51	Pakistan	91	Cameroon
12	South Africa	52	South Africa	92	Cameroon
13	South Africa	53	Uganda	93	Cameroon
14	South Africa	54	Uganda	94	Cameroon
15	South Africa	55	Kenya	95	Cameroon
16	Iran	56	Kenya	96	Cameroon
17	Afghanistan	57	Kenya	97	India
18	South Africa	58	Sudan	98	India
19	Egypt	59	Thailand	99	India
20	Nigeria	60	Chad	100	Ethiopia
21	Kenya	61	Chad	101	Sudan
22	South Africa	62	USA	102	Sudan
23	India	63	Ethiopia	103	Bangladesh
24	India	64	Ethiopia	104	Botswana
25	India	65	Ethiopia	105	Botswana
26	India	66	Ethiopia	106	Zimbabwe
27	India	67	Zimbabwe	107	India
28	India	68	Sudan	108	Senegal
29	India	69	Australia	109	Niger
30	India	70	USA	110	Niger
31	India	71	Yemen	111	USA
32	India	72	Turkey	112	USA
33	India	73	India	113	USA
34	India	74	Ethiopia	114	USA
35	India	75	Nicaragua	115	USA
36	India	76	Cuba	116	USA
37	India	77	Venezuela	117	USA
38	India	78	Mexico	118	USA
39	India	79	South Africa	119	USA
40	India	80	South Africa	120	Indonesia

Table 1. $CuSO_4 \cdot 5H_2O$ concentration tested in the calli induction.

Medium No.	$CuSO_4 \cdot 5H_2O$ concentration (mg/L)
1	1.875
2	2,375
3	2.875
4	3.375
5	3.875

Table 2. BAP and IAA combinations tested in the regeneration media.

Medium No.	BAP (mg/L)	IAA (mg/L)
1	0	0
2	0	1
3	1	0
4	1	1

Subculture medium: The same as callus induction medium but different in 2,4-D concentration(2 mg/L or 5 mg/L). Regeneration medium: MS medium with 0.249 mg/L $CuSO_4 \cdot 5H_2O$ different combinations of BAP and IAA (Table 2). Rooting medium: MS medium with 0.249 mg/L $CuSO_4 \cdot 5H_2O$, 1 mg/L IAA, 1 mg/L IBA, and 1 mg/L NAA.

Experimental design: Experiments were performed to learn the effects of medium components or culture factors to optimize culture conditions. Five different $CuSO_4 \cdot 5H_2O$ concentrations (Table 1) were tested in callus induction; 2 mg/L or 5 mg/L 2,4-D was tested in subculture; BAP and IAA in different combinations of concentration (Table 2) were tested in regeneration; finally, three subculture times (0, 1, and 2 weeks) were tested using medium with the best combination of IAA and BAP learned in the previous experiment.

Calculation and statistical analysis: Two weeks afterexplants were initiated on induction medium, callus induction rates were recorded. The embryogenic callus induction rates were evaluated after subculture according to the experimental design. Calluses were transferred ontoregeneration media after three weeks, and the percentage of regeneration and browning rate were calculated.

$$\text{Callus induction rate} = \frac{\text{Number of callus}}{\text{Number of seeds}} \times 100\ \%$$

$$\text{Embryogenic callus rate} = \frac{\text{Number of embryogenic callus}}{\text{Total number of callus}} \times 100\ \%$$

$$\text{Regeneration rate} = \frac{\text{Number of regenerated callus}}{\text{Total number of callus}} \times 100\ \%$$

$$\text{Browning rate} = \frac{\text{Browning number}}{\text{Total number of callus}} \times 100\ \%$$

DPS software (Tang & Zhang, 2013) was usedfor statistical analyses. Data were subjected to ANOVA followed byFisher's least significant difference test (p<0.05).

Results

Callus induction test of the 120 sorghum varieties: In order to select an appropriate sorghum line for tissue culture, 120 sorghum varieties were tested using induction medium (Fig. 1). The results showed that 79.17% of the varieties could not be induced to produce callus; 8.33% gave high percentages of browning callus; and 11.67% displayed low callus induction rates and long shoots. Only one variety (No. 32) produced loose, soft and yellowish primary calluses from mature embryos within two weeks on induction medium (Table 3). Therefore, the variety No.32 was selected for further study.

Table 3. Callus induction results of the 120 sorghum varieties.

Induction result	Number of varieties	Percentage (%)
Seed germination with no callus	95	79.17
Low percentage of callus and long shoots	14	11.67
High percentage of browning calluses	10	8.33
Loose and yellowish calluses	1	0.83

Table 4. The embryogenic callus induction rates in different $CuSO_4 \cdot 5H_2O$ concentration treatments of the induction medium of sorghum.

$CuSO_4 \cdot 5H_2O$ concentration (mg/L)	Embryogenic callus induction rate (%)
1.875	32.33 c
2.375	58.58 a[1]
2.875	41.25 bc
3.375	48.61 b
3.875	37.33 c

[1] Means within a column followed by the same letter do not differ from each other according to Fisher's least significant difference test p<0.05

Callus induction rate in different $CuSO_4 \cdot 5H_2O$ concentration treatments: $CuSO_4 \cdot 5H_2O$ concentration is a very important factor in sorghum callus induction medium (Nirwan *et al.,* 2003). The induction frequency of callus ranged from 32.33% to 58.58% in the treatments with different $CuSO_4 \cdot 5H_2O$ concentrations (Table 4). The result showed that the percentage of embryogenic callus in the2.375 mg/L $CuSO_4 \cdot 5H_2O$ treatment was significantly higher than in the other treatments.

Subculture on media with different 2,4-D concentrations: The primary calluses were transferred onto fresh subculture media containing 2,4-D at different concentrations to induce embryogenic calluses. The calluses differentiated into two types after 14 days on the subculture media. One was soft, watery, bruised, and browning non-embryogenic calluses; the other was compact,irregularly shaped, light-yellow or creamy embryogenic calluses (Fig. 2). Both 2,4-D concentrations could induce embryogenic calluses, but with significantly higher percentage in 2 mg/L 2,4-D than in 5 mg/L (Table 5).

Optimization of regeneration medium: To find out the best combination of BAP and IAA, calluses were transferred to the regeneration media with different BAP and IAA concentrations (Table2). Green spots occurred on the calluses within three weeks after transfer (Fig. 3). The percentage of regeneration (35.59%) in medium No.4 was significantly higher than in the other 3 media. Notably, the percentage of browning callus was the lowest in the four treatments (Table 6). Moreover, the combining of BAP and IAA together was better than BAP or IAA alone for callus regeneration. The best treatment was 1mg/L BAP and IAA.

Table 5. The growth status and percentage of embrygenic callus on subculture media with different 2,4-D concentrations.

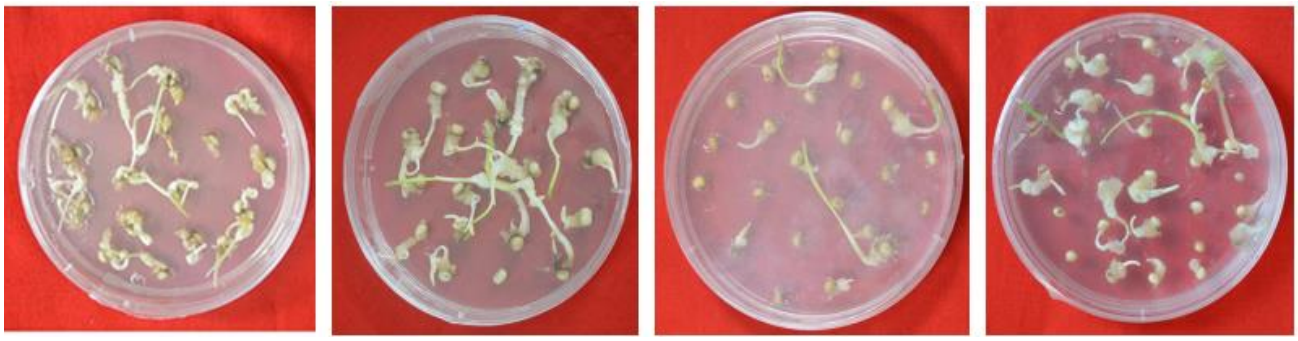
2,4-D concentration (mg/L)	Percentage of embryonic callus (%)	Callus before subculture	Callus after subculture	Growth status
2 mg/L	56.47 a[1]	Light-yellow, translucent	Yellow granular	Loose, soft and yellowish
5 mg/L	48.26 b	Light-yellow, translucent	Yellow granular	Loose, soft and yellowish

[1] Means within a column followed by the same letter do not differ from each other according to Fisher's least significant difference test p<0.05

Table 6. Regeneration results using the four regeneration media.

Medium No.	Days green spots took to occur (day)	Browning rate (%)	Percentage of regeneration (%)
1	11	19.15 a[1]	15.06 c
2	20	10.24 b	24.18 b
3	19	24 a	18.13 c
4	19	8.34 b	35.59 a

[1] Means within a column followed by the same letter do not differ from each other according to Fisher's least significant difference test p<0.05



A NO. 1 B NO. 27 C No. 29 D No. 32

Fig. 1. The induced calluses of 4 varieties from the 120 sorghum varieties

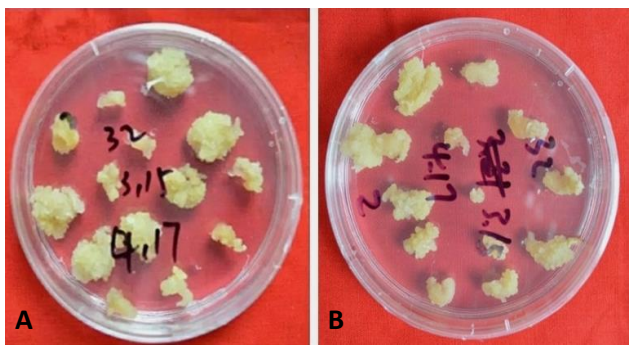


Fig. 2. Callus growth status on subculture media with different 2,4-D concentrations (A) 2 mg/L and (B) 5 mg/L.

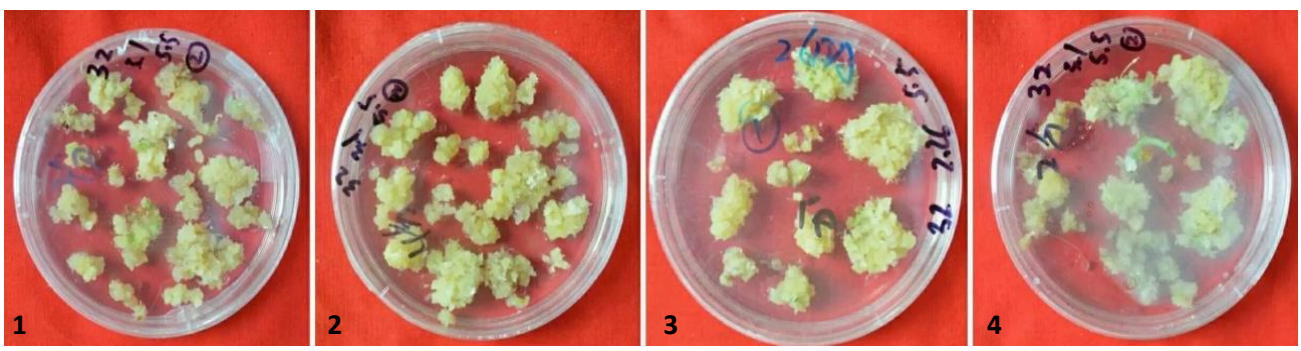


Fig. 3. Green spots showed on callus in the four treatments.

Table 7. The effects of subculture time on sorghum callus growth and regenerability.

Subculture time (week)	Regeneration rate (%)
0	67.78 a[1]
1	61.83 a
2	30.32 b

[1] Means within a column followed by the same letter do not differ from each other according to Fisher's least significant difference test $p<0.05$

Effect of subculture time: Rice embryogenic callus can maintain regenerability for over 40 week (Nabors *et al.*, 1983). To learn more about the regenerability of sorghum callus, experiments were performed on the effect of subculture time. The results showed that the appropriate subculture time for sorghum callus was no more than one week. When longer than that, the regeneration rate of sorghum callus significantly decreased (Table 7).

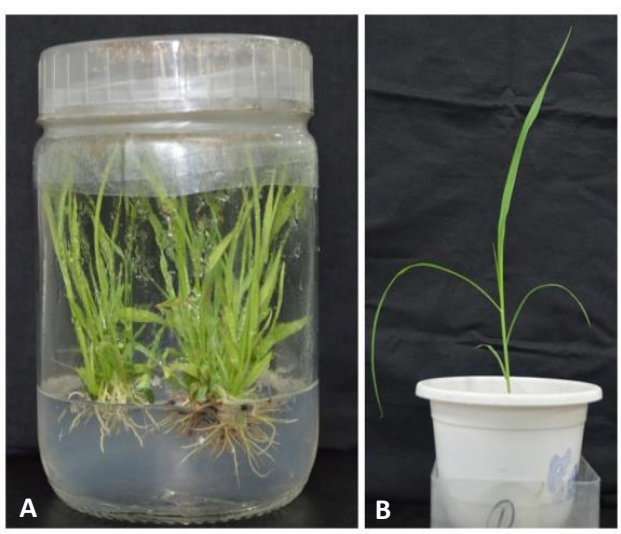


Fig. 4. *In vitro* rooting of sorghum and transferred seedlings in greenhouse.

Rooting and transplanting of regenerated plants: After shoots occurred on regeneration medium, the plantlets were transferred to rooting medium. After about 2 weeks (Fig. 4), the seedlings with well-developed roots were rinsed with water to remove the medium, transplanted into pots filled with a mixture of sterilized soil and vermiculite (v/v=1:1), placed in a growth chamber for four days, transplanted into a greenhouse, and allowed to grow to maturity.

Discussion

Callus induction is the first step of *In vitro* regeneration, and the main influence factors of callus formation are genotype, basic medium and hormon (Elkonin *et al.*, 2000), as well as the type of explant used (Carvalho *et al.*, 2011). Most researchers have focused on tissue culture using immature embryos, for example, from Tx430. However, little work has been conducted using mature embryos as explants (Wei *et al.*, 2004). An appropriate variety is the main obstacle in sorghum tissue culture and genetic transformation using mature embryos as explants. In the present research, 120 sorghum varieties were screened to select an appropriate variety for tissue culture. Our results showed that 79.17% of the varieties could not be induced to form calluses. Only one variety was found to be a good material for tissue culture. The results also revealed why there are fewer successful studies using mature embryos as explants in sorghum tissue culture.

Regeneration of callus is limited by accumulation of phenolics, low regeneration rate and short duration of regenerability (Yong *et al*., 2015). Our results showed that the highest percentage of regeneration was 35.59%, lower than that when immature embryos of Tx430 are used as explants (Liu *et al.*, 2012). The reason is that all induced calluses were transferred onto regeneration medium including embryogenic calluses and non-embryogenic calluses. Additionally, duration of regenerability also is very short for sorghum. The optimal subculture time is no more than one week. Nevertheless, these frequent manipulations of the explants (pealing) increase the contamination risk of cultures (Silva *et al*., 2015). However, studies involving strategies to avoid the browning of explants must be carried out, mainly to attempt to regenerate varieties and genotypes with lower browning rate.

Sorghum plants, in the process of tissue culture, are readily to brown; the brown phenolic substances produced impact callus growth and even lead to callus death. The concentrations of 2,4-D and copper play important roles in improving callus induction rate and plant regeneration of sorghum (Nirwan *et al.*, 2003). In the present study, the callus induction medium supplemented with 2.375 mg/L $CuSO_4 \cdot 5H_2O$ gave the best induction frequency. 2,4-D at 5 mg/L effectively inhibited the growth of shoot on induction medium but also unfavorably inhibited the growth of embryogenic calluses on subculture medium. This might be because high concentration (e.g., 5 mg/L) of 2,4-D results in a greater possibility of somatic mutation (Choi *et al.*, 2001).

Conclusions

In the present study, we obtained one appropriate sorghum variety for tissue culture using mature embryos as explants, and developed a tissue culture protocol for the variety. The protocol has the following main steps: Primary calluses are induced on induction medium supplemented with 2.375 mg/L $CuSO_4 \cdot 5H_2O$ and 5 mg/L 2,4-D for two weeks; embryogenic calluses are developed on subculture medium supplemented with 2 mg/L 2,4-D for one week; embryogenic calluses are selected and transferred onto regeneration medium containing 1.0 mg/L BAP and 1.0 mg/L IAA for three to four weeks; then, the regenerated plantlets are allowed to develop roots on rooting medium and transferred to soil for growth.

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