

## Improved Culture Media for Embryogenic Callus Generation in Sorghum [*Sorghum bicolor* (L.) Moench]

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Received: 04 June 2019; Accepted: 23 July 2019

**Abstract:** Many attempts on optimization of sorghum [*Sorghum bicolor* (L.) Moench] tissue culture induction media have been made, but the culture system remains with some bottlenecks compared to that of other crops. This study aimed at assessing the suitability of various induction media to produce embryogenic callus (yellow and friable) with high induction rates and reduced phenolic exudation. The six culture medium modifications: 3 based on Murashige and Skoog (MS) medium and one each based on Chu N6, Gamborg B5 and 190-2 media respectively were applied in the culture of mature embryos from 10 sorghum genotypes. Although there was a genotype influence on the attainment of a yellow callus, friability of the callus was determined to be dependent on the culture medium and not the genotype. Half strength MS medium with 0.2 mg/l 2,4-D with 2.8 g/l Gelrite® as the gelling agent modified with 1.0 g/l KH<sub>2</sub>PO<sub>4</sub>, 1.0 g/l L-proline, 1.0 g/l L-asparagine and 0.16 mg/l CuSO<sub>4</sub>·5H<sub>2</sub>O (type E) was found to be the most effective resulting in about 60% yellow coloured callus induction with 25% friability. Addition of CuSO<sub>4</sub>·5H<sub>2</sub>O, KH<sub>2</sub>PO<sub>4</sub>, L-proline and L-asparagine significantly reduced the phenolic production. Half strength MS medium was observed to contribute to quality callus production when compared to full strength MS media modified with the compounds. The half strength MS medium was also observed to suppress phenolic production. Medium 190-2 produced the highest regeneration frequency (40%) among the 3-regeneration media tested. The results provide information on a suitable sorghum callus induction medium necessary for embryogenesis.

**Keywords:** Sorghum; medium; embryogenic; callus; half-strength MS

### 1 Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a water-stress tolerant plant with a C<sub>4</sub> photosynthesis system [1,2]. It belongs to the family *Poaceae*, tribe *Adropoganeae* and genus *Sorghum* [3], and has its center of diversity in northeast Africa [2]. The crop, which is a dietary staple for more than 500 million people in over 30 countries [4] of Semi-Arid Tropics (SAT), especially due to its drought tolerance trait [2], is the fifth of the most consumed food crops in the world [4].

The crop's genetic variation in perenniality, sugar storage, tillering, stalk reserve retention [5] coupled with high level of inbreeding makes its breeding interesting, similarly to other perennial cellulose-rich biomass crops [2]. Climate change resilient, abiotic and biotic stress tolerant crops are other products that sorghum breeding must accelerate to deliver [6]. Technologies that utilize tissue culture techniques such as doubled haploidy assure some of the fastest ways to produce the improved crop [7,8].

Sorghum is however one of the most recalcitrant crops for tissue cultures [9]. The success has been restricted by low rate of callus induction and regeneration, high level of pigment or phenolic compound



exudation from somatic tissues and appearance of undesirable albino plantlets among regenerants [10]. Several studies have described callus initiation protocols for sorghum although with little reproducibility [11,12]. While it has been reported that the success of sorghum embryogenesis is genotype dependent, other factors such as explant type [13] and different culture medium modifications have been reported to contribute to varying degrees of callus formation [14-16]. The explant type plays a great role in minimizing phenolic exudates and increasing the regeneration rates [13].

The most remarkable stride in attainment of a quality sorghum somatic callus reported [13] an induction rate of 78.7% on full strength Murashige and Skoog (MS) medium [17] supplemented with L-proline, L-asparagine,  $\text{KH}_2\text{PO}_4$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . The effect of MS strength on the tissue culture of sorghum has however not been studied. In a study on flavonoid production in *Hydrocotyle bonariensis* tissue cultures [18] it was observed that the strength of the media and variations in its components have effect on cell growth in culture. In a later study, maximum callus growth was reported from *Costus pictus* explants on half-strength MS medium supplemented with growth hormones IAA, BAP, 2, 4-D and kinetin, whereas no response was observed on the explants on full strength MS medium supplemented with the same growth regulators which indicated that the explants were sensitive to high nutrient concentration [19].

An earlier study [13] pointed out, addition of amino acids L-proline and L-asparagine have been used to reduce phenolics together with  $\text{KH}_2\text{PO}_4$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and improve callus induction rate. The positive contributions of the amino acids had been reported [20] where high concentrations of L-asparagine and L-proline in culture induction media were observed to increase the proliferation of sorghum embryogenic callus and diminish the production of toxic pigments. Further it was observed that while embryogenic callus was obtained after addition of the amino acids to both MS and N6 media, the embryogenic callus became more friable on the latter [20].

Friability, which is the formation of nodulations on the callus is one of the attributes of an embryogenic callus [21]. This callus proliferation was observed to be positively influenced by addition of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  [13]. Addition of  $\text{KH}_2\text{PO}_4$  which was found to increase the percentage of water in the callus [22] was also observed to contribute to reduction of phenolics [13]. Earlier studies [23,24] described an embryogenic callus further to be a white compact or creamy to yellowish embryogenic callus. As has been the case with induction, sorghum calli regeneration has not been reproducible. A regeneration medium comprising of MS medium supplemented with 1 mg/l 6-benzylaminopurine (BAP), 1 g/l indole-3-acetic acid (IAA) and 0.16 mg/l  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  gave a regeneration frequency of 70% with variety T × 430 [13]. Another study reported that a high regeneration frequency (21.4 plants per embryogenic callus) was observed when 4 mg/l BAP was included in the regeneration medium [16]. Therefore, the aim of this study was to investigate the difference in the effect of full strength and half-strength MS media modified with amino acids L-proline and L-asparagine,  $\text{KH}_2\text{PO}_4$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in sorghum somatic embryogenesis and compare their efficiency in quality callus induction thereof with the induction on non-modified half strength MS [17], N6, B5 and 190-2 media [25-27].

## 2 Materials and Methods

### 2.1 Plant Materials

Ten sorghum [*Sorghum bicolor* (L.) Moench] genotypes namely hybrids ‘Alföldi 1’, ‘GK Emese’, ‘GK Zsófia’, ‘Róna 1’, ‘GK Áron’, ‘GK Erick’, ‘GK Csaba’, and candidates ‘ARET × VSZ21KKD’, ‘(A119 × KS60B) × SMRIL’, ‘AIL-1 × B119 × Va-Cir’ as varieties V01, V02, V03, V09, V10, V12, V16, V05, V14 and V15 respectively were obtained from a breeding program at Cereal Research Non-Profit Ltd., Szeged, Hungary.

### 2.2 Sterilization of Plant Explants

A set of 100 mature seeds from each genotype was sterilized separately by soaking them in 70% ethanol for 2 minutes and followed by agitation in 50 ml of 4% sodium hypochlorite and two drops of tween 20 for 30 minutes. This was followed by 3 washes with sterilized distilled water in a laminar air

flow cabinet. The seeds were dried on sterile filter papers before placing them on a half strength MS [17] basic medium for germination in light.

### 2.3 Comparison of the Induction Media

To test the efficacy of the six medium types (Tab. 1) in callus induction, twenty 1 mm pieces of mesocotyls from 1.5-day old germinating embryos were placed on each media type contained in 90 mm × 15 mm plastic Petri dishes (Sarstedt, Newton, MA, USA). Each Petri dish was a treatment in the Randomized Complete Block Design (RCBD) experiment, comprising of 3 replications. All the medium types were autoclaved at 121°C for 20 mins after appropriate pH adjustment. The explants were incubated for four weeks in a dark thermostat at 28°C.

**Table 1:** The callus induction and regeneration medium types under trial denoted with letters A-F and R1-R3 respectively

Medium type	Constituents
A	½ MS -2.2 g/l MS powder (MS0222), 2 mg/l 2,4-D, 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.7
B	½ 190-2 (Zhuang and Xu 1983), 2 mg/L 2,4-D, 20 g/l sucrose, 7 g/l agar, pH 5.8
C	½ N6 (Chu et al.1975) with 950 mg/l KNO <sub>3</sub> and 825 mg/l NH <sub>4</sub> NO <sub>3</sub> , 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.8
D	½ B5 (Gamborg <i>et al.</i> 1968) powder (1.58 g), 2 mg/l 2,4-D, 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.8
E	A (½ MS -2.2 g/l MS powder (MS0222), 2 mg/l 2,4-D, 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.7), 1.0 g/l KH <sub>2</sub> PO <sub>4</sub> , (1.0 g/l L-proline, 1.0 g/l L-asparagine and 0.16 mg/l CuSO <sub>4</sub> ·5H <sub>2</sub> O) - filter sterilized
F- Control	MS- 4.4 g/l MS powder (MS0222), 2 mg/l 2,4-D, 20g/l sucrose, 2.8 g/l Gelrite, pH 5.7 1.0 g/l KH <sub>2</sub> PO <sub>4</sub> , (1.0 g/l L-proline, 1.0 g/l L-asparagine and 0.16 mg/l CuSO <sub>4</sub> ·5H <sub>2</sub> O) – filter sterilized
Liu <i>et al.</i> 2015	
R1	4.4 g MS powder (MS0222), 30 g sucrose, 2.8 g Gelrite®, pH 5.7, 1.0 g zeatin, 1.0 g 6-benzylaminopurine (BAP), 0.16 mg CuSO <sub>4</sub> × 5H <sub>2</sub> O
R2	4.4 g MS powder (MS0222), 30 g sucrose, 2.8 g Gelrite®, pH 5.7, 1.0 g indole-3-acetic acid (IAA), 1.0 g 6-benzylaminopurine (BAP), 0.16 mg CuSO <sub>4</sub> × 5H <sub>2</sub> O
R3	190-2

Counts of the yellow and brown calli were recorded as a percentage of the total explants in the Petri dish. The same procedure was applied for all the friable yellow coloured calli.

### 2.4 Regeneration

Embryogenic calli from 3 of the 10 genotypes under study namely ‘GK Emese’, ‘GK Zsófia’ and ‘Róna 1’ were incubated under light in regeneration media MS supplemented with cytokinin hormones zeatin and 6-benzylaminopurine (BAP) and CuSO<sub>4</sub>×5H<sub>2</sub>O, MS supplemented with auxin indole-3-acetic acid (IAA), BAP and CuSO<sub>4</sub> × 5H<sub>2</sub>O (all the supplements filter sterilized into the medium after autoclaving, following a published protocol [13] ) and 190-2 medium [28] denoted as R1, R2 and R3 respectively (Tab. 1) in 90 × 15 mm plastic Petri dishes (Sarstedt, Newton, MA, USA). This was done 4 weeks after induction. The regenerated shoots were subsequently transferred to 190-2 medium in glass tubes and incubated under light for rooting induction. Data of the regenerants in the three-regeneration media was analyzed with R Commander X64 3.4.4 edition software. The regenerants were then acclimatized in a greenhouse for 3 weeks before being transplanted to a field nursery.

### 2.5 Statistical Analyses

Means of yellow, brown and friable calli with the six treatments and 10 varieties were calculated and compared by Duncan’s Multiple Range Test and two-way Analysis of Variance (ANOVA) using

RStudio and R software edition x64 3.4.4 software (Tab. 2). Plots showing the means of yellow, brown and friable calli as well as those depicting differences in the means with the six treatments were made using RStudio software.

### 3 Results

The six media showed significantly different ( $p < 0.05$ ) average yellow callus percentages. Medium type E (Tab. 1) had a significantly higher average yellow callus percentage at 59.7% than medium D, A, B and C (in decreasing order) with average percentages 31.9, 27.6, 22.6 and 19.3 respectively (Tab. 2). The control medium type F had a lower average yellow percentage than medium type E, but these two were not significantly different.

**Table 2:** Comparison of the average yellow, friable and brown calli across the six medium types and 10 genotypes. The different letters mark the significantly different values

Medium	Avg. Yellow calli %	Avg. Friable calli %	Avg. Brown calli %
A	27.6 “a”	22.1 “b”	59.8 “ab”
B	22.6 “a”	5.6 “a”	60.5 “ab”
C	19.3 “a”	1.0 “a”	71.5 “b”
D	31.9 “a”	0.7 “a”	59.8 “ab”
E	59.7 “b”	25.1 “b”	40.3 “a”
F	40.0 “ab”	19.0 “b”	58.3 “ab”
P value	0.000104 ***	0.0000000697 ***	0.0217 *
Genotype			
V01	42.4 “ab”	20.2 “a”	50.8 “ab”
V02	51.7 “b”	18.2 “a”	41.4 “a”
V03	41.5 “ab”	17.7 “a”	46.7 “a”
V05	32.3 “ab”	11.1 “a”	57.9 “ab”
V09	30.8 “ab”	15.7 “a”	61.2 “ab”
V10	53.7 “b”	20.6 “a”	40.8 “a”
V12	23.1 “ab”	3.2 “a”	68.1 “ab”
V14	21.2 “ab”	6.2 “a”	69.7 “ab”
V15	12.5 “a”	2.6 “a”	80.0 “b”
V16	26.0 “ab”	6.9 “a”	67.2 “ab”

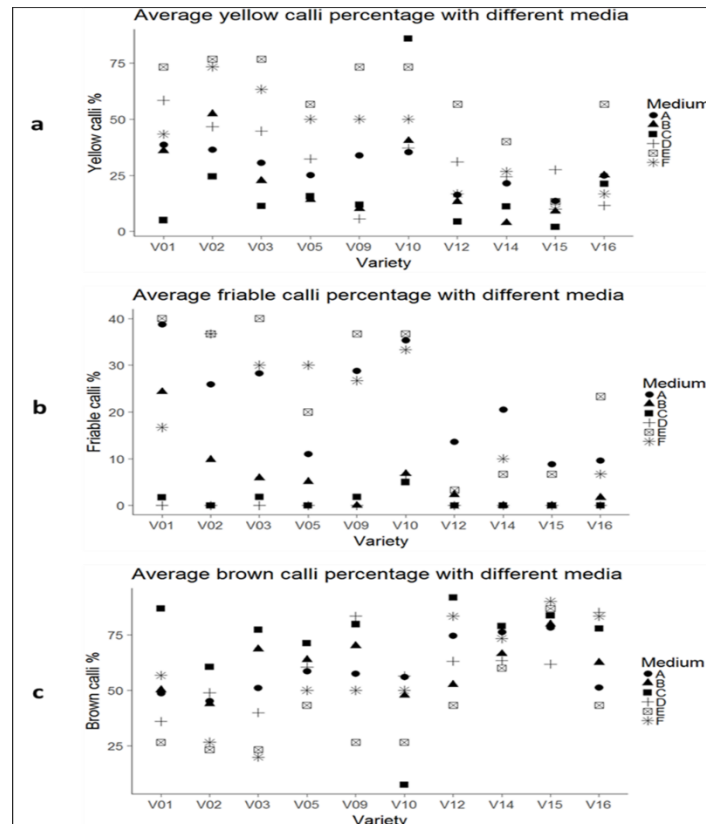
\*\*\*- significant at 0.001 \*- significant at 0.05

All the MS-based media types had higher friable callus percentages which were significantly different from the non-MS-based types B, C and D (Tab. 2). The half-strength MS media type E and A had the highest average friable callus percentages at 25.1% and 22.1% respectively, which were not significantly different from that of the control medium F which had an average of 19.0%. The non-MS-based media types had very low average friable callus percentages at 5.6% for medium B, 1.0% for medium C and 0.7% for medium D.

Medium E had a significantly lower average brown callus percentage at 40.3% than medium C which exhibited the highest average (Tab. 2). Occurrence of brown callus was seen to be influenced by the genotype, with candidate ‘AIL-1 × B119 × Va-Cir’ having the highest at 80.2%. Hybrid ‘GK Áron’ had the highest average yellow callus frequency at 53.7% and the least average brown callus at 40.8%. On the contrary, friability of the callus (Tab. 2) was not influenced by genotypes ( $p > 0.05$ ). Medium E generally had the highest average yellow callus across the genotypes (Fig. 1(a)) followed by the control medium type

F. All the MS-based medium types had generally the highest average friable callus percentage across the 10 genotypes, with medium type E having a higher quantity callus in 6 of the 10 genotypes (Fig. 1(b)), compared to the control which had a higher average on only one genotype. Medium type E tied with control medium F for the highest average friable calli percentage score for variety ‘GK Emese’.

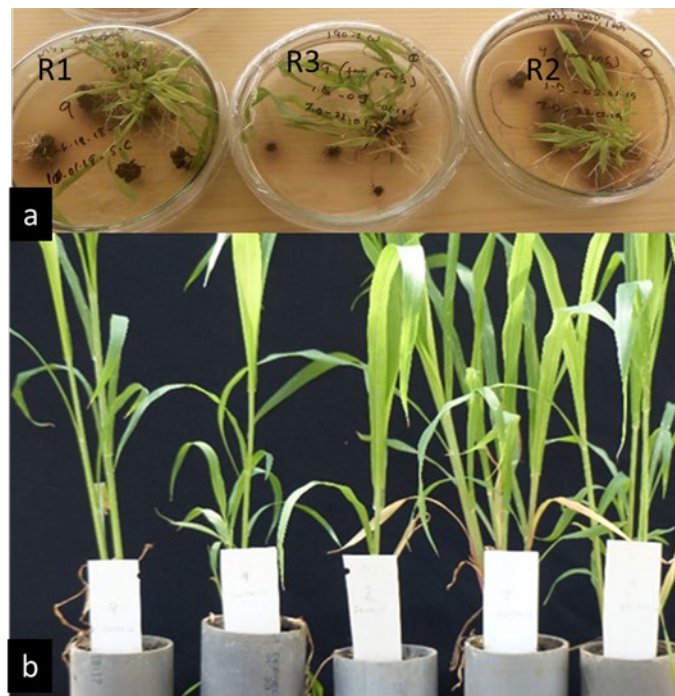
Regeneration frequency was highest with genotype ‘Róna 1’ in 190-2 medium at 40%, followed by ‘GK Zsófia’ in the same medium at 33%. ‘GK Zsófia’ had the highest average shoots per explant (5) in medium R2 followed by ‘Róna 1’ with 4 shoots per explant in medium R3. The latter also produced the highest number of roots per explant across the genotypes (Tab. 3) and therefore selected as the preferred rooting medium among the three. From the in vitro culture, the regenerants (Fig. 2) were transplanted to the glasshouse, where the plantlets were acclimatized very well and quickly.



**Figure 1:** Scatter plots of callus percentages (a) average percentages of yellow (b) friable and (c) brown calli with the six medium types across the 10 genotypes

**Table 3:** Regeneration frequency, roots and shoots per explant with 3 regeneration media across 3 genotypes

Genotype	Medium	Regeneration Frequency (%)	Shoots per explant	Roots per explant
GK Emese	R1	13.3	1	1
	R2	6.7	1	1
	R3	0	0	0
GK Zsófia	R1	6.7	3	3
	R2	26.7	5	5
	R3	33.3	2	3
Róna 1	R1	13.3	1	1
	R2	20.0	1	3
	R3	40.0	4	7



**Figure 2:** Regenerants of variety ‘Róna 1’ (a) plantlets on regeneration media R1, R2 and R3 (b) acclimatization of regenerants in the greenhouse prior to transplanting

#### 4 Discussion

This study explored the effect of six callus induction media (referred to in this study as types A, B, C, D, E and F; Tab. 1) on their effect in generation of embryogenic callus from mature embryo explants of 10 Hungarian sorghum genotypes (hybrids ‘Alföldi 1’, ‘GK Emese’, ‘GK Zsófia’, ‘Róna 1’, ‘GK Áron’, ‘GK Erick’, ‘GK Csaba’, and candidates ‘ARET × VSZ21KKD’, ‘(A119 × KS60B) × SMRIL’, ‘AIL-1 × B119 × Va-Cir’). The study allowed an investigation on the difference in the effect of full strength and half-strength MS media modified with amino acids L-proline and L-asparagine,  $\text{KH}_2\text{PO}_4$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in sorghum somatic embryogenesis and to compare their efficiency in quality callus induction thereof with the induction on non-modified half strength MS, Chu N6, Gamborg B5 and 190-2 media. The information generated would provide clarity in sorghum somatic cultures, necessary for upstream processes such as doubled-haploid production, genetic transformation and others.

An embryogenic callus [23] which is yellow, compact and friable is influenced by the medium type [21] and the constituents of the media. This study observed that half strength MS media substituted with amino acids L-proline and L-asparagine,  $\text{KH}_2\text{PO}_4$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  gave more yellow, and friable sorghum calli compared to full-strength media substituted with the same components. This result gives more credence to the earlier findings that MS based media resulted in high callus induction rates [13], but also highlight the strength of MS media’s effect on quality of the sorghum calli. The observation agrees with earlier findings [19], where sensitivity of explants on the strength of MS media was reported, although the work was on *Costus pictus*.

The addition of components amino acids L-proline and L-asparagine,  $\text{KH}_2\text{PO}_4$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  contributed significantly in maintaining yellow colour of the calli, a finding that corroborates the observation that addition of the components to MS media contributed to reduction of phenolics in sorghum tissue culture [13,15] and led to increased callus induction rates. Indeed, proline has been shown to have a strong antioxidant effect [29], which may have contributed to phenolic production inhibition.

This observation is notable by the difference of medium type A which was MS medium without the components and medium types E and F, both of which had the additional components. The MS based media regardless of the strength were seen to produce friable calli, unlike the non-MS based media, although half strength medium types E and A resulted in slightly more friable calli than full strength medium type F. This result contrasts the findings [20] that friable (embryogenic) callus resulted on the addition of amino acids L-proline and L-asparagine, since there was no significant difference on average friable calli percentage between medium type A and medium types E, F considering that the former did not have the amino acids. Further, our findings diverge from the observation that Chu N6 medium resulted in more embryogenic calli than MS based media [20], although the Chu N6 medium used in this study did not contain the amino acids.

This study found that average yellow callus percentage was influenced by genotype whereby there were significant differences observed among both the varieties and culture media and therefore partly agree with the findings that sorghum embryogenesis is genotype dependent [13]. This result is however true only as far as the callus colour is concerned and not the average friable calli percentage, where there was no difference as a result of the varieties. Our study provides the much-needed clarity in sorghum embryogenesis, regarding the effectiveness of MS based media as compared to non-MS types on sorghum calli induction, as well as the sensitivity of sorghum explants to the strength of MS media, making half strength MS media more effective in quality calli generation.

As aforementioned many authors have reported varied degrees of successful regeneration with different medium types and varieties for sorghum [13,15,16]. These results however, as it has been observed [14], have not been reproducible across different varieties for sorghum. Our study gave an average regeneration frequency of 40% with medium 190-2 and variety 'Róna 1' higher than the results of the other two tested medium types, which had produced high regeneration frequencies [13] in earlier reports.

**Acknowledgement:** We are grateful to the Stipendium Hungaricum Scholarship under whose support this work was undertaken. This work was also supported by grants from the Higher Education Institutional Excellence Program of the Ministry of Human Capacities in Hungary within the framework of plant breeding and plant protection researches of Szent István University ((20430--3/2018/FEKUTSTRAT), 2017-1.3.1-VKE-2017-0030 and the EFOP-3.6.3-VEKOP-16-2017-00008 project (co-financed by the European Union and the European Social Fund).

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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