International Research Journal of plant and Crop Sciences ISSN: 1711-3490 Vol. 1(1), pp. 005-014, November, 2013. Available online at www.advancedscholarsjournals.org © Advanced Scholars Journals

Full Length Research Paper

Development of a highly efficient *in vitro* Culture system for Ugandan adapted sorghum Genotypes

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Accepted 22 October, 2013

World over sorghum genetic transformation is limited by its recalcitrance to tissue culture and genotype dependent regeneration and Ugandan adapted sorghum genotypes may not be exceptional. This study intended to develop an efficient *in vitro* culture system for Ugandan adapted sorghum genotypes and identify genotypes amenable to *in vitro* culture under different test conditions. Ages of IZE and phytohormone callus induction medium composition were optimized. Twenty Ugandan adapted sorghum genotypes were screened on two medium types; Murashige and Skoog and L3 basal medium. Optimum *in vitro* culture responses were achieved at 2, 4-D level of 2.5 mg/l supplemented with 0.5 mg/l of kinetin hormone. Inclusion of kinetin hormone in callus induction medium at low 2, 4-D levels gradually reduced the callus induction frequency while higher 2, 4-D levels exhibited inhibitory effects. The best age of IZE for *in vitro* culture was between 16-18 days post anthesis. IZE physiological state of different genotypes at the same age varied. Strong genotype by medium type influenced *in vitro* culture responses rather than the genetic control only. MUC007/194 and MUC007/193 have been earmarked as model genotypes for *in vitro* culture and can be systems to introgress useful genes into recalcitrant but commercially viable genotypes.

Key words: Phenolic compounds, 2, 4-Dichlorophenoxyacetic acid (2, 4-D), Kinetin, explants age, genetic transformation.

INTRODUCTION

Sorghum (*Sorghum bicolor* (L) Moench) belongs to the family Poaceae and genus *Sorghum* which is taxonomically separated into five subgenera; *Eu-sorghum*, *Chaetosorghum*, *Heterosorghum*, *Para-sorghum* and *Stiposorghum* (Garber, 1950). All cultivated sorghum races and varieties belong to the *Eu-sorghum* section as *Sorghum bicolor* subsp. *bicolor* (Dogget, 1988). Sorghum is the most important crop in the semi-arid tropical and sub-tropical Africa, and parts of Asia where persistent drought, high temperatures and poor soil nutrient availability are common (Dicko *et al*., 2006;Chakauya *et al*., 2006; O'Kennedy *et al*., 2006). The crop has a great potential as human food, animal feed and industrial raw material owing to its adaptability to drought and water logged conditions. However, sorghum crop suffers significantly from an array biotic and abiotic stresses. Sorghum also has poor nutritive quality due to low calcium content, lysine deficiency and reduced protein digestibility.

Sorghum improvement has relied on traditional breeding approaches for many years, but these avenues can only exploit variation present in species that can be crossed with sorghum (O'Kennedy *et al*., 2006). Important genes (for example striga resistance, stem borer resistance, drought tolerance, better nutritive quality), for further sorghum improvement exist in species outside *Eu-sorghum* section (Sharma and Franzmann,2001; Kamala *et al*.,2002). These genes have not been used because of hybridisation failures (Price *et al*., 2005b; Hodnett *et al*., 2005). Genetic transformation which is underpinned by tissue culture offers a direct access to this vast pool of useful genes. A high frequency of plant regeneration is a prerequisite for successful genetic transformation of cereal crops (Rachmawati and Anzai, 2006). Nonetheless, world over sorghum transformation has been lagging behind other cereals due to its recalcitrance to tissue culture and genotype dependent regeneration

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and Ugandan adapted sorghums may not be exceptional (Godwin and Gray, 2000; Jogeswar *et al*., 2007). Various factors have been reported to influence *in vitro* cereal regeneration frequency including sorghum namely: choice of explants, medium composition, culturing and regeneration conditions (Vasil, 1994). Sorghum *in vitro* culture system development has lagged behind other cereals possibly because of the extremely high levels and diversity of phenolic compounds present in sorghum compared to other cereals (Dicko *et al*., 2006). This challenge underpins research and development strategies for sorghum and the focus of this study was to develop efficient *in vitro* culture techniques for Ugandan adapted sorghum genotypes as well as select candidate genotypes highly amenable to tissue culture under different test conditions.

MATERIALS AND METHODS

Sorghum plant materials used and Explant culture

Twenty genetically distant Ugandan elite sorghum lines were selected among the Makerere University sorghum collections of 2007 (MUC007) based on quality characteristics (high protein and low tannin content). The selected elite sorghum lines were grown in sterile potted forest loam soil in a screen house but were later moved outside two weeks after germination. The potted seedlings were thinned to two plants per pot and poultry manure was applied into the plastic pots. The sorghum plants were sprayed against aphids and sorghum shoot fly using Dimethoate 40EC. At the booting stage, bagging was used to ensure controlled self-pollination. The sorghum plants were grown at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK) located in Lake Victoria crescent agro ecological zone of Uganda at about 1200 m above sea level. It lies between 00^{0}28'N latitude and 32^{0}37'N longitude.

Immature seeds were removed from panicles of each test sorghum accession harvested 15 days post anthesis using a pair of tweezers and placed in a glass Petri dish. The seeds were surface sterilised in 70 % ethanol for 2-3 min and then immersed in a 2.5 % Sodium hypochlorite (Reckitt Benckiser, Kenya) solution in which 3-4 drops of commercial detergent has been added as described by Grootboom *et al*., (2008). After this treatment the seeds were carefully rinsed 3-4 times in sterile double distilled water. Twenty immature zygotic embryos were aseptically isolated from seeds and cultured on callus induction medium with the scutellum side facing up. Sub-culturing was done after every two weeks to medium of the same composition. After, a period of 3-4weeks, the callus was transferred to callus maturation medium for 2 weeks. The callusing frequency was determined 4 weeks post initiation of culture while frequency of embryogenesis was determined after the following two weeks. After 2 weeks, the calli with developed somatic embryos were transferred to shoot induction medium for another 3 weeks. Finally the developed shoots were transferred to a rooting medium for four weeks.

Data on callus induction frequency which was taken as a percentage of cultured immature zygotic embryos forming callus, callus growth score on a visual scale of (0 = no callus growth, 1 = poor growth with large dark coloured parts, 2 = slight growth with some darkened parts, 3 = compact, white callus with some darkened and loose parts, 4 = compact, hard, white callus with some darkened parts, 5 = white, compact, and hard callus without dark parts), somatic embryogenesis frequency considered as the percentage of cultured embryos showing germinated embryos, and number of shoots per 5 explants selected at random was recorded.

Optimisation of regeneration medium phytohormone composition

Preliminary studies to optimise phytohormone concentrations in callus induction medium and rooting medium were conducted using an immature zygotic embryo regeneration system (Hagio, 2002). The callus induction medium was composed of MS basal salts + vitamins mg/l [0.5 nicotinic acid +0.5 pyridoxine HCl +1 mg/l thiamine HCl + 0.1 g/l myo-inositol +30 g/l sucrose+1 % Polyvinypyrolidone+ 3 g/l Gelrite + varying concentrations of 2,4-D (0, 1, 2, 2.5 and 3 mg/l) in combination with varying concentrations of kinetin (0, 0.1, 0.2 0.5 mg/l) pH 5.8 was autoclaved at 121^{o}C for 15min and dispensed into pre-autoclaved glass Petri-dishes or glass jars under sterile conditions. From the preliminary study carried out, a combination of 2-3 mgl^{-1} 2, 4-D and 0.5 mgl^{-1}kinetin was the best in inducing regenerable callus. The callus maturation medium used was of the same composition as the callus induction medium, except that it had reduced concentration of 2, 4-D of 2.0 mg/l and 0.5 mg/l kinetin. The shoot induction medium was of the same composition as callus induction medium without 2, 4-D but with kinetin concentration maintained at 0.5 mgl^{-1}. The effect of Naphthalene Acetic Acid (NAA), Indole Butylic Acid (IBA), and Indole -3-Acetic Acid (IAA) in varying combinations of 0 mgl^{-1}growth hormones, 1 mg/l NAA, 1 mg/l IBA, 0.2 mg/l (NAA+IBA+IAA), 0.5 mg/l (IBA+NAA) and 0.5 mg/l (NAA+IAA) in rooting medium were tested on induced shoots (data not shown). The rooting medium with 1 mg/l NAA induced roots formation quickly on regenerated shoots and all shoots cultured formed roots. All the phytohormones, Vitamins, Gelrite and Polyvinylpyrolidone and amino acids used in this study were supplied by Duchefa Biochemie, Germany. Micro and macro salt components were supplied by Sigma Aldrich, USA.

Optimisation of developmental stage of immature zygotic embryo

Panicles were harvested from sorghum plants of genotypes MUC007/193, MUC007/194, and MUC007/178

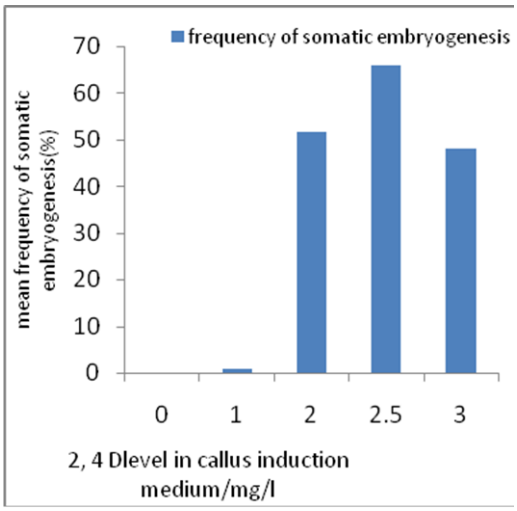


Figure 1A

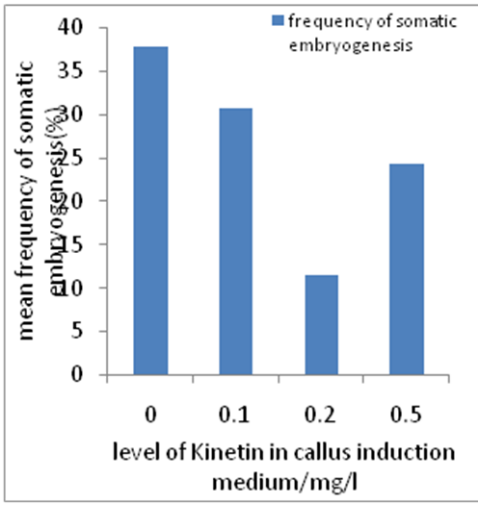


Figure 1B

Figure 1. Effect of phytohormone levels in callus induction medium on somatic embryogenesis.The figure on the left marked 1A for 2, 4-D and on the right marked 1B for kinetin.

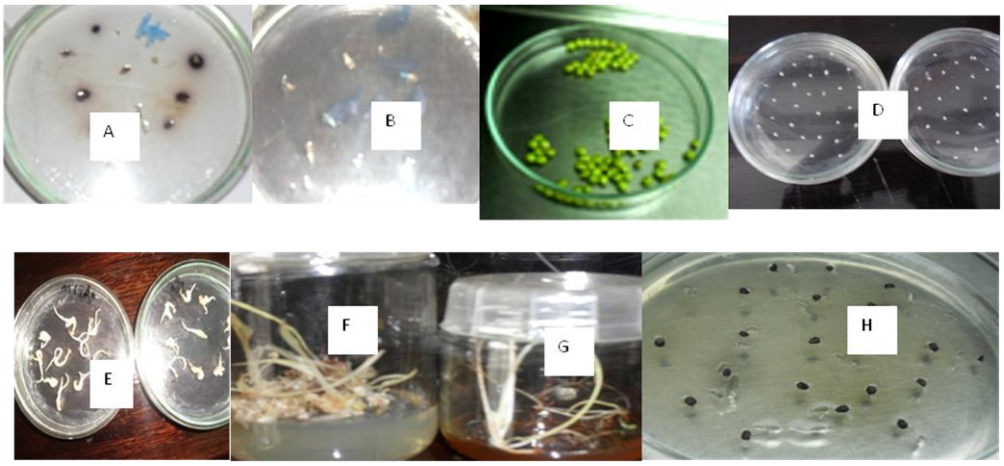


Plate 2. Sorghum *explant*s used and response of Ugandan adapted sorghum genotypes to different *in vitro* conditions (A) Black phenolic compounds inhibited immature embryo, (B) Immature embryo exhibited growth response on PVP (antioxidant) supplemented callus induction medium, (C) Sorghum seeds isolated from panicle, (D) Aseptically isolated immature zygotic embryos on CIM, (E) Callus initiation 7 days post culture (F) Callus initiated on 2,4-D supplemented medium (G)Shoot initiated from Immature zygotic embryos cultured on 2,4-D free medium, and (H) Response of Immature zygotic embryo isolated 14 days post anthesis.

grown in pots at 14, 16, 18 and 20 days post anthesis to optimize the best developmental stage of Immature Zygotic Embryo for producing morphogenic callus and sorghum regeneration. The seeds were surface sterilized in 70 % ethanol for 2-3 minutes and then immersed in a 2.5 % Sodium hypochlorite solution in which 3-4 drops of commercial detergent had been added as described by Grootboom *et al*. (2008). After this treatment the seeds were carefully rinsed 3-4 times in sterile double distilled water. Twenty immature zygotic embryos were aseptically isolated from seeds and cultured on callus induction medium with the scutellum side facing up. Sub-culturing was done after every two weeks or less (depending on level of phenolic compounds produced) to culture medium of the same composition. After, a period of 3-4 weeks, the callus was transferred to callus maturation medium depending on genotype for 2 weeks. The callusing frequency was determined 4 weeks post initiation of culture while frequency of embryogenesis was determined after the following two weeks. After 2 weeks, the calli with developed somatic embryos were transferred to shoot induction medium for further 3 weeks.

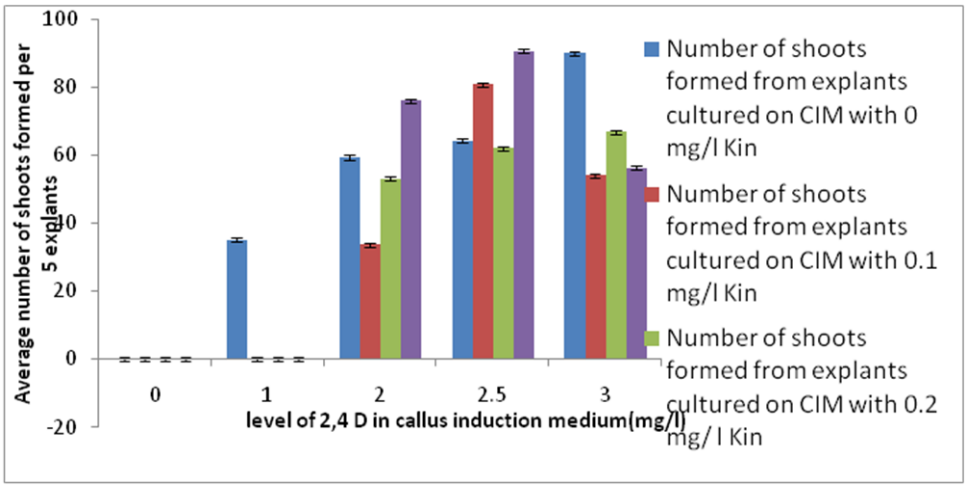


Figure 2. Effect of 2, 4-D and kinetin level in callus induction medium on number of shoots formed.

The developed shoots were transferred to a rooting medium that was half strength Murashige and Skoog (MS) medium supplemented with 1 mg/l naphthalene acetic acid for further four weeks. Data on callus induction frequency, somatic embryogenesis frequency, and number of shoots per 5 *explant*s selected at random was recorded.

Identification of elite Ugandan sorghum genotypes with high potential of embryogenic callus formation and regeneration

Ugandan sorghum genotypes selected among the Makerere University sorghum collections of 2007 were screened for their *in vitro* response on two medium types: Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) and L3 basal medium (Jähne *et al*., 1991). L3 medium differs from Murashige and Skoog with respect to ammonium nitrate, vitamins and amino acids. L3 medium has been recommended for callus induction and regeneration of recalcitrant crops and cultures for example microspores of cereals (Mordhorst and Lörz, 1993). The two medium types Murashige and Skoog based and L3 based medium were used with optimised phytohormone concentrations of 2.5 mg/l 2, 4-D with 0.5 mg/l kinetin in callus induction medium to screen the regeneration potential of twenty sorghum genotypes selected. The rooting medium used was comprised of half strength salts and supplemented with 1 mg/l NAA in both L3 and MS basal medium. Data on callus induction time (number of days post culture initiation to callusing), callusing frequency, somatic embryogenesis frequency, and number of regenerants formed was collected as previous described.

Data Analysis

The data collected in this study as frequencies and counts was subjected to arcsine and square root transformation respectively before Analysis of variance (ANOVA) to normalize the data (Steel *et al*., 1997; McDonald, 2009). Where significant differences were found, means were separated using Least Significant difference test at $P \leq 0.05$. The analysis of data was carried out using GenStat discovery edition 3.

RESULTS AND DISCUSSION

RESULTS

Effect of 2, 4-D and kinetin hormone levels in callus induction medium on in vitro culture responses of Ugandan adapted sorghum genotypes

The callus induction frequency of sorghum was significantly influenced by the level of kinetin hormone ($p \leq 0.05$) and 2, 4-D ($p<0.001$) in the callus induction medium. However, there was no significant multiplicative effect between the two hormones on callus formation ability of sorghum. A significantly high callus induction frequency was observed at a 2, 4-D level of 2.5 mg/l (75.54 %) and 0 mg/l of kinetin (47.6 %). Embryos cultured on medium supplemented with either 1 mg/l 2, 4-D or without 2, 4-D germinated to form shoots and no callus was formed. Generally, addition of kinetin hormone in the callus induction medium at low levels of 2, 4-D significantly reduced the callus induction frequency of sorghum. The combined effects of kinetin and 2, 4-D in callus induction medium, highly significantly affected ($p<0.001$) callus growth. Significantly good callus growth was also observed on medium supplemented with 3 mg/l 2, 4-D without kinetin, 2.0 mg/l 2, 4-D together with 0.5 mg/l kinetin or no kinetin and 2.5 mg/l 2, 4-D with, 0, 0.1 or 0.5 mg/l kinetin. A significant poor growth was observed on callus from medium supplemented with high levels of kinetin at 2, 4-D level of 3 mg/l. No callus growth was observed on callus induction medium supplemented

Figure 3A

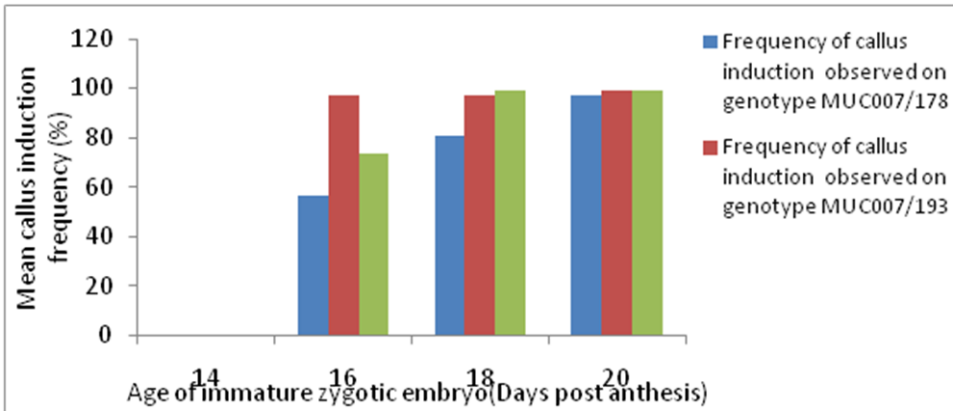
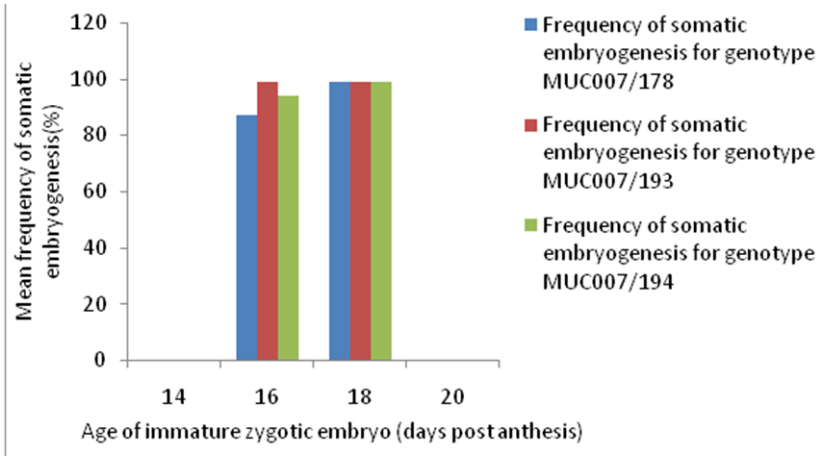


Figure 3B



with 1 mg/l of 2, 4-D and kinetin hormone. This was probably due to the fact that the effect of kinetin outweighed the 2, 4-D effect on embryos resulting into induction of shoots instead of callus.

Different levels of 2, 4-D and Kinetin hormones in callus induction medium greatly influenced ($p<0.001$) the frequency of somatic embryo formation and number of shoot formation on calli cultured on maturation medium.

Significantly high frequency of somatic embryogenesis was obtained on *explant*s cultured on callus induction medium supplemented with 2.5 mg/l 2, 4-D. Addition of Kinetin hormone resulted in a drastic reduction in frequency of somatic embryogenesis in an ambiguous trend (Figure 1). Similarly, addition of kinetin hormone in callus induction medium supplemented with 1 mg/l 2, 4-D inhibited plant regeneration through somatic embryogenesis and no regenerants were obtained. The immature zygotic embryos cultured on this callus induction medium germinated to form shoots and roots instead of forming callus (Plate 2(G)).

The number of shoots formed were highly influenced ($p<0.001$) by the interaction of kinetin and 2, 4-D hormones in callus induction medium. The largest number of regenerants was achieved on calli from embryos cultured on callus induction media supplemented with 3 mg/l 2, 4-D without kinetin, and 2.5 mg/l 2, 4-D together with 0.5 mg/l kinetin hormone (Figure 2).

Effect of growth and developmental stage of sorghum embryos on callus induction frequency, somatic embryogenesis and number of regenerants

The age of sorghum panicles has always been used to make harvesting decisions for immature zygotic embryo used *in vitro* culture. In this study, the developmental stage of the cultured immature zygotic embryo highly influenced ($p<0.001$) the callus induction frequency, somatic embryogenesis and number of regenerants formed among the three genotypes used. The callus induction frequency, somatic embryo formation frequency, and number of regenerants formed were significantly different ($p<0.001$) among the three used so-

Figure 3C

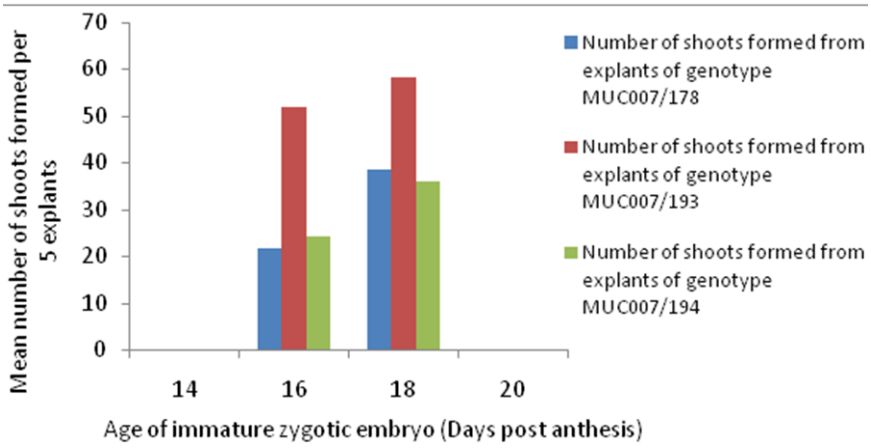


Figure 3. Effect of age of zygotic embryo on callus induction and regeneration among three candidate sorghum genotypes. The figure on top marked 3A shows effect on callus induction frequency; figure in the middle marked 3B shows effect on frequency of somatic embryogenesis whereas figure marked 3C indicates the effect on number of shoots formed.

rghum genotypes. Although genotype and developmental stage of embryo cultured independently affected *in vitro* culture responses, their interaction greatly influenced these responses. This suggests physiological difference among immature zygotic embryos of different genotypes at the same harvesting age. The embryos harvested 14 days post anthesis did not form callus among the three candidate genotypes because the embryos died before germinating (Plate 2 (H)). The genotypes MUC007/194 and MUC007/178 had the highest callus induction frequency when their embryos cultured were harvested at 18 and 20 days post flowering while genotype MUC007/193 had the highest callus induction frequency when embryos cultured were harvested above 14 days post flowering (Figure 3). Although, the callus induction frequency was highest for the three genotypes at the harvesting age of 20 days, all the calli formed were non embryogenic hence no somatic embryos were obtained. The major challenge remains on how to produce somatic embryos from mature embryos of sorghum in Uganda. Genotypes MUC007/178 and MUC007/194 had the highest formation of embryogenic calli when embryos were harvested at 18 days after flowering while genotype MUC007/193 showed formation of embryogenic calli at both 16 and 18 days (Figure 3B). The largest number of regenerants was significantly formed among the three genotypes when their embryos were harvested at 18 days post flowering and no regenerants were achieved at harvesting time of 14 and 20 days post flowering (Figure 3C).

Ugandan adapted sorghum genotypes with high potential of embryogenic callus formation and regeneration

Twenty sorghum genotypes used in this study showed significant differences ($p<0.001$) in terms of callus induction time and frequency, callus growth, somatic embryo formation frequency, and shoot formation. Although the callus induction time and frequency, and frequency of somatic embryogenesis of sorghum genotypes was significantly different ($P\leq0.05$) between the two media types used, growth of induced calli and shoot formation was not influenced by medium type. All sorghum *in vitro* culture parameters considered were highly affected ($p<0.001$) by the interaction between genotype and medium type.

The genotypes MUC007/102, MUC007/107, MUC007/111, MUC007/114, MUC007/127, MUC007/224, MUC007/162, MUC007/167, MUC007/178, MUC007/192, MUC007/194 and MUC007/63 showed a significantly higher callus induction frequency on L3 medium (Jähne *et al*., 1991) than on Murashige and Skoog medium (Murashige and Skoog, 1962) while genotypes MUC007/124 and MUC007/193 had the highest callus induction frequency on Murashige and Skoog compared to L3 medium. On L3 medium genotypes MUC007/111 and MUC007/194 had the highest callus induction frequency of 96.73 % while on MS medium, genotype MUC007/193 showed the highest callus induction frequency of 98.75 % followed by genotypes MUC007/194 (90 %) and MUC007/124 (80.69%). The sorghum genotypes MUC007/80, MUC007/88, MUC007/93, MUC007/99, and MUC007/189 showed the lowest callus induction frequency on both medium types (Table 1).

The callus induction time estimated as the number of days at which callus is formed post culture initiation was highly influenced by the multiplicative effect of medium and sorghum genotype. Genotypes MUC007/194 and MUC007/193 formed callus faster on Murashige and Skoog medium (7 days post culture initiation) than on L3

Table 1. Mean in *vitro* responses among the 20 elite Ugandan sorghum genotypes on two different medium types.

Sorghum genotype	Mean callus induction frequency		Mean number of days to callus initiation		Mean callus growth score		Mean somatic embryogenesis frequency		Mean number of regenerants formed per 5 *explants*	
	L3 medium	MS medium	L3 medium	MS medium	L3 medium	MS medium	L3 medium	MS medium	L3 medium	MS medium
MUC007/100	68.37	76.82	17	16	1.33	2	46.65	56.69	43.44	42.56
MUC007/102	86.99	70.33	10	14	3	2.33	83.64	63.55	95.01	27.07
MUC007/107	74.67	22.15	14	15	1	1	27.96	13.01	20.04	27.01
MUC007/111	96.73	70.33	14	13	1.33	2	83.64	70.33	34.20	25.68
MUC007/114	89.02	68.37	13	13	1.33	1	80.39	42.80	30.24	22.62
MUC007/124	46.50	80.69	14	16	1	2.67	13.01	74.56	14.98	56.42
MUC007/127	63.90	25.44	18	16	1	1	56.84	22.15	11.90	36.15
MUC007/224	91.32	80.69	10	13	1.67	2	53.35	74.56	21.99	37.97
MUC007/162	93.79	67.09	8	12	1.67	1	91.32	60.14	46.77	31.24
MUC007/167	77.53	46.65	12	14	1	1	53.50	13.01	34.52	23.20
MUC007/178	90.00	56.69	14	14	2	1.33	90.00	39.86	45.00	34.83
MUC007/189	36.10	43.31	14	10	1	1	21.02	33.26	37.20	21.78
MUC007/192	80.39	73.48	9	10	1.33	1	65.21	63.90	25.35	25.55
MUC007/193	39.86	98.75	18	7	1	4.33	16.36	93.79	23.49	104.23
MUC007/194	96.73	90.00	10	7	4	3.33	83.64	83.64	81.53	64.16
MUC007/63	83.64	29.67	12.7	16	1.67	1	68.51	11.61	19.11	41.53
MUC007/80	29.67	29.67	15	17	1	1	19.31	15.72	27.30	29.16
MUC007/88	23.18	29.67	10	16	1.33	1	23.18	23.18	29.62	28.57
MUC007/93	10.00	25.44	20	14	0.33	0.67	10.00	16.74	1.11	10.33
MUC007/99	46.65	19.31	15	16	1	1	36.10	4.53	54.63	27.90
$LSD_{0.05}$	6.18	6.18	0.30	0.30	0.63	0.63	8.82	8.82	4.19	4.19
CV %	18.8	18.8	1.4	1.4			24.5	24.5	22.1	22.1

LSD = Fishers protected Least significant difference test calculated at $P \leq 0.05$ (Steel *et al*., 1997).

medium while genotypes MUC007/162 and MUC007/192 formed callus much faster on L3 medium than on MS medium. The genotypes MUC007/102 and MUC007/194 showed better callus growth on L3 medium while on MS medium, genotype MUC007/193 showed the best callus growth (Plate 3). The growth of callus from genotype MUC007/124 was better on MS than on L3 medium.

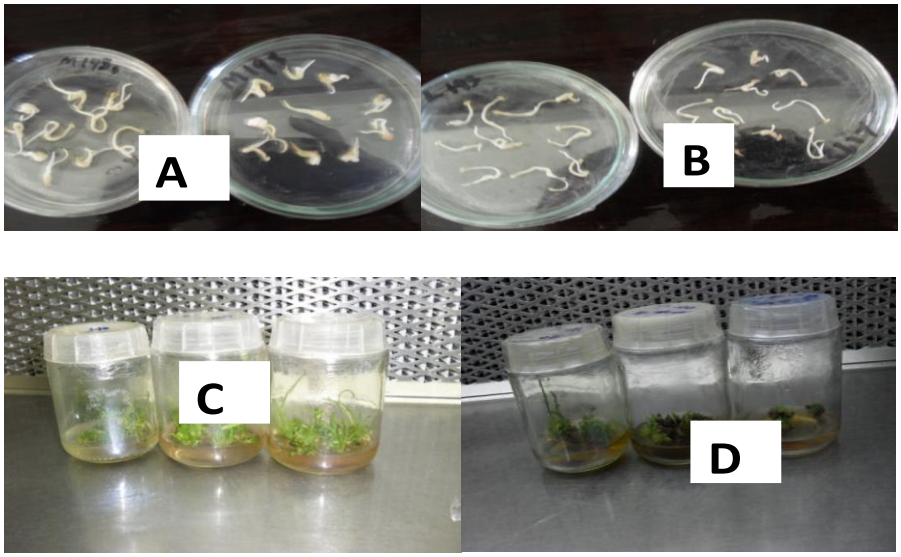


Plate 3. Response of Ugandan adapted sorghum genotypes on different types of *in vitro* culture medium (A)Response of sorghum genotype MUC007/193 on MS callus induction medium (B) Callusing Response of MUC007/193 on L3 callus induction medium, (C) Brown phenolic compounds produced in culture (D) Black phenolic compounds produced in culture.

The frequency of somatic embryogenesis indirectly measures the frequency of embryogenic callus formation among sorghum genotypes. On L3 medium, the sorghum genotypes showed the somatic embryogenesis frequency ranging from 10 % up to 91.31 % with genotypes MUC007/102, MUC007/111, MUC007/114, MUC007/162, MUC007/178, and MUC007/194 showing a somatic embryogenesis frequency above 80 %. On MS medium, only eight genotypes showed a frequency of somatic embryogenesis above 60 % with the genotypes MUC007/194 (93.8 %) and MUC007/193 (83.6 %) showing the highest frequency of somatic embryogenesis. In terms of frequency of somatic embryo formation, genotypes MUC007/80, MUC007/88, MUC007/93, MUC007/99, and MUC007/189 were ranked the worst (Table 1).

The genotype by medium interaction greatly affected the number of regenerants formed among selected sorghum genotypes. Genotype MUC007/193 formed the highest number of regenerants on Murashige and Skoog medium (20.9 shoots per *explant*) while sorghum genotypes MUC007/102 (19 shoots) and MUC007/194 (16.3 shoots per *explant*) showed the highest number of regenerants on L3 medium. Although genotype MUC007/99 had the lowest callus induction and somatic embryogenic frequency, it had a reasonably higher number of regenernts than most genotypes that were considered to perform better in terms of callus induction frequency and somatic embryo formation frequency (Table 1). This was possibly due to the differences in number of germinating embryos per *explant*.

Overall, the response of selected Ugandan sorghum genotypes was highly influenced by a multiplicative effect of genotype and medium and to some degree genotype dependent. Genotypes MUC007/193, MUC007/194, and MUC007/124 were highly regenerable on Murashige and Skoog medium with MUC007/193 and MUC007/194 forming callus faster than all other genotypes. They resulted in callus induction frequency above 90 % on MS medium while on L3 medium genotypes MUC007/102, MUC007/162, MUC007/178, MUC007/99, and MUC007/194 were highly regenerable.

DISCUSSION

Effect of 2, 4-D and kinetin hormone levels in callus induction medium on sorghum in vitro culture responses

Plant growth regulators play a vital role in cereal tissue culture. Several plant growth hormones have been developed and tested. Among them auxin and cytokinin products that are also natural plant growth regulators are being used. 2, 4-Dichlorophenoxyacetic acid hormone (2, 4-D) is one of the most widely used auxins for induction of callus and somatic embryogenesis in both monocots and dicot plant species (Nguyen *et al*., 1998; Saradamani *et al*., 2003; Jogeswar *et al*., 2007). In this study, callus induction frequency of sorghum was highly influenced by level of 2, 4-D and kinetin included in the media. An optimum callus induction frequency was obtained at a 2,4-D level of 2.5 mg/l and addition of kinetin hormone resulted into a drastic reduction in callus induction and somatic embryogenesis. Other studies have also observed that high levels of cytokinins indeed reduce callus induction (Oldach *et al*., 2001). Embryos cultured on callus induction medium supplemented with 2, 4-D lev-

el above 2.5 mg/l resulted in a substantial decline in callus induction and somatic embryogenesis. This suggests an inhibitory effect of increased levels of 2, 4-D on callus induction, growth, shoot induction and number of shoots per callus (Baskaran *et al*., 2005; Gupta *et al*., 2006). Inclusion of kinetin in callus induction medium however improves these responses. In other studies, a high frequency of somatic embryogenesis has been achieved on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 2 mg/l 2, 4-D and 0.5 mg/l of kinetin, which overall enhanced the formation of somatic embryos from explants (Jogeswar *et al*., 2007).

In this study, a substantial good callus growth and high number of regenerants was achieved from explants cultured on callus induction medium supplemented with 2, 4-D ranging from 2.0 to 3.0 mg/l. It was further observed that addition of kinetin hormone to callus induction medium with 2.0 and 2.5 mg/l of 2, 4-D further improved sorghum regeneration. Therefore the use of 2, 4-D and kinetin in subsequent studies at a level of 2.5 and 0.5 mg/l respectively was opted for due to the quality of callus induced coupled with increased number of regenerants obtained.

Effect of age of sorghum embryo on *in vitro* culture responses

In this study, the age of immature zygotic embryo influenced in vitro culture responses. The optimum age of sorghum immature zygotic embryo varied among different sorghum genotypes investigated in this study. The embryos isolated from panicles of all genotypes harvested at 14 days after anthesis were too small (about <0.5mm) and neither did they germinate nor form callus. Similarly embryos isolated from panicles of all the genotypes harvested at 20 days post anthesis were too long (about 2.5mm long). These embryos germinated after 3 days and started forming callus however, the calli formed was non embryogenic. Embryos isolated from the panicles at 16 to 18 days after flowering, were in a range of about 0.9-1.8 mm long and produced calli that regenerated into plantlets. The physiological state of the embryos of different sorghum genotypes at the same age appeared to be different and thus judging the harvesting time of the panicles basing on the age may be erroneous. This possibly showed that other characteristics especially size of the embryo and consistence of the seed should be used in making decision of when to harvest panicles. In similar studies elsewhere immature zygotic embryos of 8-18 days post anthesis were most suitable for *in-vitro* studies of sorghum although this was also genotype dependent (Indra Arulselvi and Krishnaveni, 2009). In other studies the size of the immature embryo of *Sorghum sudanenses* (sudan grass) influenced callus formation and plant regeneration with embryos of 0.7-1.5 initiating callus quickly, inducing fast growing callus that frequently forms more shoots (Gupta *et al*., 2004). In this study, it was observed that either too young or mature embryos were less responsive to *in vitro* culture. In maize, a gramineae relative of sorghum, embryo size influences callus induction with a large decline in frequency of callus induction occurring when immature embryos are harvested at 10 and 24 days post pollination (Binott *et al*., 2008). These studies on sorghum *in vitro* culture and other gramineae all indicate that age and size of embryo influence the responses expected *in vitro*. Indeed, various reports all confirm this position, with successful *in vitro* responses on embryos harvested 14 days after flowering (Hagio, 2002), 12- 15 days after anthesis (Taddesse *et al*., 2003), and 12-14 days after flowering (Grootboom *et al*., 2008; 2010). Overall, this study shows that the developmental stage of immature zygotic embryo affects the *in vitro* culture responses of sorghum. The age of sorghum immature zygotic embryo to be used should thus not only be based on number of days post flowering or pollination but also on embryo size. The best immature embryo to be cultured should neither be too small (<0.5mm long) nor big (>2mm long).

Response of different Ugandan sorghum genotypes to *in vitro* culture

Callus induction and regeneration parameters varied significantly among the selected Ugandan elite sorghum genotypes and this suggests a strong genotypic control of sorghum *in vitro* culture response among Ugandan sorghum genotype. The sorghum genotypes used showed variation in phenolic compound production on culture medium in terms of colour (brown to black) and quantity (Plate 3). Genotype dependence of sorghum *in vitro* culture has been widely reported by many researchers (Indra Arulselvi and Krishnaveni, 2009; Grootboom *et al*., 2008; Hagio, 2002) and this genetic control has been reported in other cereals such as sugarcane (Gandonou *et al*., 2005), wheat (Malik *et al*., 2004), and maize (Binott *et al*., 2008; Omer *et al*., 2008). In this study, medium composition greatly influenced callus induction time,callus induction frequency and somatic embryogenesis frequency but not shoot formation and callus growth. Callus induction and regeneration of Ugandan sorghums was influenced by genotype by medium interaction and thus model genotypes for transformation studies were identified. On Murashige and Skoog basal medium, genotypes MUC007/193, MUC007/194 and MUC007/124 were highly amenable to *in vitro* analyses. On L3 basal medium, most of the genotypes exhibited high levels of regeneration and these included genotypes MUC007/194, MUC007/102, and MUC007/162, MUC007/99, and MUC007/100. A similar phenomenon of genotype by medium interaction in cereal *in vitro* culture was reported by Grootboom *et al*.

(2008). In this study L3 culture medium proved to be the best basal medium for most of less *in vitro* culture responsive sorghum genotypes.

CONCLUSION AND RECOMMENDATIONS

The main objective of this study was to develop an efficient *in vitro* culture system for selected Ugandan elite sorghum genotypes.This study developed an efficient *in vitro* culture system the first of its kind for Ugandan adapted sorghum genotypes. It was found that the level of 2, 4-D and kinetin hormones in callus induction medium greatly influence the *in vitro* culture responses of sorghum. A combination of 2.5 mg/l 2, 4-D and 0.5 mg/l Kinetin was outstanding for sorghum regeneration. It was also found that too immature and too old zygotic embryos did not regenerate into sorghum plants under the test conditions. However, a regeneration system using old zygotic embryos as *explant*s would be desirable since mature embryos would be available throughout the year. This study found that the age of immature zygotic embryos of sorghum is not a good parameter for making harvesting decisions but this should be supplemented with other parameters such as embryo size and seed consistency. This study found that the *in vitro* culture responses of Ugandan sorghum genotypes were not only influenced by genotypic differences but also strong genotype by medium interaction. Thus model Ugandan sorghum genotype on each medium type was identified with genotype MUC007/193 and MUC007/194 highly amenable to tissue culture on MS basal medium.

Based on the results of this study, an efficient in *vitro* culture system the first of its kind developed for Ugandan adapted sorghum genotypes paves way for further sorghum genetic improvement. However, there is a need to characterize the tissue culture generated sorghum plants to estimate the frequency of somaclonal variation among adapted Ugandan sorghum genotypes. Finally candidate Ugandan sorghum genotypes identified should be used to study the possibility of Ugandan sorghum stable transformation with transgene for agronomic traits**.**

ACKNOWLEDGEMENTS

Authors greatly acknowledge the Uganda National Council of science and Technology (UNSCT) and World Bank through Millennium science initiative Cereals project (MSI cereals project) for funding this study.

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