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In vitro Culture and Plant Regeneration of Sorghum Genotypes Using Immature Zygotic Embryos as Explant Source

[1,2]A.W. Grootboom, [1]M.M. O'Kennedy, [1]N.L. Mkhonza, [2]K. Kunert, [1]E. Chakauya and [1,2]R.K. Chikwamba
[1]CSIR Biosciences, Meiring Naude Road, Brummeria, Pretoria, 0001, South Africa
[2]Department of Plant Science, University of Pretoria, Lynnwood Road, Pretoria, 0001, South Africa

Abstract: Regeneration capacity of five sorghum genotypes on three different solid nutrient media viz., medium J, Tadesse's and CAPD were investigated. The study analysed the ability of genotypes to form totipotent callus cultures and plantlets within the shortest culture period *in vitro*. We observed that regeneration of plants was mainly through somatic embryogenesis via type 1 callus. The genotype P898012 gave the best callus induction of 98%, the highest regeneration potential of 6.13 regenerants/explants and the shortest tissue culturing period of 74 days on medium J. We are now using this genotype and medium combination for subsequent transformation and regeneration of sorghum transgenics with improved nutrition.

Key words: Immature zygotic embryos, *in vitro* plant regeneration, somatic embryogenesis, callus, sorghum

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is a principal crop in the semi-arid tropical and sub-tropical Africa, India and China where persistent drought, high temperatures and poor soil nutrient availability are endemic (Chakauya *et al*., 2006; O'Kennedy *et al*., 2006). World annual sorghum production is over 60 million tonnes, of which Africa produces about 20 million tonnes. This makes sorghum quantitatively the second most important cereal grain in Africa after maize (FAO, 2007). As a crop, it plays a critical role in human nutrition. In Africa it is the only viable food grain for many of the world's most food insecure people because of its adaptation to drought and its ability to withstand periods of water-logging. Moreover, the soaring food prices in the past year have necessitated increased food production with particular emphasis on neglected and utilised species (NUS) such as sorghum and millets. Globally, the crop finds utility in animal feed stocks and industrial products such as adhesives and waxes. Furthermore, sorghum has great potential in the emerging biofuel industry, especially the sweet genotypes.

Unfortunately, low yields as a result of unimproved germplasm (Mujaju and Chakauya, 2008) and poor nutritional quality have hampered the development of sorghum as an international cash crop. Moreover, there is an urgent need to focus attention on improving crops relevant to the smallholder and resource-poor farmers if the goal of food security is to be achieved. Interestingly, the area of advanced molecular biology, genetic engineering and plant tissue culture has ushered new tools for direct access to a vast pool of useful genes (O'Kennedy *et al*., 2006) and precision breeding via marker-assisted breeding and targeted manipulation of specific genes and pathways. In commercial crops, such as maize and soybean, these techniques have greatly complemented traditional breeding. Sorghum has the potential to play a much greater role in human nutrition and poverty alleviation, the livestock feed and other industries. To realize this potential, it is necessary to harness the new technologies in genetic improvement of the crop and subsequent product processing.

Until the advent of the tissue culture technology in the 1970s, the improvement of sorghum for agronomic and quality traits has been accomplished through traditional plant breeding and improved culture management practices (O'Kennedy *et al*., 2006). Sorghum has been classified as one of the most recalcitrant plant species to manipulate through tissue culture and transformation (Zhao *et al*., 2000). In fact, it took a long time from the first reports of *in vitro* culture of sorghum (Battraw and Hall, 1991; Hagio *et al*., 1991) to successful transformation and regeneration using the particle bombardment-mediated transformation approach (Casas *et al*., 1993, 1997; Zhu *et al*., 1998). Later, *Agrobacterium*-mediated transformation of sorghum was achieved in the last decade (Zhao *et al*., 2000; Able *et al*., 2001; Jeoung *et al*., 2002).

Corresponding Author: R. Chikwamba, CSIR Biosciences, Meiring Naude Road, Brummeria, Pretoria, 0001, South Africa
Tel: +27 12 8412177 Fax: +27 12 8413651

High frequency plant regeneration from cultured explant material is a prerequisite for successful transformation of most cereal crops. One of the key limiting steps in the development of genetic engineering protocols for the improvement of cereal crops through biolistic and *Agrobacterium*-mediated transformation is the *in vitro* plant regeneration process. *In vitro* culture of cereals shows strong genotype dependence and production of the appropriate culture is generally limited to selected genotypes (Morocz *et al*., 1990). There is also strong evidence to suggest that sorghum is no exception to this genetic control (Jogeswar *et al*., 2007).

In the current study, we investigated callus initiation and regeneration potential of five sorghum genotypes originating from major sorghum growing areas in Africa on specific nutrient media with the aim to determine the best genotype-nutrient medium combination that results in satisfactory regenerability.

MATERIALS AND METHODS

Sorghum genotypes and explant: Five sorghum [*Sorghum bicolor* L. Moench] genotypes chosen for tissue culture amenability screening were Kapaala (Indian), Kadaga (Ghanaian), SA 2861, SA 4322 (both South African genotypes) and P898012 (American). The genotypes were chosen on the basis of agronomic and functional attributes ranging from good food and brewing qualities (Kapaala and Kadaga) to stem borer and aphid resistance (SA 2861 and SA 4322, respectively) and transformability (P898012).

Plant material and nutrient media: Immature zygotic embryos (IZEs) ranging from 0.8-1.2 mm in length were used as explants. These were derived from sorghum seeds harvested 12-15 days post anthesis. The immature seeds were surface-sterilized in 70% (v/v) ethanol for 3 min and 15 min in 2.5% sodium hypochlorite solution containing 0.1% Tween-20 before a thorough rinse with sterile distilled water. Tissue culture experiments were performed under aseptic conditions. As a rule cultures were transferred to fresh callus induction medium (CIM) every 2 weeks until the onset of somatic embryogenesis. The IZEs were placed on CIM with the scutellum cells facing up and the embryogenic axis in contact with the callus induction medium for somatic embryo formation. After somatic embryos formation, the calli were transferred to callus maintenance medium (maturation medium) before transfer to regeneration media for plantlet production (Table 1-3).

The CIM for Medium J (O'Kennedy *et al*., 2004) contains L3 based salts and vitamins, 2.5 mg L^{-1} of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), the carbon source maltose, 4 g L^{-1} Gelrite as the gelling agent and 20 mM L-proline. In the regeneration and rooting medium (RRM), 2,4-D and L-proline were not included. A 4-week culture on initiation medium was followed by a 2-weeks period on callus maturation medium, prior to regeneration and rooting regimes/phases. The maturation medium contained double the amount of carbohydrate, which in this case was maltose (O'Kennedy *et al*., 2004).

The CAPD medium was described by Casas *et al*. (1993). This medium was used with the following modifications: 1 g L^{-1} asparagine and 2 mg L^{-1} 2,4-D in the CIM. After 14 days on CAPD2 and 7 days on CAPD1 (CIM) to initiate somatic embryoids, the cultures were transferred to callus maintenance medium (CCM) for 4-7 days followed by subsequent culture for 2-6 weeks on regeneration medium. The callus regenerating medium (CMR) was responsible for shoot formation within

Table 1: Composition of tissue culture medium J

Nutrient	J CIM	J maturation medium	J regeneration medium
L3 macro- and micro elements	+	+	+
MS-Fe source	+	+	+
HL2 vitamins	+	+	+
2,4-D	2.5 mg L^{-1}	-	-
Maltose	30 g L^{-1}	60 g L^{-1}	30 g L^{-1}
pH	5.8	5.8	5.8
Gelrite	4 g L^{-1}	4 g L^{-1}	4 g L^{-1}
L-Proline	20 mM	-	-

+: Nutrients included at standard reported concentration, -: Not included

Table 2: Composition of Tadesse's tissue culture medium

Nutrient	Tadesse's CIM	Tadesse's modified CIM	Tadesse's shoot induction medium	Tadesse's root induction medium
MS macro- and micro elements	+	+	+	½ strength
MS-Fe source	+	+	+	½ strength
Jacobs Vitamins	+	+	+	+
2,4-D	2.5 mg L^{-1}	2 mg L^{-1}	-	-
Kinetin	0.2 mg L^{-1}	0.5 mg L^{-1}	0.5 mg L^{-1}	-
IBA	-	-	-	0.5 mg L^{-1}
NAA	-	-	-	0.5 mg L^{-1}
Sucrose	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	20 g L^{-1}
pH	5.8	5.8	5.8	5.8
Agar	8 g L^{-1}	8 g L^{-1}	8 g L^{-1}	8 g L^{-1}

+: nutrients included at standard reported concentration, -: Not included

Table 3: Composition of CAPD2 tissue culture medium

Nutrient	CAPD2	CAPD1	CCM	CMR	CSE	C root	CRE
MS Macro-, micro-and Fe source	+	+	+	+	+	½ strength	½ strength
B5 modified Vitamin (Ca-pantothenate)	+	+	+	+	+	+	+
2,4-D	2 mg L^{-1}	1 mg L^{-1}	1 mg L^{-1}	-	-	-	-
Kinetin	-	-	0.5 mg L^{-1}	0.5 mg L^{-1}	0.5 mg L^{-1}	-	-
IAA	-	-	-	1 mg L^{-1}	1 mg L^{-1}		
IBA	-	-	-	-	-	0.5 mg L^{-1}	-
NAA	-	-	-	-	-	0.5 mg L^{-1}	-
NH_4NO_3	3.3 g L^{-1}	3.3 g L^{-1}	-	-	-	-	-
Proline	2 g L^{-1}	2 g L^{-1}	-	-	-	-	-
Asparagine	1 g L^{-1}	1 g L^{-1}	-	-	-	-	-
$AgNO_3$	-	-	-	10 mg L^{-1}	-	-	-
Coconut water	100 ml L^{-1}	100 ml L^{-1}	-	-	-	-	-
Sucrose	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	20 g L^{-1}	20 g L^{-1}
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Agar	8 g L^{-1}	8 g L^{-1}	8 g L^{-1}	8 g L^{-1}	8 g L^{-1}	8 g L^{-1}	8 g L^{-1}

+: Included at standard reported concentration, -: Not included

14-28 days. The Callus Shoot Elongation (CSE) medium cultures take 10-14 days before Casas Rooting Medium (CROOT)-14 days and Casas Root Elongation (CRE)-7-14 days.

For Tadesse's medium, somatic embryogenic calli formed within 4 weeks of culture on CIM were transferred to a modified CIM with reduction in 2,4-D (2.5-2.0 mg L^{-1}) and increment in kinetin (0.2-0.5 mg L^{-1}) until somatic embryos were ready to germinate (Tadesse *et al*., 2003). The somatic embryos were then transferred to Shoot Induction Medium (SIM) until shoots developed and subsequently to root induction medium (RIM).

In total, 100 to a 150 IZEs (10 embryos per petri dish) were cultured per genotype and tissue culture media. The IZE explants consisted of three biological replicates i.e., independent panicles and harvest dates, with 15-30 explants per replication.

Data collection and statistical analysis: The incubation conditions for all cultures were at 24-25°C under low-light conditions (1.8 Me m^{-2} sec^{-1}), except regeneration shoots (≥ 5 cm), which were incubated under light conditions of 18 µE m^{-2} sec^{-1}. The rooted plantlets with a height of 5-10 cm were recorded as regenerants. A random selection of regenerants was hardened off to be assessed for fertility in the greenhouse. Data on percentage callusing and regeneration ratio was entered into Excel and Statistical analysis was carried out using the MINITAB software Release version 12.21 (MINITAB Inc., 1998). The experiment was a two-factor factorial experiment (genotype and media) in a Completely Randomized Design with replication where the genotype and media are the factors. The Tukey's Test was used to perform a pair-wise comparison of the means of the genotypes.

RESULTS

Sorghum proliferated to produce two types of calli, a very soft and watery non-embryogenic callus (Fig. 1a) and

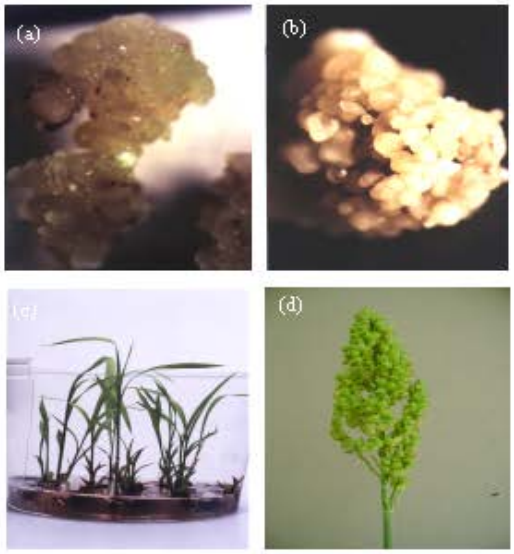

Fig. 1: *In vitro* plant regeneration from immature zygotic embryos of sorghum. (a) Non-regenerable, soft and watery callus produced/formed by some embryos. (b) White, compact embryogenic type I tissue derived from cultured IZEs of genotype P898012 on tissue culture medium J within two weeks. (c) Sorghum genotype P898012 plantlets shooting and rooting on regime J regeneration medium. These plantlets resulted in fertile T0 plants. (d) Mature Sorghum head from tissue culture plants

a highly embryogenic totipotent type I callus that is harder and white in appearance (Fig. 1b). Table 4 shows the regeneration data for the genotypes under different media conditions. Statistical analysis of the data showed that the response to both callus induction and regeneration were influenced by medium and genotype independently and thus their interaction had a stronger effect (Table 5). Overall,

Table 4: Results of plant regeneration observed for each of the genotypes on the three media tested

Genotype	Medium	No. of explants (IZEs)	No. of somatic embryos produced	No. of reg./expl.	Days in tissue culture
P898012	J	146	143	6.13	74-100
SA 4322	CAPD	135	86	3.56	100-140
SA 2861	CAPD	134	107	1.71	100-140
P898012	Tadesse's	113	70	1.40	100-144
SA2861	J	150	80	1.01	120-140
SA4322	Tadesse's	135	9	0.77	100-144
SA2861	Tadesse's	132	121	0.63	120-144
Kapaala	Tadesse's	147	37	0.17	120-140
Kapaala	CAPD	100	39	0.10	120-140
Kapaala	J	147	16	0.02	120-140

Table 5: ANOVA for regeneration statistics (a) callus induction in sorghum genotypes and (b) regeneration in sorghum genotypes

Source	df	Mean Square	F-value	Probability
(a)				
Replication	9	69.90	0.72	0.686
Genotype	4	20153.80	208.53	0.000*
Media	2	4141.60	42.85	0.000*
Genotype x Media	8	3750.90	38.81	0.000*
Error	113	96.60		
(b)				
Replication	9	0.148	0.84	0.582
Genotype	4	44.169	250.32	0.000*
Media	2	10.274	58.23	0.000*
Genotype x Media	8	22.315	126.47	0.000*
Error mean square	113	0.176		

* Shows statistical significance at 5% level, Coefficient of variation: (a) 18.8, (b) 6.1%

pair-wise comparison ranked the genotypes in the order of P898012>SA2861>Kadaga>SA4322 for callus induction on the three media. Moreover the coefficient of variation for the callus induction and regeneration (18.8 and 6.1%) suggested that the experiment was well managed with reduced experimental error. A dissection of the influence of media on the genotypes revealed some clear differences. On medium J the genotype P898012 had the highest callus induction (98±1.14%) while SA 4322 had no induction at all but was not significantly different ($p>0.05$) from Kapaala. Furthermore, 50% of the IZEs of genotype Kadaga proliferated, while genotype SA 4322 did not proliferate to form calli at all on medium J.

The regeneration potential of the five genotypes was calculated as the number of regenerants divided by the number of explants cultured and the results are shown in Fig. 2 and Table 4. Overall, the genotype responsiveness were ranked in the order of P898012>SA 2861>SA4322>Kapaala>Kadaga in all three media tested. P898012 was found to be the most regenerable genotype at 6.13 regenerants/explants (reg./expl.) on medium J, followed by genotype SA 2861 at 1.01 reg./expl. On Tadesse's medium, P898012 was also found to be superior yielding 1.4 reg./expl... On CAPD medium, the two South African genotypes performed better, yielding 3.56 reg./expl. for SA 4322 and 1.71

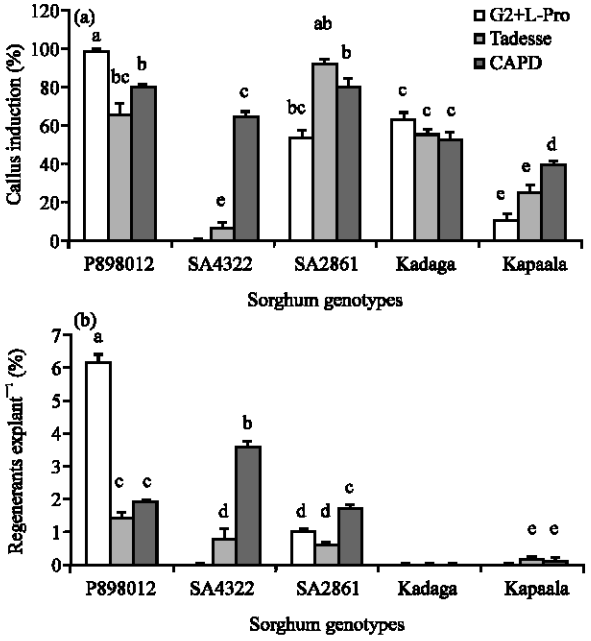

Fig. 2: The effect of culture media on callus induction (a) and plant regeneration (b) of 5 sorghum genotypes from zygotic embryos. The most totipotent calli were produced by P898012 on J medium which resulted in a 6.13 regenerants per explant. Bars with the same letter(s) are not significantly different ($p>0.05$) and error bars represent standard error

reg./expl. for SA 2861. Kapaala showed poor regenerability (between 0.01 and 0.17 reg./expl.) while no regeneration was obtained for Kadaga in all three media tested. All F_0 sorghum genotypes that were hardened-off showed phenotypes with normal vegetative growth and viable F_1 seeds. The nutrient medium J produced plantlets much faster (75-90 days) when compared to the other two (100-140 days).

DISCUSSION

We utilized in this study our knowledge in plant tissue culture to test the response of five genotypes, of which two were from South Africa, cultured on three different media. The sorghum genotypes displayed different responses to *in vitro* culture where P898012 showed the highest regeneration capacity on medium J, supplemented with L-Proline. These varied responses show clear interactions of the genotypes with the different nutrient media. Observed genotype medium interactions are consistent with what has been observed for other cereals. This has resulted in the identification of model genotypes for transformation

purposes in other cereals, such as genotypes T309 and IR72 in rice (Datta *et al*., 1992), the A188 in maize (Ishida *et al*., 1996), Golden Promise in barley (Tingay *et al*., 1997) and Bobwhite genotypes in wheat (Wu *et al*., 2003).

The different callus responses and subsequent plant regeneration potential displayed by different genotypes on the same nutrient medium is likely due to the genetic versus environmental relationship. Conversely, the same environmental conditions (medium) gave different callus responses and this could be a result of different genetic control factors (Tomes and Smith, 1985; Morocz *et al*., 1990). Other factors that could affect callus totipotency were eliminated by experimental design. These factors include the size and physiological state of the explants. All explants were carefully chosen to exclude explant sizes that could either be too small or too big. Each seed batch of a particular genotype was equally divided by culturing on all three nutrient media. This ensured that a uniform callus response from explant to explant and plate to plate was achieved (Table 5). However, a concern is that the experimental design does not address the question of clones i.e., it does not determine the No. of clones produced per explant. Nevertheless, the data produced in this study gave a clear indication of which genotype-nutrient medium combination to focus on for transformation purposes.

The period of *in vitro* culturing, indicated by days to regeneration, is also a useful indicator for rapid tissue proliferation. The most regenerable line also produced the shortest *in vitro* culturing i.e., earliest plantlets produced after 74 days with P898012 on medium J. This is especially crucial in the production of healthy transgenic plants as the stress imposed by tissue culture is minimized and so is the opportunity for somaclonal variation.

In conclusion, the sorghum genotypes tested showed a variation of genotypic responses to *in vitro* culture. From these results, the chosen sorghum genotypes for transformation experiments were, in their order of priority, P898012 on medium J, followed by SA 4322 and SA 2861 on CAPD. These combinations displayed superior sorghum regeneration potential and it is anticipated that it should significantly increase the probability of producing transgenic sorghum plants. The response of these genotype-media combination to biolistic transformation is currently tested. In short the study provided the foundation for transforming sorghum in the lab in an effort to improve the nutrition of the crop.

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