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Somatic embryogenesis induction from immature embryos of *Sorghum bicolor* L. (Moench)

Inducción de embriogénesis somática a partir de embriones inmaduros de *Sorghum bicolor* L. (Moench)

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Abstract. In this study, we established an *in vitro* regeneration system from immature embryos of four varieties of Sweet Sorghum with biotechnological potential. A total of 17 treatments were assesses in the induction of somatic embryogenic calli. We analyzed the effect of Murashige y Skoog media supplemented with different concentrations of plant growth regulators such as auxins and cytokinins. The callus induction response was evaluated using three immature embryos development stages and based on the number of embryos with callus induction. Different responses were observed in the embryos depending of the embryo age, culture condition, media composition and variety. The results showed that embryos of the middle stage had the best response in terms of calli induction. Interestingly, more embryos responded to calli induction in light presence. Complex media showed better response that minimal media to callus induction, media complemented with 4 to 6 mg/L of 2,4-D combined with 1 mg/L of BAP were more efficient to callus induction. With respect to variety, the best response to callus induction was obtained with Atlas variety, followed by Keller and Kansas, whereas Fortuna variety had lower response. In this work also, it was obtained an efficient culture medium to shoots development because until 90% of calli response to shoots regeneration. This study reports relevant information to tissue culture protocols that can be used in cereals in vitro cultivation as tool for biotechnological applications.

Keywords: *Sorghum bicolor*; Immature embryos; Somatic embryogenesis.

Resumen. En este estudio se estableció un sistema de regeneración *in vitro* de embriones inmaduros de cuatro variedades de sorgo dulce con potencial biotecnológico. Un total de 17 tratamientos fueron utilizados en la inducción de embriogénesis somática. Analizamos el efecto de medios Murashige y Skoog suplementados con diferentes concentraciones de reguladores tales como auxinas y citocininas. La respuesta de inducción de callo fue evaluada utilizando tres estados de desarrollo de embriones inmaduros y basados en el número de callos inducidos. Se observaron diferentes respuestas dependiendo de la edad del embrión, condiciones de cultivo, composición del medio y la variedad. Los resultados mostraron que embriones de mediana edad tuvieron mejor respuesta en términos de inducción de callos. Interesantemente, más embriones respondieron a la inducción de callo en presencia de luz. Los medios complejos tuvieron mejor respuesta que los medios mínimos en la inducción de callo, los medios suplementados con 4 a 6 mg/L de 2,4-D combinado con 1 mg/L de BAP fueron más eficientes en la inducción de callos. En cuanto a la variedad, la mejor respuesta de inducción de callo fue obtenida con la variedad Atlas, seguida por Keller y Kansas, mientras que la variedad Fortuna tuvo menor respuesta. En este trabajo también encontramos un eficiente medio de cultivo para regeneración de plántulas debido a que los callos inducidos respondieron eficientemente hasta en un 90% a la regeneración de brotes. Este estudio reporta información relevante para protocolos de cultivo de tejidos que pueden ser utilizados en cereales como herramientas para aplicaciones biotecnológicas.

Palabras clave: *Sorghum bicolor*; Embriones inmaduros; Embriogénesis somática

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INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] has positioned as an important crop worldwide due to their adaptation capacity to poor soil, nutritional quality, antioxidant capacity and bioactive compound. Their grains are used for human and domestic animal feed and for biofuel production. In this sense, it is the fifth cereal crop in the world behind corn, rice, wheat, and barley (Ejeta & Knoll, 2007; Murray et al., 2009; Singh et al., 2011; Wu et al., 2013; Meryemoğlu et al., 2014). Also, this crop shows high tolerance to biotic and abiotic stresses such as drought and heat (Arulselvi & Krishnaveni, 2009; Borghi et al., 2013). The adaptation to different ecosystems has allowed to obtain different genotypes but eventual climatic changes may affect their grain production (Borghi et al., 2013; Grossi et al., 2013). *In vitro* propagation is a potential tool to mass propagation or to obtain a germplasm bank; however, the high quantity of phenolic compound and the subsequent necrosis are constraint to its *in vitro* tissue culture (Sudhakar et al., 2008). Nevertheless, there are several reports to somatic embryogenesis in cereals. The results vary between crops, genotypes, localities and environmental factors which may differ in their regenerative potential (Casas et al., 1997; Baskaran & Jayabalan, 2005; Murray et al., 2009; Arulselvi & Krishnaveni, 2009). *In vitro* regeneration is an important objective to perform sorghum genetic improvement programs. However, in this sense, is considered one of the most recalcitrant species to *in vitro* responses (Rathus et al., 2004; Pola & Sarada, 2006; Gurel et al., 2009; Lu et al., 2009; Raghuwanshi & Birch, 2010). *In vitro* propagation in sorghum is highly dependent on tissue culture response, genotype, explant type (seedling, inflorescence, shoot apex and immature embryo), media composition and plant growth regulators (Baskaran & Jayabalan, 2005; Pola & Sarada, 2006; Sudhakar et al., 2008; Arulselvi & Krishnaveni, 2009; Amali et al., 2014). Plant *in vitro* regeneration in sorghum has been implemented using different explants as hypocotyls seedlings, immature inflorescences, shoot apexes, leaf segment and immature embryos (Baskaran & Jayabalan, 2005; Pola & Sarada, 2006; Arulselvi & Krishnaveni, 2009). It is important to evaluate the age explant and culture conditions (Ma et al., 1987; Sudhakar et al., 2008; Arulselvi & Krishnaveni, 2009). In the present work, we reported somatic embryogenesis from immature embryos of four Sweet Sorghum varieties with biotechnological potential evaluating the effect of media compositions, culture conditions and age of immature embryos to induce somatic embryogenesis.

MATERIALS AND METHODS

Plant material. Seeds from *Sorghum bicolor* var. Keller, Kansas, Atlas and Fortuna were obtained from the Sweet Sorghum Project of the Agronomy Faculty of UANL, Nuevo León, México. The seeds were collected and stored at 4 °C refrigeration until use.

Preparation of immature embryos. Seeds of four sorghum cultivars were cleaned by washings with a commercial liquid detergent. To evaluate the explants age, three developmental morphology stages of immature embryos were used: early stage embryos from 8-10 days after pollination (DAP) (1.3-2.0 mm long), middle stage embryos from 10-14 DAP (2.0-3.0 mm long) and mature stage embryos from 15-20 DAP (2.8-4.0 mm long). All embryos were excised and disinfected by soaking them in ethanol 100% and Tween® 20, followed by five rinses with sterile distilled water. These embryos were placed in a sterile solution of citric acid 0.01% (Sigma) to prevent oxidation. Finally, to evaluate the callus induction response, the embryos were placed on different calli induction media: some under a specific photoperiod (16 h light/8 h dark), and others under completely darkness conditions at 27 °C.

Callus induction media. A total of 100 immature embryos of each variety previously sterilized were used on each treatment. Seventeen treatments were performed containing Murashige and Skoog (MS) as basal medium (Murashige & Skoog, 1962). The mediums were supplemented with different vitamins, amino acids and growth regulator concentrations: *2,4-Dichlorophenoxyacetic acid* (2,4-D) (Sigma), 6-Benzylaminopurine (BAP) (Sigma), Naphthalene Acetic Acid (NAA) (Sigma), Proline (Sigma), Casein hydrolysates (*Fluka*® Chemie GmbH, *Steinheim*, Germany), Pyridoxine (Sigma), Thiamine (Sigma), myo-inositol (Sigma), Pantothenic acid (Sigma), $CuSO_4$, KH_2PO_4 and 8 g/L bacto agar. All media were adjusted to pH 5.6 - 5.8 and then sterilized by 20 min at 121 °C.

Callus maintenance and shoot induction media. To callus maintenance, the callus were sub-cultured on their respective induction media to generate callus masses during 60 days in a photoperiod 16 hour light and 8 hour h darkness (photon flux= 7 $\mu mol/m^2/s$) at 27 ± 1 °C and 13% relative humidity. To determinate the capacity of the callus regeneration, a regeneration medium was performed containing MS medium supplemented with 1 mg/L of Pantothenic acid, 0.5 mg/L Kinetin, 1.0 mg/L Indole Acetic Acid (IAA) (Sigma) and 2 mg/L of Casein hydrolysates.

Statistical analyses. The response of callus induction was evaluated depending on the significant differences between varieties, media treatments, culture conditions, through one-way analysis of variance. Means were compared using the Least Significant Difference using the statistical program SPSS ver. 17.

RESULTS

Effect of explant age and incubation conditions on the callus induction response. Four different varieties of sweet Sorghum: Kansas, Keller, Atlas and Fortuna were used to analyze the response of callus induction. A total of 100 immature embryos were used by treatment. Initially, we evaluated the re-

sponse of immature embryos in the early (8-10 DAP), middle (10-14 DAP) and mature developmental morphology stage (15-18 DAP) to induce somatic embryogenic callus. The results showed that embryos of the early stage presented a tendency to necrotize after three days on culture medium without callus formation, and all varieties died by dehydration after fifteen days, independently of incubation conditions. Embryos of middle stage showed high tendency to callus formation depending of the formulated media and the incubation conditions whereas embryos in mature stage formed callus and other germinated. Due to this result, embryos from the middle stage were used to evaluate the response under either light or dark conditions on seventeen embryogenic callus induction media (MICE): MICE1, MICE2, MICE3A, MICE3B, MICE3C, MICE5A, MICE5B, MICE5C, MICE5D, MICE8A, MICE8B, MICE8C, MICE8D, MICE9A, MICE9B, MICE9C and MICE9D, with different concentration of growth regulators (Table 1). The results showed that minimal medium (MICE1, MICE2, MICE3A and MICE3B) non-responded to callus formation; however, MICES3C medium containing the same formulation of nutrients but with 2.0 mg/L casein hydrolyzed showed callus formation of 2/1 (in light and dark, respectively) in Fortuna variety without any response in other varieties. MICES5A medium showed response in Keller and Fortuna varieties forming 4/1 (light/dark) and 4/4 calluses, respectively (Table 2). MICE5B and C were the best culture medium to callus induction; this culture medium contained 2,4-D hormone added at 4 and 6 mg/L combined with 1 mg/L of BAP (MICE5B and MICE5C, respectively). However, the use of 2,4-D hormone at 8 mg/L, decreased the callus formation (MICE5D), indicating that 2,4-D hormone is an important factor to callus development (Fig. 1A, B). On the other hand, MICE8 medium enhanced the callus development when the BAP and 2,4-D hormones were inversely proportional favoring the increase of 2,4-D hormone until 4 mg/L (MICE8C) because of low concentrations decreased the callus formation (Fig. 1C, D). The results obtained in MICE9 medium supplemented with 2,4-D and NAA hormones showed that the substitution of BAP by NAA decrease the formation of callus, although the concentration of 2,4-D was optimal (Fig. 1E, F). This observation suggested that, to improve the callus formation, the 2,4-D (4 to 6 mg/L) must be accompanied with the use of NAA and

Table 1. Formulations of culture media used to callus induction of immature embryos from Sweet Sorghum.
Tabla 1. Formulación de medios de cultivo usados para la inducción de callos a partir de embriones inmaduros de Sorgo dulce.

Components/ Treatment	MICE1	MICE2	MICE3			MICE5				MICE8				MICE9			
			A	B	C	A	B	C	D	A	B	C	D	A	B	C	D
MS SALTS/L																	
Macro Nutrients	100.0 mL																
Micro Nutrients	10.0 mL																
Sucrose	30.0 g																
FeEDTA	5.0 mL																
KH_2PO_4	-	1.2 g				-											
$CuSO_4$	-	1.2 g				-											
VITAMINS/L																	
Pyridoxine	1.0 mg					200 mg				1.0 mg				-			
Thiamine	-	1.0 mg				1.0 mg				1.0 mg				-			
Myo-inositol	-	100 mg															
Pantothenic acid	-									1.0 mg							
REGULATORS/L																	
2,4-D	2.0 mg		4.0 mg		2.0 mg	2.0 mg	4.0 mg	6.0 mg	8.0 mg	2.0 mg		4.0 mg		2.0 mg		4.0 mg	
BAP	-	1.0 mg	1.0 mg	2.0 mg	1.0 mg	1.0 mg				0.5 mg	1.5 mg	0.5 mg	1.5 mg	-			
NAA	--													0.5 mg	1.5 mg	0.5 mg	1.5 mg
AMINO ACID/L																	
Casein hydrolysates	2.0 mg	-	2.0 mg			2.0 mg				1.0 mg							
Proline	-					0.5 mg								1.0 mg			

not by BAP. In addition, mediums with casein hydrolysates and proline in concentrations of 2.0 mg and 0.5 mg, respectively, showed more callus development.

With respect to varieties, the Atlas variety had more callus development with 798-formed callus in all media, of which 550 were under light conditions, followed by Keller with 672, of which 453 were under light conditions, Kansas with 339 of which 228 were under light conditions, and Fortuna with 108 formed callus of which 62 were under light condition. Each genotype showed response at a specific medium; Kansas and Keller varieties showed more callus development in MICE5 medium, Atlas variety showed more development in MICE8 medium, whereas Fortuna variety had more induction in MICE9. With respect to the incubation conditions, the light had more response with a total of 1293 developed calluses, in contrast to the darkness conditions with a total of 624-formed calluses; the embryos incubated in darkness had less tendencies to oxidation and die by dehydration (Table 2).

To contrast the formation of callus coming from seed, green leaf tissue was also cultivated in medium to callus induction under light and darkness conditions. The response to callus formation occurred after 18 days of culture. Immature embryos cultured in callus induction media under light conditions had a better response, although with less proportion of callous mass with respect to green leaf tissue (Fig. 2A). In the darkness conditions, although the number of embryos forming callus was lower, the proportion of callous mass was higher and presented chlorotic leaf structures due to deficiency of light (Fig. 2B). Embryos under selection to callus formation showed descolored structures (Fig. 2C), followed by dedifferentiation as globular structure translucent callus with beige coloration and bright yellow (Fig. 2D,E,F). Respect to shoot regeneration, the regeneration media (MR1) showed high efficiency because until 90% of the callus cultured on this medium under light conditions regenerated shoots after 15 days (Fig. 3A-D).

Statistical analysis. An analysis of mean comparison was performed using the Least Significant Difference. The analysis showed that Atlas and Keller varieties were not statistically different due their response with similar development of callus, whereas Kansas and Fortuna varieties were different (Table 3). On the other hands, the MICE5 treatments was grouped in two groups MICE5C and MICE5B in one group and MICE5A and MICE5D in other group, indicating each group did not show significant differences. To MICE8 medium, all treatments were grouped in one group because no statistical differences were found. In the MICE9 medium, there did not exist a relation between MICE9A and MICE9D, but both were related to MICE9B and MICE9C (Table 4).

Table 2. Number of induced calluses by variety to different culture media under different incubation conditions.
Tabla 2. Número de callos inducidos por variedad para diferentes medios de cultivo bajo diferentes condiciones de incubación.

Treatment	KANSAS		KELLER		ATLAS		FORTUNA	
	Light	*Darkness*	*Light*	*Darkness*	*Light*	*Darkness*	*Light*	*Darkness*
MICE1	0	0	0	0	0	0	0	0
MICE2	0	0	0	0	0	0	0	0
MICE3A	0	0	0	0	0	0	0	0
MICE3B	0	0	0	0	0	0	0	0
MICE3C	0	0	0	0	0	0	2	1
MICE5A	0	0	4	1	0	0	4	4
MICE5B	70	15	61	23	68	36	1	9
MICE5C	55	23	84	60	47	34	2	5
MICE5D	0	0	0	0	0	0	2	4
MICE8A	5	2	15	8	65	28	6	3
MICE8B	14	7	68	17	72	34	10	6
MICE8C	13	6	72	45	70	30	5	1
MICE8D	11	6	36	18	51	21	5	2
MICE9A	0	0	25	6	66	26	13	9
MICE9B	6	2	37	14	39	15	5	1
MICE9C	50	50	43	26	31	10	5	1
MICE9D	4	0	8	1	41	14	2	0

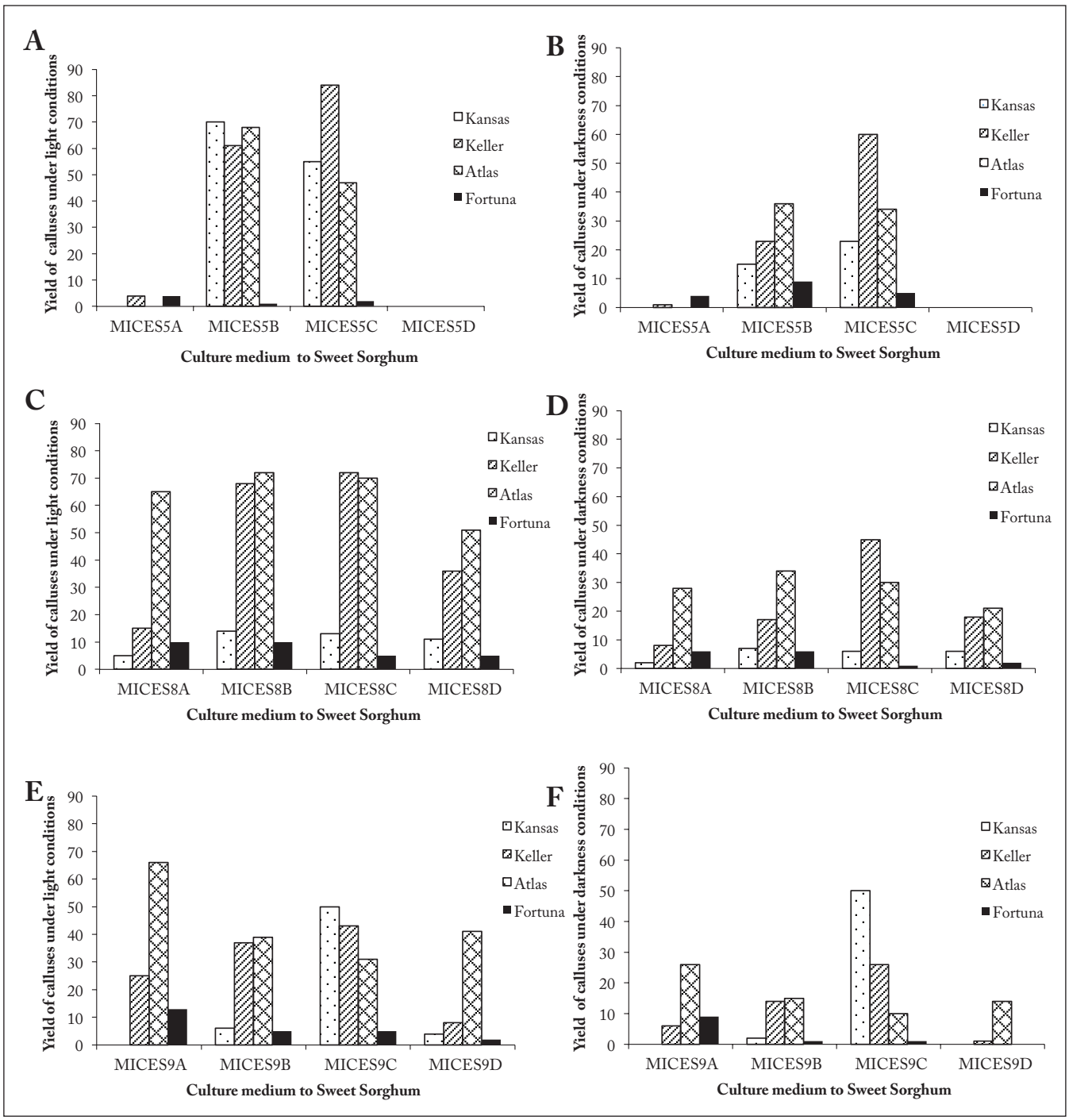

Fig. 1. Induction response of embryogenic calluses of four varieties from Sweet Sorghum under different culture media. (A) Formed calluses in MICE5 culture medium under light conditions. (B) Formed calluses in MICE5 culture medium under darkness conditions. (C) Formed calluses in MICE8 culture medium under light conditions. (D) Formed calluses in MICE8 culture medium under darkness conditions. (E) Formed calluses in MICE9 culture medium under light conditions. (F) Formed calluses in MICE9 culture medium under darkness conditions.

Fig. 1. Respuesta de inducción de callos embriogénicos de cuatro variedades de Sorgo dulce bajo diferentes medios de cultivo. (A) Callos formados en medio de cultivo MICE5 bajo condiciones de luz. (B) Callos formados en medio de cultivo MICE5 bajo condiciones de oscuridad. (C) Callos formados en medio de cultivo MICE8 bajo condiciones de luz. (D) Callos formados en medio de cultivo MICE8 bajo condiciones de oscuridad. (E) Callos formados en medio de cultivo MICE9 bajo condiciones de luz. F) Callos formados en medio de cultivo MICE9 bajo condiciones de oscuridad.

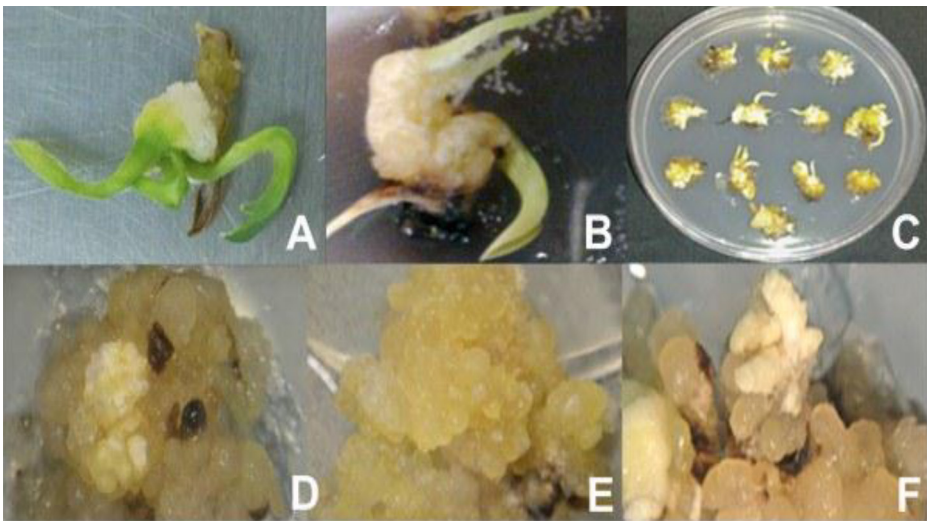

Fig. 2. Somatic embryogenesis of Sweet Sorghum Keller variety. (A) Embryo dedifferentiation after 18 days of initiation under light conditions. (B) Embryo dedifferentiation after 18 days of initiation under darkness conditions. (C) Embryogenic calluses from Keller variety after two weeks in callus culture medium in MICE5B containing 4 mg/L of 2,4-D and 1 mg/L of BAP. (D) Embryogenic calluses from globular structure. (E) Compact and translucent calluses. (F) White calluses type "*popcorn*".

Fig. 2. Embriogénesis somática de Sorgo dulce de variedad Keller. (A) Desdiferenciación de embriones después de 18 días bajo condiciones de luz. (B) Desdiferenciación de embriones después de 18 días bajo condiciones de oscuridad. (C) Callos embriogénicos de variedad Keller después de 2 semanas en medio MICE5B conteniendo 4 mg/L de 2,4-D y 1 mg/L de BAP. (D) Callos embriogénicos de estructura globular. (E) Callos compactos y traslucidos. (F) Callos blancos tipo "*popcorn*".

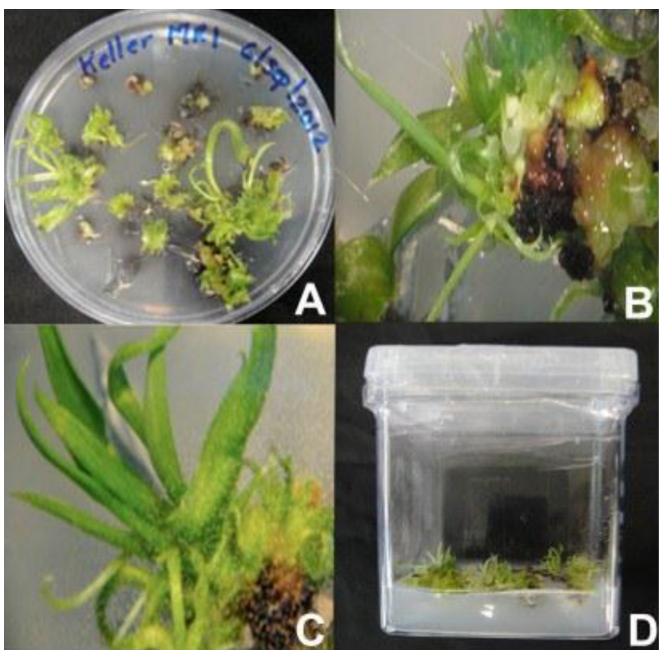

Fig. 3. Shoots generated from embryogenic calluses after six weeks of the regeneration initiation in medium MR1. (A,B) Shoots derived from embryogenesis of the Keller variety. (C,D) Shoots developed from embryos of the Atlas variety.

Fig. 3. Explantes generados a partir de callos embriogenicos despues de seis semanas de regeneracion en medio MR1. (A,B) Explantes derivados de embriogénesis de la variedad Keller. (C,D) Explantes desarrollados de embriones de variedad Atlas.

DISCUSSION

The development of an efficient and reproducible *in vitro* tissue culture and plant regeneration protocols are a prerequisite to applying modern biotechnological tools for genetic improvement of crops, such as somaclonal variants recovery, production of transgenic, pathogen-free plants and clonal propagation of desirable germoplasm, among others (Aguado-Santacruz et al., 2014). However, it is broadly reported that cereals and grasses are recalcitrant to *in vitro* culture, and the plant genotype, media constituents, type and devel-

Table 3. Difference between varieties of Sweet Sorghum exposed to callus formation with different formulations of culture media using a mean comparison by the Least significant Difference.

Tabla 3. Diferencia entre variedades de Sorgo dulce sometidas a formación de callo mediante diferentes formulaciones de medios de cultivo utilizando una comparación de medias por Diferencia Mínima Significativa.

Difference between varieties	
Atlas	5.339 a*
Keller	4.783 a
Kansas	3.170 b
Fortuna	2.217 c

*Same letter are statistically similar.

Table 4. Difference between treatments used in the callus production of four sweet Sorghum varieties using a mean comparison by the Least Significant Difference.
Tabla 4. Diferencia entre tratamientos utilizados en la producción de callos de cuatro variedades de Sorgo dulce utilizando una comparación de medias por Diferencia Mínima Significativa.

Difference between treatments					
MICE5		**MICE8**		**MICE9**	
MICE5C	5.805 a	MICE8B	4.959 a	MICE9C	4.869 a
MICE5B	5.521 a	MICE8C	4.946 a	MICE9A	3.754 ab
MICE5A	1.515 b	MICE8D	4.079 a	MICE9B	3.575 ab
MICE5D	1.246 b	MICE8A	3.667 a	MICE9D	2.592 b

*Same letter are statistically similar.

opmental morphology stage of explants and environmental conditions can affect the response of the tissue culture (Baskaran & Jayabalan, 2005; Pola & Sarada, 2006; Sudhakar et al., 2008; Arulselvi & Krishnaveni, 2009). To sorghum plant regeneration different protocols have described with multiple responses to *in vitro* culture using hypocotyls (Baskaran & Jayabalan, 2005), leaf segments (Pola & Sarada, 2006), seedlings, immature inflorescences, shoot apices (Arulselvi & Krishnaveni, 2009) and immature embryos (Ma et al., 1987; Sudhakar et al., 2008; Arulselvi & Krishnaveni, 2009). In this study, embryos were evaluated on simple and complex media that showed different behavior according to the explant age, plant genotype, media formulation and incubation conditions.

We evaluated the response of immature embryos in the early, middle and mature developmental morphology stages to induce somatic embryogenic callus from four different varieties of Sweet Sorghum: Kansas, Keller, Atlas and Fortuna to determine the optimal developmental morphology stage for callus induction. The results showed that embryos of the early stage presented a tendency to become necrotic after three days on culture medium without callus formation. In addition, all varieties died by dehydration after fifteen days, independently of incubation conditions. Embryos of middle stage showed high tendency to callus formation depending of the formulated media and the incubation conditions, whereas embryos in mature stage formed callus and others germinated. This agrees with reports of Ma et al. (1987) and Arulselvi & Krishnaveni (2009). These authors reported that immature embryos of 0.8-1.4 mm obtained after 11 to 15 days after pollination produced higher percentages of callus induction dependent on the genotype. The media for induction and proliferation of callus, particularly in cereals and grasses, requires relatively high concentrations of auxins, amino acids like casein hydrolyzed and proline. These are important for callus development; however, the callus induction is limited when these are used at high concentrations. Here we reported the use of 2,4-D and BAP hormones in a range of 4 to 6 mg/L, or 0.5 to 1 mg/L, respectively, with well callus formation. Other studies reported that concentrations of 2,4-D in the range of 1 to 3 mg/L are more efficient to induce embryogenic regenerable callus (Barro et al., 1999; Halámková et al., 2004). There are reports with immature embryos of 1-2 mm it was possible to obtain up to 95% of embryogenic callus formation and more than 70% of regenerated shoots with the use of 2,4-D, IAA, IBA, NAA and BAP as growth regulators (Liu & Godwin, 2012). The use of casein hydrolysates plays a significant role, and is recommended the use of 2 mg/L in research that include callus development with the optimal concentrations of hormones here reported. The use of low proline concentrations is recommended for callus induction. Our results are according to preview research which report that appropriate concentrations and a balance of plant growth regulators, auxins and cytokinins are crucial to induce embryogenic callus induction. The results showed that the genotype responded in differential forms because, in general, each genotype showed more response at a specific medium. Kansas and Keller varieties showed more callus development in MICE5 medium while Atlas variety showed more development in MICE8 medium. Fortuna variety had more induction in MICE9 medium. However, this variety is not recommended for doing research of *in vitro* propagation due to its lower regeneration capacity in most treatments. These results confirmed that the plant variety or genotype is a very important factor because of its different nutritive requirements (Baskaran & Jayabalan, 2005; Pola & Sarada, 2006; Sudhakar et al., 2008; Arulselvi & Krishnaveni, 2009). The relative influence of genotype on tissue response has been controversial; Vasil & Vasil (1986) suggested that the differential responses among genotypes may be due to differential expressions which in turn depend upon their spatial and temporal distribution, and their physiological and developmental morphology stage. The use of immature embryos or green leaf tissue can produce callous mass, but the number of calluses formed is greater with embryos (Baskaran & Jayabalan, 2005; Sudhakar et al., 2008) which retained high capacity to develop shoots until 90% on the regeneration media (Arulselvi & Krishnaveni, 2009). With respect to incubation conditions, light had more response to develop callus than darkness condition. Under these later conditions, calluses die by dehydration.

In this study, we demonstrated that sorghum varieties could be used to *in vitro* propagation with different media formulations for being used in genetic improvement. The results of callus induction demonstrated the capacity of our media to maintain and propagate calluses of Sweet Sorghum. Likewise, these results are important due to the need to improve *in vitro* propagation in this crop to be used in genetic improvement programs. This study contains novel information of efficient methods of tissue culture in cereal crops that can be used as tool for biotechnological applications.

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