Biotecnología Vegetal Vol. 15, No. 3: 163 - 175, julio - septiembre, 2015
ISSN 2074-8647, RNPS: 2154 (Versión electrónica)
Instituto de Biotecnología de las Plantas. UCLV. MES.

Novel explant for somatic embryogenesis in *Sorghum bicolor* (L.) Mohen

Silvio Martínez Medina[1,2]*, Raúl Collado[2], Raúl Barbón[2], Mayelin Rodríguez[2], Marta Pérez[2], Miguel Tejeda[3], Luis Rojas, Dion D. Daniels[4], Berkis Roque[2], Damaris Torres[2], Luís O. Maroto[5], Amanda L. Vitlloch[5], Rafael Gómez-Kosky[2] *Autor para correspondencia

[1]Centro de Investigaciones Agropecuarias, Universidad Central Marta Abreu de Las Villas. Carretera a Camajuaní km 5.5, Santa Clara, Cuba. CP 54 830. e-mail: silviod@uclv.edu.cu

[2]Instituto de Biotecnología de las Plantas, Universidad Central Marta Abreu de Las Villas. Carretera a Camajuaní km 5.5, Santa Clara, Cuba. CP 54 830 e-mail: silvio@ibp.co.cu

[3]Biofábrica MINAGRI, Cienfuegos. Carretera a Palmira km 1.5, Cienfuegos, Cuba.

[4]University of Belize, Belmopan, Belize C.A.

[5]Departamento de Biología, Facultad de Ciencias Agropecuarias. Universidad Central Marta Abreu de Las Villas. Carretera a Camajuaní km 5.5, Santa Clara, Cuba. CP 54 830

ABSTRACT
This work was carried out with the objective to form somatic embryos of sorghum, cv. 'CIAP 132R-05' starting from callus obtained from sections of *in vitro* shoots. For the formation of callus, different concentrations of 2,4-D were studied as well as three concentrations of ascorbic acid to eliminate the phenolic oxidation. To increase the percentage of callus formation with embryogenic structures, different segments of the shoots were used. For the formation of somatic embryos, different concentrations of 2,4-D; 6-BAP and L-Proline were added to the culture media. The greatest callus formation (50%) was obtained in the culture medium with 18.1 µM of 2,4-D. When 50 mg l^{-1} of ascorbic acid was added to the culture medium, the percentage of callus formation increased to 67.5%, and was couple with absence of oxidation of the medium and the explant. The frequency of callus formation with embryogenic structures increased to 95% with the use of segment 1 of the shoot sections *in vitro* as explant. The greatest number of somatic embryos per callus was obtained when 2,4-D was reduced to 4.52 µM, combined with 22.2 µM of 6-BAP and 500 mg l^{-1} of L-Proline (41.88). Histological analysis confirmed that structures formed in callus came from a somatic embryogenesis process. For the first time, the efficient formation of somatic embryos of sorghum section 1 of *in vitro* shoots *in vitro* was obtained.

Keywords: 2,4-D, shoots *in vitro*, callus, somatic embryos

Nuevo explante para la embriogénesis somática en *Sorghum bicolor* (L.) Mohen

RESUMEN
El trabajo fue realizado con el objetivo de formar embriones somáticos de sorgo, variedad 'CIAP 132R-05' a partir de callos obtenidos de secciones de brotes *in vitro*. En la formación de callos fueron estudiadas diferentes concentraciones de 2,4 D y tres concentraciones de ácido ascórbico para eliminar la oxidación fenólica. Para incrementar el porcentaje de formación de callos con estructuras embriogénicas fueron empleados diferentes segmentos de brote. Se añadieron diferentes concentraciones de 2,4-D, 6-BAP y L- prolina al medio de cultivo para la formación de embriones somáticos. La mayor formación de callos (50%) se obtuvo en el medio de cultivo con 18.1 µM de 2,4-D. Cuando se añadieron 50 mg l^{-1}de ácido ascórbico al medio de cultivo, el porcentaje de formación de callos se incrementó en 67.5%, unido a la ausencia de pigmentos fenólicos en el explante. La frecuencia de formación de callos con estructuras embriogénicas aumento hasta 95% con el uso del segmento 1 como explante. El mayor número de embriones somáticos por callos se obtuvo cuando 2,4-D fue reducido a 4.52 µM, combinado con 2.22 µM de 6-BAP y 500 mg l^{-1} de L-prolina (41.88). El análisis histológico confirmó que las estructuras formadas a partir de los callos provienen del proceso de embriogénesis somática. Por vez primera se obtuvo la formación eficiente de embriones somáticos de sorgo de a partir de la seccion 1 de brotes *in vitro*.

Palabras clave: 2,4-D, brotes *in vitro*, callos, embriones somáticos

Abbreviations

CIAP	Centro de Investigaciones Agropecuarias
2,4-D	2,4- dichlorophenoxyacetic acid
6-BAP	6- benzylaminopurine

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Mohen) is a plant with physiological C-4 metabolism that adapts well to an agro-ecological environment that is hot and dry, in which it is difficult to cultivate other cereals (Antonopoulou *et al*., 2008; Liu *et al*., 2009).

This species is a native *Poaceae* of the tropical and subtropical regions of Africa (Sudhakar *et al*., 2009). It is used for animal feed, human nutrition, fiber production and biocombustible ethanol (Rooney *et al*., 2007). Its cultivation is widespread, ranking fifth after wheat, rice, maize and barley (Zhao *et al*., 2010). However, its production is affected by several factors such as bird attacks, diseases, insect pests, the tannin content that affects the quality and availability of protein in the grain, as well as its poor amino acid composition. Thus, it requires of breeding programs to develop tolerant and resistant cultivar, capable of face adverse factors without affecting their yield. (Pérez *et al.* 2010).

The use of traditional methods of breeding is limited by various factors, among them: long selection periods with the utilization of morphological markers, the complexity of the trait to be improved and the influence of the environment. So that the availability of biotechnological techniques, including genetic transformation, will be very useful as a method of genetic improvement of the crop (Kumar *et al.* 2011).

The establishment of a plant regeneration system from somatic cells constitutes a prerequisite of extreme importance within the process of genetic transformation (Brandao *et al*., 2012). This way of regeneration constitutes a rapid method to produce a large number of plantlets (Baskaran *et al.,* 2006).

Tomas *et al*. (1977) and Gambor *et al.* (1977) did the first works on the regeneration of plants via somatic embryogenesis in sorghum. Various types of explants have been used for the formation of callus such as: apical buds (Maheswari *et al*., 2006); immature zygotic embryos (Gupta *et al*., 2006); immature inflorescence (Jogeswar *et al*., 2007); mature zygotic embryos (Zhao *et al*., 2008; 2010). However, with these explants, the frequency of callus formation with embryogenic structures has been low (Zhao *et al*., 2010). Besides, due to photoperiod sensitivity, most of these types of explants are not available in sorghum all year (Kishore *et al*., 2006). It is known that one of the problems described in the different protocols for plant regeneration in sorghum is the production of phenolic compounds that can affect the formation of callus and thereafter obtaining plants, especially in the red-seed genotypes (Nguyen *et al*., 2007).

Even though plant regeneration protocols via somatic embryogenesis exist for *Sorghum bicolor*, these have low regeneration efficiencies and are dependent on the genotypes. These factors are fundamentally related with the type of explants used, the influence of the genotype and the occurrence of phenolic compounds that cause a low frequency of callus formation with embryogenic structures, which all affect plant regeneration. Although genetic transformation have been achieved in sorghum, to date persist inefficiencies in calli with embryogenic structures formation, big amount of initial explants (7000 immature zygotic embryos) are needed for obtaining low (848 calli) callus formation (Wu *et al*., 2014). These limitations make sorghum one of the most recalcitrant species for *in vitro* culture and genetic transformation (Gupta *et al*., 2006; Maheshwari *et al*., 2006).

The objective of this work was to form somatic embryos from callus developed from sections of *in vitro* shoot sections of *Sorghum bicolor* cv. 'CIAP 132R'. This paper constitutes the first report on obtaining somatic embryos of sorghum using this explant.

MATERIALS AND METHODS

Plant material

Mature seeds of sorghum cv. 'CIAP 132R-05' (red sorghum) were used as starting material.

Donor plants were grown under controlled conditions (shade houses), whose panicles from emission were covered with wax paper bags. To obtain *in vitro* shoots, seeds were disinfected and germinated according to the methodology proposed by Baskaran and Jayabalan (2005). *In vitro* shoots regenerated via direct organogenesis and thickened using the protocol described by Martínez *et al.* (2012) were used for callus formation.

Callus formation

Effect of 2,4-D

Different concentrations of 2,4-D (9.05, 18.1 and 27.14 µM) were evaluated for callus formation. Cross sections above the base of the *in vitro* shoot of 0.5 cm long, were used as explants for callus formation (Figure 1 A, B). The explants were placed in a culture medium of Murashige and Skoog (1962) (Duchefa) MS (4.32 g l^{-1}), MS vitamins, supplemented with 100 mg l^{-1} myo-inositol, 50 mg l^{-1} L-Proline, and 3% sucrose. As gelling agent in the different culture media used it was, 8.0 g l^{-1} Agar (BIOCEN, Cuba). For the experiments, glass culture vessels 250 ml total capacity with plastic cap (polycarbonate) were used and 30 ml of culture media were added in each flask. The pH of the different culture media was adjusted to 5.7 with NaOH or HCl before autoclaving.

The plant material was placed in a growth chamber at 27 ± 2°C and constant darkness. Visual observations were performed every seven days for the presence of phenolic compounds in the culture media and the explant. After 30 days of culture, the number of explants forming callus, expressed as a percentage of callus per culture vessel was assessed; and the number of explants phenolized.

Effect of ascorbic acid on the phenolic oxidation

To reduce phenolic oxidation during callus formation, different concentrations of ascorbic acid (20, 50 and 80 mg l^{-1}) in the culture media were evaluated. The culture medium was composed of MS salts, MS vitamins modified with a thiamine concentration of 0.4 mg l^{-1}, 100 mg l^{-1} myo-inositol and the concentrations of 2,4-D that gave the best results in the experiment above was used as control.

Observations were made to determine the onset of callus formation, presence of dark pigments in the culture media as well as on the explant. After 30 days of culture the number of explants forming callus and expressed in percentage was evaluated.

Influence of different sections of in vitro *shoots on callus formation*

The experiment was conducted with the objective of increasing the percentage of callus formation. For this, different types of thickened shoot sections obtained over 0.3 mm diameter were studied, whose shoot was cut and divided into three sectios as described below and shown in Figure 1C: I) section 1 (unlike the explant used in the experiment where the effect of 2,4-D was studied in the formation of callus, this section contains a part of the base where the meristematic zone of growth is located); II) section 2 (above 1, a cauline section of about 0.5 cm long was taken; III) section 3 (above 2, a cauline section of about 0.5 cm long was taken).

The sections were placed on culture medium containing MS salts, MS vitamins modified with a concentration of thiamine 0.4 mg l^{-1}, supplemented with 100 mg l^{-1} myo-inositol, with concentrations of 2,4-D and ascorbic acid determined in previous experiments. After 30 days of culture, the number of explants forming callus was evaluated and expressed in percentage.

Formation and differentiation of somatic embryos

Influence of 2,4-D and 6-BAP

To form and differentiate somatic embryos, light yellow, compact and friable calli with four multiplication subcultures were used. Calli fragments of approximately 0.5 cm in size were transferred to callus culture medium containing MS salts, MS vitamins modified with a thiamine concentration of 0.4 mg l^{-1}, supplemented with 100 mg l^{-1} myo-inositol, and 20 mg l^{-1} ascorbic acid. The effect of different concentrations of 2,4-D (2.26, 9.05 and 18.1 µM) and 6-BAP (1.11, 2.22 and 4.44 µM) in the formation and differentiation of somatic embryos was studied. As a control the culture medium described previously, free of growth regulators was used.

For all assessment of *in vitro* responses of shoot sections or calli on the various media tested, a total of ten replicated culture vessels with four explants each, i.e. 40 replicated shoot sections or callus pieces, were used. Observations were made on all treatments for the presence of somatic embryos. After 30 days of culture, the number of somatic embryos per callus was evaluated and the somatic embryos formed by developmental stage were classified.

Observations made during the formation and differentiation of the somatic embryos were done using a Leica brand (WILD M8 model, Germany) with 100x magnification and a manual counter stereomicroscope.

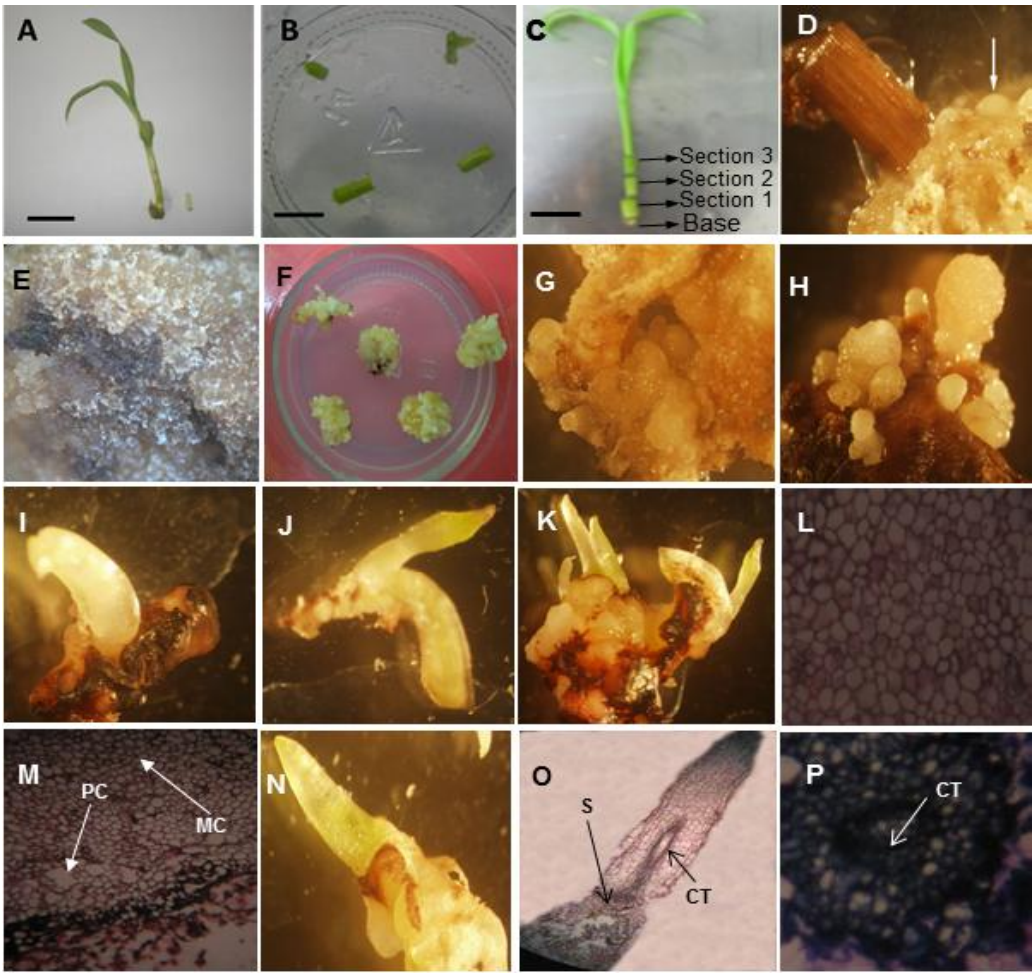


Figure 1. Calli and somatic embryos formed from the section of *in vitro* shoots *Sorghum bicolor* (L) Moench in MS medium and 2,4-D (18.1 µM) after 30 days of culture (Bar = 1.0 cm). (A) *in vitro* thickened shoots, located above the basal part where the growth zone is, (B) sections in MS medium for callus formation, located above the basal part where the growth zone is, (C) section dissection from *in vitro* thickened shoots. (D) Compact callus with smooth, compact, friable, light yellow surfaces and embryogenic, (E) Soft callus dark colored, cream yellow, non-embryogenic, (F) Callus formed in culture medium MS salts and 50 mg l^{-1} of ascorbic acid, (G) Globular stage somatic embryos, (H) groups of somatic embryos at various stages of histodifferentiation, (I) Coleoptile stage somatic embryo, (J) germinating somatic embryos, (K) somatic embryosat various stages of histodiferenciation, (L) Cells from embryogenic callus, (M) Different types of embryogenic callus cells, (N) coleoptile stage somatic embryo, (O) Longitudinal section of somatic embryo stage coleoptile, (P) Cross section of a somatic embryo stage coleoptile (WPS), (MC) meristematic cells, (PC) parenchymal cells, CT conductive tissues, S = suspensor.

Effect of L-Proline

With the objective of increasing the number and quality of somatic embryos, L-Proline was added to the culture medium. The concentration of 2,4-D and 6-BAP that gave the greatest number of somatic embryos formed were used. Light yellow, compact, friable calli with embryogenic appearance were used. Callus fragments were transferred to MS medium with the best combination of growth regulators in the above experiment, and the effect of three concentrations of L-Proline (250, 500, 750 mg l^{-1}) was evaluated. As a control the culture medium described previously, free of L-Proline was used.

For all assessment of *in vitro* responses of shoot sections or calli on the various media tested, a total of ten replicated culture vessels with four explants each, i.e. 40 replicated shoot segments or callus pieces, were used. Observations were conducted to determine the presence of embryogenic structures in all treatments at 30 days of culture. The following variables were evaluated:

- number of somatic embryos per callus,
- stage of development of somatic embryos formed.

Histological analysis of callus with embryogenic estrutures

Histological analysis was performed in order to check whether structures formed in calli were derived from a somatic embryogenic process in this monocotyledonous plant species. For this analysis calli with embryogenic structures that came from a culture medium containing 4.52 µM 2,4-D, 2.22 µM 6-BAP and 500 mg l^{-1} L-Proline were used. These were placed for 24 hours in a fixing solution containing 37% formaldehyde (v/v), glacial acetic acid 100% (v/v) and ethyl alcohol 70% (v/v), in a 1:1:18 ratio. Progressive dehydration of the samples was performed in ethanol solutions of increasing concentrations starting with 70% up to the absolute ethyl ethanol and the inclusion was made into paraffin blocks (Kraus and Arduin, 1997). The cuts were made at 10 µm with a rotary microtome HM 320 Heidelberg and subsequently stained with safranin 0.1% and toluidine blue 0.1% for the general examination of the tissue (Jensen, 1962). The samples were examined with a microscope (Olympus SZX7, USA) and photographed with a Canon camera (Power Shot A 630).

Experimental design and data analysis

The experimental design was completely randomized. The statistical analysis of the experimental data was performed using the software package SPSS version 18.0 in Microsoft Windows operating system. In all experiments evaluated, the data obtained underwent assumption checks of normality and homogeneity of variance (Lindman, 1974). The experimental data that did not meet the above assumptions were processed using the nonparametric Kruskal-Wallis test. The comparison of means was performed according to Mann Whitney nonparametric test with a level of significance $p \leq 0.05$. Experimental data that met the assumptions above were performed by analysis of variance (ANOVA) and two-factor simple classification. In each experiment, the tests carried out are outlined.

RESULTS AND DISCUSSIONS

Callus formation

Effect of 2,4-D

After 10 days of culture, the start of callus formation at the border closest to the base of *in vitro* shoot sections in all treatments tested (Figure 1D) was observed. After 30 days of culture, the callus had covered the base of the section. Two types of calli were formed; one compact, nodular, friable, light yellow with embryogenic appearance (Figure 1D) and the other watery, with dark and creamy yellow coloration, and non-embryogenic (Figure 1E).

In this context, and in line with our results, Zhao *et al*. (2010) described the formation of two types of calli during this stage of plant regeneration via somatic embryogenesis in sorghum; one compact, shiny yellow, friable and nodular with embryogenic appearance and the other watery and friable with non-embryogenic appearance.

At all concentrations of 2,4-D evaluated, there was callus formation. The highest percentage of callus with embryogenic structures (50%) was formed with 18.1 µM 2,4-D, with significant differences to the other treatments (Figure 2).

The lowest callus formation frequencies was obtained with 27.14 µM 2,4-D (30%). It was noted that many explants became dark brown and no callus formed in all treatments. The largest exudation by explant of dark brown pigments occurred with a concentration of 27.14 µM 2,4-D, observed in 100% of the explants and in the culture medium.

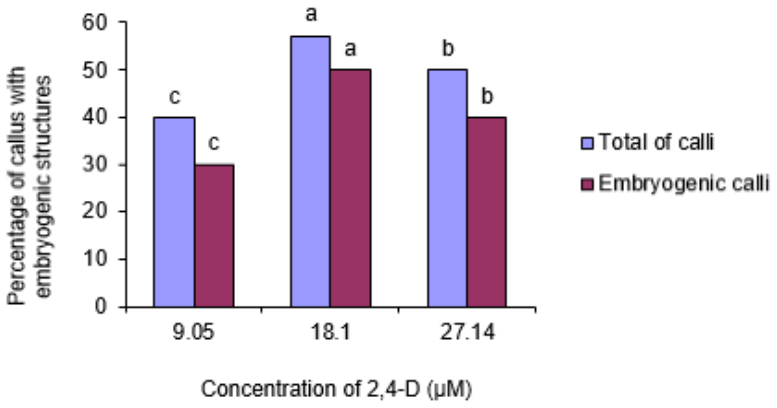
The low efficiency of the protocols for somatic embryogenesis in sorghum is one of the main difficulties in the development of genetic transformation in this species. In this study, various factors are optimized to improve the efficiency of somatic embryogenesis in sorghum. In the formation of callus, the growth regulator used plays a major role (von Arnold *et al*., 2002). The growth regulator most widely used in cereal for tissue culture in the formation of calli with embryogenic structures is 2,4-D (Bhaskaran and Smith, 1990; Vikra and Rashid, 2003; Jogeswar *et al*., 2007). This auxin has been proven to be very useful for the induction of calli with embryogenic structures in sorghum (Sudhakar *et al*., 2009; Zhao *et al*., 2010). In this work, the addition of 2,4-D to the culture medium favored the formation of callus from shoot segments grown *in vitro*. In the culture medium without growth regulator, no shoot segments with callus were observed.

The 2,4-D concentration for callus formation varied with the genotypes and explants used (Gupta *et al*., 2006; Arulselvi and Krishnaveni, 2009). In this work, a genotype and explant not previously studied, were used, and hence different concentrations of 2,4-D were added to the culture medium. Optimum callus formation occurred in the culture medium with 18.1 µM of 2,4-D. With concentrations of 9.05 and 27.14 µM there were low percentages of callus formation. In line with our results Lateefat *et al*. (2014) in a red grain mutant sorghum also achieved the highest frequency of callus induction with 18.1 µM 2,4-D in the culture medium, but used mature seeds as initial explant.

Effect of ascorbic acid on the phenolic oxidation

The addition of ascorbic acid to the culture medium reduced necrosis of explants. In the culture medium with this antioxidant, the formation of dark purple pigment was decreased. With the concentration of 50 mg l^{-1} ascorbic acid, necrotic explants were not observed, neither were dark purple pigments in the culture medium (Figure 1F).

The use of this concentration of ascorbic acid significantly increased the frequency of callus formation up to 68%. This result may have been caused by the removal of phenolic



Letters different on the bars indicated differences among means according to the Kruskal Wallis and Mann Whitney tests, $p \leq 0.05$

Figure 2. Effect of 2,4-D concentration on callus formation from section of *in vitro* shoots of sorghum (*Sorghum bicolor* (L.) Moench) cv. 'CIAP 132R-05' after 30 days of culture. n=40

compounds excreted from the explants, which should increase the availability of the components in the culture medium. With the concentration of 20 mg l^{-1}, though reduced, the formation of these compounds relative to the control was not adequate to achieve greater inhibition of the formation of the phenolic compounds from the explants. With the addition of 80 mg l^{-1} to the culture medium, it was not possible to increase the control of phenolic oxidation. This may be given by the toxic effect of high concentrations of ascorbic acid produces to the tissue, which prevented the control of the activity of the polyphenol oxidase enzyme (Table 1).

Phenolic oxidation is one of the main obstacles for the development of somatic embryogenesis in sorghum (Sadia *et al.,* 2010; Brandão *et al*., 2012). In this study, the presence of phenols in the culture medium around the explant caused mortality and negatively influenced the formation of callus. Thus, the addition of ascorbic acid to the culture medium was necessary. The use of 50 mg l^{-1} of ascorbic acid resulted in a significant reduction of phenolic oxidation, which increased callus formation by 17%. The concentration of this antioxidant, which resulted adequate (50 mg l^{-1}) in this experiment using 'CIAP 132R-05' cv., differs from that reported by Lu *et al*. (2009) in line P898012, which used 10 mg l^{-1} of ascorbic acid for diminishing phenolic oxidation during callus induction stage. The need to vary the concentration of ascorbic acid for controlling phenolic oxidation when different varieties are cultured, shows that the emission of phenols by the explants to the culture medium is highly influenced by the genotype.

Influence of different sections of in vitro *shoots on callus formation*

Callus formation was achieved only in the treatments where section 1 (section containing part of the base where the meristematic zone of growth is located) was used as explant. This result was related to the presence of the vegetative meristem at the base of the explant. The use of this type of explant increased the frequency of embryogenic callus formation to 95%. Callus formation from section 1 began at the base of the explant where the zone of active cell division is located. After 30 days of culture, the callus formed covered the entire base of the explant (Figure 1D). Explants of sections 2 and 3 did not form callus. They became necrotic and also delivered phenolic compounds to the culture medium. This indicates that the presence of this zone of active cell division is critical for callus formation from *in vitro* shoot sections in sorghum, at least for the conditions established in this experiment. The different response of *in vitro* shoot sections suggests that the cells that make up the apical meristem are better able to achieve dedifferentiation than other cell types present in these explants.

An important factor to consider for the establishment of an efficient regeneration protocol is the type of initial explant. Different types of explants have been described for the development of somatic embryogenesis in sorghum. Among the explants most widely used are those resulting from the process of sexual reproduction such as inflorescences, zygotic embryos and apices of germinated seeds *in vitro* (Arulselvi and Krishnavenl, 2009).

Table 1 Effect of ascorbic acid on callus formation of *Sorghum bicolor* (L.) Moench cv. 'CIAP 132R' using as explant cauline section segments of *in vitro* shoots after 30 days of culture.

Concentration of ascorbic acid (mg l^{-1})	Callus formation (%)	Mean rank	
0 (Control)	55.00	54.10	c
20	65.00	85.33	b
50	75.00	112.90	a
80	60.00	69.70	b

Means with different letters in the same column differ by Tukey test for $p \leq 0.05$ n=40

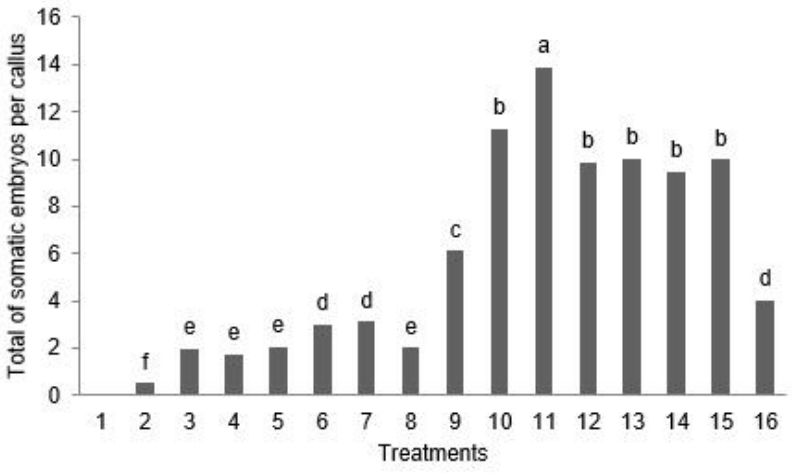
According Zhao *et al*. (2008) a high calli induction frequency has been achieved using mature seeds and zygotic embryos. However, with these explants the frequency of callus formation was lower than 40%. The same researcher group increased the formation of embryogenic calli up to 67% using germinating seeds as explant (Zhao *et al.*, 2010). In this work, calli were formed from sections of *in vitro* shoots, which have not been used previously for somatic embryogenesis in sorghum. The callus with embryogenic structure formation frequency that we obtained with this novel explant is high compared with the results previously reported for Zhao *et al.* (2008; 2010).

Formation and differentiation of somatic embryos

Influence of 2,4-D and 6-BAP

After 30 days of culture, somatic embryos were observed in various stages of development in all treatments. Groups of proembryos and somatic embryos in the globular stage with round and translucent appearance (Figure 1G) were observed. Some of the globular somatic embryos showed an intussusception, which represents a transition towards the scutellar stage (Figure 1H). Seventy two percent of the somatic embryos were in the globular stage and 25% in the scutellar stage. Also embryos in the coleoptile stage were observed in treatments with 4.52 µM 2,4-D, in combination with 2.22 µM 6-BAP (Figure 1I). Besides, some somatic embryos germinated in the same culture medium that were formed (Figure 1J).

The highest percentage of callus that formed somatic embryos were obtained in the culture medium containing 4.52 µM 2,4-D, in combination with 2.22 µM 6-BAP. Usually, in the treatments where the concentration of 2,4-D were superior to 4.52 µM, the most number of somatic embryos per callus was achieved. This value was significantly higher in treatment 11 with 13.84 somatic embryos per callus (Figure 3).



Letters different on the bars indicated differences among means according to the Kruskal Wallis and Mann Whitney tests, $p \leq 0.05$

Figure 3. Effect of 2,4-D and 6-BAP in the formation of somatic embryos of sorghum (*Sorghum bicolor* (L.) Moench) cv. 'CIAP 132R' after 30 days of culture. Treatments: 1(0 2,4-D and 0 6-BAP), 2(6-BAP 1.11 µM), 3(6-BAP 2.26 µM), 4(6-BAP 4.44 µM), 5 (2,4-D 2.26 µM), 6 (2,4-D 2.26 µM, 6-BAP 1.11 µM), 7(2,4-D 2.26 µM, 6-BAP 2.22 µM), 8(2,4-D 2.26 µM, 6-BAP 4.44 µM), 9 (2,4-D 4.52 µM), 10(2,4-D 4.52 µM, 6-BAP 1.11 µM), 11(2,4-D 4.52 µM, 6-BAP 2.22 µM), 12 (2,4-D 4.52 µM, 6-BAP 4.44 µM), 13(2,4-D 9.05 µM), 14(2,4-D 9.05 µM, 6-BAP 1.11 µM), 15 (2,4-D 5.05 µM, 6-BAP 2.22 µM), 16 (2,4-D 9.05 µM, 6-BAP 4.44 µM).

Seetharama *et al*. (2000) and Arulselvi and Krishnaveni (2009) described the regeneration of sorghum plants via somatic embryogenesis. However, the information on the formation of somatic embryos and the description of the different stages of development of these embryos is limited. Generally, to stimulate the regeneration of plants from embryogenic callus of sorghum, auxins and cytokinins are used, but the type and concentration of such growth regulators are varied. The use of certain growth regulators for regeneration of sorghum plants is subject to the genotype and the type of explant used for callus formatiòn. A reduction of the concentrations of 2,4-D used during callus formation combined with low concentrations of 6-BAP favored the formation of somatic embryos of sorghum in this study.

It is known that the addition of plant growth regulators in the culture medium affects the auxin/cytokinin endogenous balance (Del Pozo *et al*., 2005). Arulselvi and Krishnaveni (2009) in four genotypes of *Sorghum bicolor* added kinetin as the growth regulator and it increased the formation of somatic embryos. In this study, the combination of 4.52 µM 2,4-D with 2.22 µM 6-BAP reacted with the endogenous balance of auxin/cytokinin causing an increase in the formation of somatic embryos. In other investigation, Sudhakar *et al.* (2008) obtained the highest number of somatic embryos when 6-BAP, at concentrations of 8.88 µM, was used as cytokinin.

Embryogenic cells develop when the experimental conditions allow them to express their potential. Generally, this occurs when the amounts of auxin-cytokinin ratio is reduced. This can be demonstrated with *in vitro* systems where plant somatic cells can regain their totipotency and form embryos through the development of somatic embryogenesis (Raghavan, 2000).

Souter and Lindsey (2000) mentioned that the phase of formation and differentiation of somatic embryos is crucial. It is here where the patterns of apical-basal polarity is determined and specified for developing the cauline shoots and root apices of the somatic embryo.

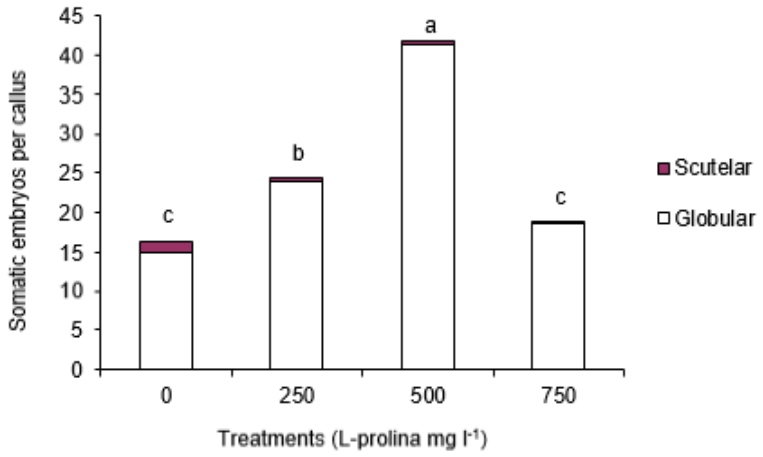
Lateefat *et al.* (2014) in a red grain mutant sorghum (Karandafi), observed after three weeks the formation of somatic embryos on most of the callus transferred to culture media containing low concentrations of 2,4-D. Reducing the amounts of 2,4-D combined with 6-BAP used in this experiment is apparently the appropriate auxin-cytokinin ratio for this sorghum variety and type of explant. The appropriate relationship between these growth regulators allows somatic cells of this variety to retain their totipotency and form somatic embryos through the development of somatic embryogenesis.

The combination of growth regulators needed to form somatic embryos depends on the type of cells present in the explant, embryogenic cell either predetermined (ECPDs) or embryogenic cell induced (ECIs). For ECIs tissues, the use of high concentrations of auxin is necessary to induce cell embryonic stage and subsequently decreasing the threshold of these auxins or cytokinins added to form and develop the embryos (Merkle *et al.*,1995). The results revealed that the calli formed in this study presented ECIs, because with only the addition of a cytokinin, somatic embryos (Figure 3) were formed. Besides, the formation of somatic embryos increased with the combination of auxin and cytokinin in the culture medium, which revealed that in most of the callus cells present ECIs.

Effect of L-Proline

In the present study, after 21 days of culture, groups of embryos at different development stages (Figure 1K), were observed. Some of the globular somatic embryos showed an intussusception, which represents a transition stage towards a more advanced stage in the development of somatic embryo (Jayasankar *et al*., 2003). After 30 days of culture, the formation of somatic embryos at various stages of development in all treatments was observed.

The greatest number of somatic embryos formed was in the treatment three in a culture medium with 4.52 µM 2,4-D, 2.22 µM 6-BAP and 500 mg l^{-1} L-Proline (Figure 4).



Letters different on the bars indicated differences among means according to the Kruskal Wallis and Mann Whitney tests, p≤0.05

Figure 4. Effect of L-proline in the formation of somatic embryos of sorghum (*Sorghum bicolor* (L.) Moench) cv. 'CIAP 132R' after 30 days of culture. Treatments: 1 (2,4-D 4.52 µM, 6-BAP 1.11 µM), 2 (2,4-D 4.52 µM, 6-BAP 1.11 µM, L-proline 250 mg l^{-1}), 3 (2,4-D 4.52 µM, 6-BAP 1.11 µM, L-proline 500 mg l^{-1}), 4 (2,4-D 4.52 µM, 6-BAP 1.11 µM, L-proline 750 mg l^{-1}).

The role of amino acids as an immediately available source of nitrogen to plant cells is well known. The uptake may be much faster than that coming from inorganic nitrogen in the same culture medium (Thom *et al*., 1981). Monnier (1990) noted that reduced nitrogen is required for induction of somatic embryo, as these young embryos lack the enzyme nitrate reductase, responsible for the reduction of nitrate to nitrite. Organic nitrogen can be built quickly, stimulating the growth of cells, even when the mechanisms of absorption of ammonium and nitrate are not fully functioning (Pola *et al*., 2007). As has been described by several authors, L-Proline promotes the formation of embryogenic callus and plant regeneration capacity of sorghum (Armstrong and Green, 1985; Rao *et al*., 1995).

Sorghum is a crop that requires high amounts of nitrogen. L-Proline is an important amino acid for the development of somatic embryogenesis in this monocot. The presence of L-Proline in the culture medium can influence the formation and differentiation of somatic embryos of sorghum. Thus, Sudhakar *et al*. (2008) reported that L-Proline (1000 mg l^{-1}) promotes the development of somatic embryos of sorghum. In this study the addition of L-Proline in the culture medium up to 500 mg l^{-1} caused an increase in the formation of somatic embryos. Other authors such as Neguyen *et al.* (2007) also achieved high efficiency plant regeneration with the addition of 500 mg l^{-1} of L-Proline to the culture medium. Similar effects were reported by Zhao *et al*. (2010) who achieved the highest efficiency of calli with embryogenic structures and plant regeneration via somatic embryogenesis of sorghum varieties 'Yuantian No.1' and 'M81E' with 650 mg l^{-1} of L-Proline in the culture medium.

The role of individual amino acids or mixtures of them is controversial in the literature, and sometimes they were reported to have no effect or a different one dependent on the species studied (Thorpe *et al.,* 2008). L-Proline is more stimulating compared with the other two amino acids such as L-glutamine and L-arginine (Amali *et al*., 2014). These authors, in their study, were able to increase the number up to 33.3 somatic embryos per callus in a growth medium where callus with embryogenic structure was subcultured on MS salts with 11.31 µM 2,4-D, 0.25 mg l^{-1} kinetin, and 500 mg l^{-1} casein hydrolysate and 500 mg l^{-1} L-Proline. In the present investigation, the addition of 500 mg l^{-1} L-Proline into a culture medium containing 9.05 µM 2,4-D and 1.11 µM 6-BAP caused a

significant increase in the number of somatic embryos per callus (41.88), which showed the influence of this amino acid in the formation and differentiation of somatic embryos of sorghum.

Histology of embryogenic callus and somatic embryos

In *S. bicolor* cv. 'CIAP 132R-05', two types of calli with embryogenic and non-embryogenic structures were formed. It was observed that one type of callus also had the formation of embryogenic structures. These embryogenic calli were characterized as compact, friable and of a light yellow color. Histological analysis of this embryogenic callus was found to be composed mainly of meristematic cell type that is isodiametric cell with no well-defined nucleus and vacuoles.

A histological section of a callus with embryogenic structures revealed the presence of different cells characterized by their sizes and densities of the cytoplasmic, small well-defined intercellular spaces and cell walls are present (Figure 1L). In this type of callus, meristematic cells and others in smaller numbers, with parenchymatic characteristics were observed. The latter is located further inside the callus; while the meristematic cells are located on the surface regions of the callus (Figure 1M). The parenchymal cells (PC) could be derived from meristematic cell differentiation during the initial period of callus formation. It is characterized by a larger, more elongated shape or a higher level of cell differentiation (Figure 1M). While meristematic cells, of smaller size, were grouped into the surface region of the callus (Figure 1M).

In histological sections, the presence of somatic embryos in the coleoptile stage (Figure 1N) was observed. These somatic embryos had well-defined vascular bundles along the central axis of the somatic embryo, which is characteristic in the morphogenetic development phase (Figure 1O). It was observed in the histological analysis that somatic embryos that had formed coleoptile stage remained with suspending structures. Also in the cross section, a well-defined structure of the conductive tissue was observed (Figure 1P).The histological studies are important for the confirmation of somatic embryogenesis and have been used for this purpose. In *Sorghum bicolor* Kishore *et al.* (2006) described meristematic and non-meristemetic cells, from embryogenic calli, with similar characteristics to the cells observed in this investigation.

CONCLUSIONS

The use of section 1 from *in vitro* shoots as explant that has not been utilized before for the somatic embryogenesis in sorghum was an important factor for the somatic embryos formation in this crop. The formation of callus with embryogenic structures was influenced by 2,4-D concentration, the antioxidant effect of ascorbic acid and the type of explant. The formation of somatic embryos depended on the growth regulators added to the culture medium. The rate of exogenous auxin/ citokinine played a determinant role on the increase of somatic-embryo formation. The L-Proline provoked a significant increase of the average number of somatic embryos per callus (41.88). It evidenced the influence of this aminoacid on the somatic embryos formation and differentiation in sorghum. The histological analysis confirmed that the structures formed from callus are derived of somatic embryogenesis morphogenetic process.

REFERENCES

Amali P, Kingsley SJ, Ignacimuthu S (2014) Enhanced plant regeneration involving somatic embryogenesis from shoot tip explants of *Sorghum bicolor* (L. Moench). Asian Journal of Plant Science and Research 4: 26-34

Armstrong CL, Green CE (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-Proline. Planta 164: 207-214

Antonopoulou G, Gavala H, Skiadas I, Angelopoulos K, Lyberatos G (2008) Biofuels generation from sweet sorghum: Fermentative hydrogen production and digestion of the remaining biomass. Bioresour Technology 99: 110-119

Arulselvi I, Krishnaveni S (2009) Effect hormones, explants and genotypes in *in vitro* culturing of sorghum. J Biochem Tech 1: 96-103

Bhaskaran S, Smith RH (1990) Regeneration in cereal tissue culture: A review. Crop Sci 30: 1328-1336

Baskaran P, Jayabalan N (2005) Simple approach to improve plant regeneration from callus culture of

Sorghum bicolor for crop improvement. Journal of Agricultural Biotechnology 1: 179-192

Baskaran P, Raja Rajeswari B, Jayabalan N (2006) Development of an *in vitro* Regeneration System in Sorghum [*Sorghum bicolor* (L.) Moench] Using Root Transverse Thin Cell Layers (tTCLs). Turk J Bot 30: 1-9

Brandão RL, Carneiro NP, Oliveira AC, Coelho G, Almeida A (2012) Genetic Transformation of Immature Sorghum Inflorescence via Microprojectile Bombardment. En: Özden Çiftçi Y (Ed.) Transgenic Plants - Advances and Limitations, InTech, [En línea] En: http://www.intechopen.com/books/transgenic-plants-advances-and-limitations/genetic-transformation-of-immature-sorghum-inflorescence-via-microprojectile-bombardment. Consultado el 15 de diciembre de 2014

Del Pozo JC, López-Matas MA, Ramírez-Parra E, Gutiérrez C (2005) Hormonal control of the plant cell cycle. Physiologia Plantarum 123: 173-183

Gupta S, Khanna V, Singh R, Garg G (2006) Strategies for overcoming genotypic limitations of *in vitro* regeneration and determination of genetic components of variability of plant regeneration traits in sorghum. Plant Cell Tiss Organ Cult 86: 379-388

Jayasankar S, Bondada RB, Li A, Gray JD (2003) Comparative anatomy and morphology of *Vitis vinifera* (*Vitaceace*) somatic embryos from solid and liquid culture derived proembryogenic masses. Amer J Bot 90: 973-979

Jensen WA (1962) Botanical Histochemistry. Principles and practice. University of California, Berkeley. WH Freeman and Company. San Francisco. pp. 23-56

Jogeswar G, Ranadheer D, Anjaiah V, KaviKishor PB (2007) High frequency somatic embryogenesis and regeneration in different genotypes of *Sorghum bicolor* (L.) Moench from immature inflorescence explants. *In vitro* Cell Developmental Biology Plant 43: 159-166

Kishore K, Visarad K, Aravinda Y, Pashupatinath E, Rao S, Seetharama N (2006) *In vitro* culture methods in sorghum with shoot as the explant. Plant Cell Report 25: 174-182

Kraus JE, Arduin M (1997) Manual Básico de Métodos em Morfologia vegetal. Editora da Universidade Federal Rural do Rio de Janeiro. Rio de Janeiro

Lateefat BH, Inuwa SU, Katung MD, Bugaje SM (2014) Optimum Protocol for Shoot Formation in *karandafi* Red Sorghum (*Sorghum bicolor* (L.) Moench) through Somatic Embryogenesis Using Mature Embryo. American Journal of Plant Sciences 5: 671-675

Liu G S, Zhou QY, Song S Q, Jing H C, Gu W B, Li X F, Su M, Srinivasan R (2009) Researchs advances into germoplasm resources and molecular biology of the energy crop sweet sorghum. Bulletin of Botany 44: 253-261

Maheswari M, Lakshmi N J, Yaday S K, Varalaxmi Y, Lakshmi A V, Vanaja M (2006) Efficient plant regeneration from shoot apices of sorghum. Biology Plant 50: 741-744

Martínez S, Gómez Kosky R, Barbón R, Posada L, Acosta M, Reyes M, Pérez M, Torres D, Pons M, La O M, Aguilera A, Tejeda M (2012) Efecto de dos citoquininas, ácido ascórbico y sacarosa en la obtención de plantas *in vitro* de *Sorghum bicolor* para la formación de callos. Rev. Colombiana. Biotecnología 14: 101-110

Merkle SA, Parrott WA, Flinn BS (1995) Morphogenic aspects of somatic embryogenesis. En: TA Thorpe (Ed.) *In vitro* embryogenesis in plants, pp. 155–203. Kluwer Academic Press. Dordrecht

Monnier M (1990) Zygotic embryo culture. En: Bhojwani SS (Ed.) Plant tissue culture: applications and limitations, pp. 366-393. Elsevier. Amsterdam

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco tissue culture. Physiologia Plantarum 15: 473-497

Nguyen TV, Thanh Thu T, Claeys M, Angenon G (2007) *Agrobacterium*- mediated transformation of sorghum (*Sorghum bicolor* (L.) Moench) using an improved *in vitro* regeneration system. Plant Cell Tiss Organ Cult 91: 155-164

Pérez A, Saucedo O, Iglesias J, Wencomo H, Reyes F, Oquendo G, Milán I (2010) Caracterización y potencialidades del grano de sorgo (*Sorghum bicolor* (L.)Moench.) Pastos y Forrajes 33: 1-17

Pola S, Saradamani N, Ramana T (2007) Enhanced shoot regeneration in tissue culture studies of *Sorghum bicolor*. Journal of Agricultural Technology 3: 275-286

Raghavan V (2000) Developmental Biology of Flowering Plants. Springer-Verlag. New York

Rao AM, Sree KP, Kishor PBK (1995) Enhanced plant regeneration in grain and sweet sorghum by asparagine, Proline and cefotaxime. Plant Cell Rep 15: 72-75

Rooney WL, Blumenthal J, Bean B, Mollet JE (2007) Designing sorghum as a dedicatec bioenergy feedstock. Biofuels, Bioprod and Bioref 1: 147-157

Sadia B, Josekutty PC, Potlakayala SD, Patel P, Goldman S, Rudrabhatla SV (2010) An efficient protocol for culturing meristems of sorghum hybrids. FYTON 79: 177-181

Seetharama N, Sairam RV, Rani TS (2000) Regeneration of sorghum shoot apex cultures and field performance of the progeny. Plant Cell Tissue Organ Cult 61: 169–173

Souter M, Lindsey K (2000) Polarity and signaling in plant embryogenesis. J Exp. Bot 51: 971-983

Sudhakar P, Sarada MN, Ramana T (2008) Plant tissue culture studies in *Sorghum bicolor*: immature embryo explants as the source material. International Journal of Plant Production 2: 1-14

Sudhakar N, Sarada MN, Ramana T (2009) Mature embryos as source material for efficient regeneration response in sorghum (*Sorghum bicolor* L. Moench). Sjemenartvo 26: 93-104

Thom M, Maretzki A, Komor E, Sakari WS (1981) Nutrient uptake and accumulation by sugarcane cell cultures in relation to the growth cycle. Plant Cell Tissue Organ Cult. 1: 3-14

Thorpe T, Stasolla C, Yeung EC, de Klerk GJ, Roberts A, George EF (2008) The Components of Plant Tissue Culture Media II: Organic Additions, Osmotic and pH Effects, and Support Systems. En: George EF, Hall MA, De Klerk GR (Eds.) Plant Propagation by tissue culture (3rd Edition) Volume 1, Chapter IV, pp.115-174. Springer. Dordrecht

Vikra L, Rashid A (2003) Somatic embryogenesis or shoot formation following high 2,4-D pulse-treatment of mature embryos of *Paspalum scrobiculatum*. Biol Plant 46: 297-300

von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tiss Organ Cult. 69: 233-249

Wu E, Lenderts B, Glassman K, Berezowska-Kaniewska M, Christensen H, Asmus T, Zhen S, Chu U, Cho MJ, Zhao ZY (2014) Optimized *Agrobacterium*-mediated sorghum transformation protocol and molecular data of transgenic sorghum plants. *In vitro* Cell Dev. Biol. Plant 50: 9-18

Zhao L, Liu S, Song S (2008) Efficient induction of callus and plant regeneration from seeds and mature embryos of sweet sorghum. Chinese Bulletin of Botany 25: 465-468

Zhao L, Liu S, Song S (2010) Optimization of callus induction and plant regeneration from germinating seeds of sweet sorghum (*Sorghum bicolor* L. Moench). African Journal of Biotechnology 9: 2367-2374

Recibido: 14-7-2015
Aceptado: 07-10-2015