

An efficient protocol for culturing meristems of sorghum hybrids

Un protocolo eficiente para el cultivo de meristemas híbridos de sorgo

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Abstract. A robust protocol for culturing meristems of *Sorghum* is required to assist with rapid genetic improvement of the genus. Through meristem culture, an efficient method for rapid micropropagation was developed for *Sorghum bicolor* (L.) Moench. hybrids, namely NC+262, NC+6C21 and NC+6B50. Complete plants were regenerated directly from shoot meristems without an intervening callus phase. Regeneration frequencies varied between the studied genotypes and according to the growth regulator combinations present in the medium. The combination of BAP and TDZ showed a synergistic effect on shoot multiplication. The highest number of shoots per meristem (68 ± 2) was recorded for the genotype NC+262, followed by NC+6C21 (31.3 ± 0.8), and NC+6B50 (16 ± 1.7). MS medium supplemented with 3 mg/L BAP and 1 mg/L TDZ was the most effective hormone combination for multiple shoot production. Regenerated shoots were rooted on MS basal medium. Once rooted, plants were transferred to the greenhouse, where 80% survived to reach maturity.

Keywords: Micropropagation; *Sorghum bicolor*; Tissue culture; Genetic improvement; Genotypes.

Abbreviations: BAP: 6- Benzylaminopurine. TDZ: Thidiazuron. MS: Murashige and Skoog, PGR: Plant growth regulator(s).

Resumen. Se requiere un buen protocolo para el cultivo de meristemas de *Sorghum* para asistir en una rápida mejora genética del género. A través del cultivo de meristemas, se desarrolló un método eficiente para la rápida micropropagación de híbridos de *Sorghum bicolor* (L.) Moench, específicamente NC+262, NC+6C21 y NC+6B50. Se obtuvieron plantas completas directamente a partir de meristemas del tallo sin la intervención de una fase de callo. Las frecuencias de regeneración variaron entre los genotipos estudiados y de acuerdo a las combinaciones de los reguladores de crecimiento presentes en el medio. La combinación de BAP y TDZ mostraron un efecto sinérgico en la multiplicación del tallo. El mayor número de tallos por meristema (68 ± 2) se registró para el genotipo NC+262, seguido por NC+6C21 (31.3 ± 0.8), y NC+6B50 (16 ± 1.7). El medio MS suplementado con 3 mg/L BAP y 1 mg/L TDZ fue la combinación hormonal más efectiva para la producción múltiple de tallos. Los tallos regenerados enraizaron en medio basal MS. Una vez enraizadas, las plantas fueron transferidas al invernáculo, donde el 80% sobrevivió hasta alcanzar la madurez.

Palabras clave: Micropropagación; *Sorghum bicolor*; Cultivo de tejido; Mejoramiento genético; Genotipos.

Abreviaturas: BAP: 6- Bencilaminopurina. TDZ: Thidiazuron. MS: Murashige y Skoog, PGR: Regulador(es) del crecimiento vegetal.

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INTRODUCTION

Sorghum bicolor (L) Moench. is grown in 98 countries across Africa, America and Asia. It is the fifth most important cereal crop in the world, and is the dietary staple of more than half a billion people in over 30 countries (ICRISAT, 2010). World-wide production of sorghum is 57–63 million tons grown on approximately 45 million hectares (ha) of land (Maqbool et al., 2001). Sweet sorghum varieties are popular feedstock for cellulosic ethanol production (Murray et al., 2008).

Improvement of sorghum for superior agronomic traits has been carried out using traditional plant breeding methods (Casas et al., 1993; Maqbool et al., 2001). Traditional breeding for genetic improvement depends largely on the genetic variability available among the crops, and may require many years of cross-breeding to develop an improved plant. Genetic engineering allows for the generation of novel varieties of a certain crop by introducing genes from sources outside that crop. Genetic transformation with specific genes that would confer disease and pest resistance in sorghum is hampered by the recalcitrancy of the species to transformation (Zhu et al., 1998; Maqbool et al., 2001; Pandey et al., 2010). To achieve efficient genetic transformation in sorghum, the development of an *in vitro* regeneration system specific to this crop is required.

Explants such as immature inflorescences (Brettel et al., 1980; Boyes & Vasil 1984; Eapen & George, 1990), immature embryos (Ma et al., 1987; Oldach et al., 2001), mature embryos from seeds (MacKinnon et al., 1987; Zapata et al., 2004), and shoot tips (Baskaran & Smith, 1992; Seetharama et al., 2000; Maheswari et al., 2006) were used as explants for tissue culture of sorghum. Maheswari et al. (2006) noted that major hurdles in the micropropagation of sorghum to be the species' genotype dependant response, its very low rate of regeneration, the production of phenolics, and its problems in acclimation. The frequency of callus production, subsequent growth rate of callus, and the rate of plant regeneration from callus of sorghum are lower than tissue cultures of many other cereals (Lusardi & Lupotto, 1990). Obtaining embryogenic callus from mature seeds of cultivated sorghum genotypes is difficult (Hagio, 1994). Hence, the immature embryo is usually the explant used for genetic transformation of cereals (Casas et al., 1993). Collecting immature embryos, however, is time consuming and laborious, requiring time to grow donor mother plants in soil at least until the development of immature embryos. An alternative method is to use shoot tips as explants for transformation and plant regeneration. Using such shoot tips is also problematic, yielding only a few sites per explant for genetic manipulation (Baskaran & Smith, 1988).

Zhong et al. (1992) reported high yield of somatic embryos from cultured maize shoot meristem explants by allowing proliferation of their shoot meristems *in vitro*. Sairam et al. (2002 and 2003) developed efficient protocols for the induction of high frequency shoot proliferation from *in vitro*-cultured

shoot meristems of *Tripsacum* and corn. These shoots were shown to arise either by direct organogenesis or by somatic embryogenesis.

The objective of the present study was to develop an efficient protocol for micropropagation of sorghum hybrids from shoot meristem explants. In this communication, we report production of a robust, *in vitro* propagation protocol for three sorghum hybrids (NC+6C21, NC+6B50 and NC+262) starting with shoot meristem explants.

MATERIALS AND METHODS

Sorghum hybrids, namely, NC+6C21, NC+262, and NC+6B50 were used in the current study. The seeds were washed in running tap water with a few drops of soap for 10 min, and surface sterilized in 70% (v/v) ethanol for 1 min followed by 10 min incubation in 10% (v/v) commercial bleach with intermittent shaking. The seeds were subsequently rinsed three times in sterile, distilled water. Surface sterilized seeds were germinated on hormone free MS medium (Murashige & Skoog, 1962), pH 5.8, and gelled with 8 g/l agar in Petri dishes (100 x 50 mm) incubated in darkness at 24 ± 2 °C.

Three days after germination, 3–4 mm long shoot apices (consisting of the shoot meristem and part of the mesocotyl) were isolated and cultured on MS medium containing 3 mg/L BAP for one week. After a week, shoot meristems were transferred to six different culture media: modified MS medium supplemented with BAP (3 mg/L) alone or in combination with various concentrations of TDZ (0.1, 0.5, 1.0 and 2.0 mg/L). The basal medium was supplemented with 30 g/l sucrose and 8 g/l agar. The medium pH was adjusted to 5.8 before autoclaving. Ten shoot meristems were transferred to each Petri dish (100 x 15 mm) containing 20 ml solidified medium. All shoot meristems were incubated at 24 ± 2 °C under 16 h photoperiod provided by cool-white fluorescent lights at a quantum flux density of 30 µmol/s/m². Hundred explants were produced for each genotype and media combination. This process was repeated three times.

Elongating leaves were removed from the growing shoot meristems and the secondary meristems were subcultured every two weeks to fresh medium for 10 weeks. Emerging secondary shoot meristems from each primary shoot meristem were maintained separately. Multiple shoot clumps produced thereof were carefully separated during the subculture. After 10 weeks in culture, the relative frequency of shoot proliferation was calculated by dividing the total number of shoots generated with the original number of shoot meristem explants used for every genotype.

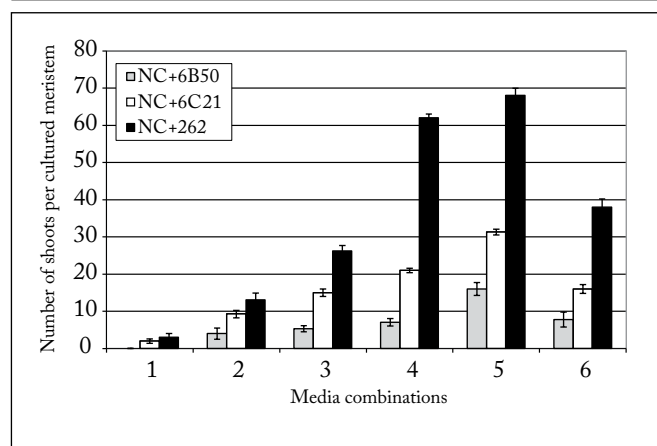
Individual shoots (4–5 cm) were isolated and transferred to MS basal medium containing 2% (w/v) sucrose to induce rooting. Rooted plants were subsequently transferred to soil and maintained in greenhouse for further growth and development.

Statistical analysis. All values provided in Figure 1 are means of 300 observations per study treatment (effect of the media on shoot multiplication) and genotypes. Error bars indicate standard deviation of the means.

Fig. 1. Comparison of Sorghum hybrids for multiple shoot formation. Media Codes: (1) MS basal medium, (2) MS + 3 mg/L BAP, (3) MS + 3 mg/L BAP + 0.1 mg/L TDZ, (4) MS + 3 mg/L BAP + 0.5 mg/L TDZ, (5) MS + 3 mg/L BAP + 1 mg/L TDZ, (6) MS + 3 mg/L BAP + 2.0 mg/L TDZ. All values are mean of 300 observations per treatment per genotype. Error bars indicate S.E.

Fig. 1. Comparación de híbridos de sorgo para la formación múltiple de tallos.

Códigos para los medios: (1) Medio basal MS, (2) MS + 3 mg/L BAP, (3) MS + 3 mg/L BAP + 0.1 mg/L TDZ, (4) MS + 3 mg/L BAP + 0.5 mg/L TDZ, (5) MS + 3 mg/L BAP + 1 mg/L TDZ, (6) MS + 3 mg/L BAP + 2.0 mg/L TDZ. Todos los valores son promedio de 300 observaciones por tratamiento y genotipo. Las barras de error indican ± 1 error estándar.

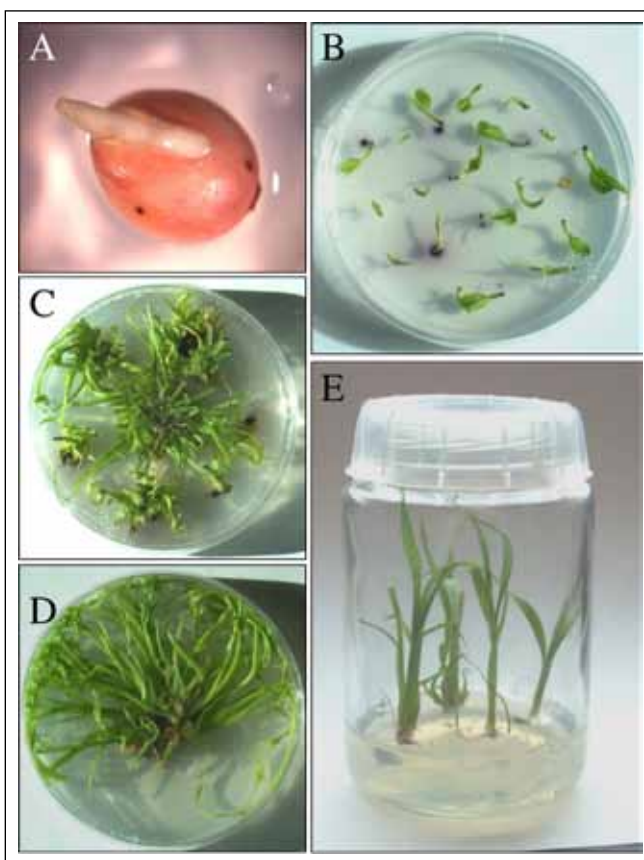


RESULTS

All the media combinations tested in this study produced multiple shoots from meristems of the three sorghum genotypes cultured *in vitro*. Sorghum hybrid NC+262 yielded the highest number of shoots (68.0 ± 2.0) per cultured meristem. Hybrid NC+6C21 generated 31.3 ± 0.8 shoots/meristem, while NC+6B50 produced only 16 ± 1.7 shoots/meristem explant. MS medium supplemented with a combination of plant growth regulators (3.0 mg/L BAP and 1 mg/L TDZ) produced the best rate of shoot proliferation in all the three genotypes studied, as shown in Figure 1. Each multiple shoot clump produced six to seven shoots, and, depending on the genotype, approximately 18-68 shoots were recovered from each original shoot meristem within 10 weeks of culture initiation, as shown in Figure 2. Most of the regenerated shoots rooted in a hormone-free MS medium. Rooted plants were transferred to the greenhouse, out of which 80% plants subsequently developed to maturity.

Fig. 2. Stages in the meristem culture of sorghum hybrids. A: Germinating seeds of sorghum hybrid NC+262 on 3rd day of incubation in MS medium. B: Shoots developing from cultured meristems of hybrid NC+262. C: Multiple shoots developed from meristems of NC+262 (two weeks after culture in shoot multiplication medium). D: Multiple shoots developed from single meristem of hybrid NC+262 (6th week of culture in shoot multiplication medium). E: *In vitro* rooted plants.

Fig. 2. Estadios en el cultivo de meristemas de híbridos de sorgo. A: Germinación de semillas del híbrido de sorgo NC+262, luego del 3^o día de incubación en el medio MS. B: Tallos en desarrollo desde meristemas cultivados de híbrido NC+262. C: Tallos múltiples desarrollados a partir de meristemas de NC+262 (dos semanas luego de su cultivo en el medio de multiplicación de tallos). D: Tallos múltiples desarrollados desde un único meristema del híbrido NC+262 (6^a semana de cultivo en el medio de multiplicación de tallos). E: Plantas enraizadas *in vitro*.



DISCUSSION

Shoot apex culture and subsequent regeneration through intervening callus phase in a few sorghum genotypes have been previously documented (Zhong et al., 1998; Baskaran & Jayabalan, 2005; Maheswari et al., 2006). Zhong et al. (1998) reported the differentiation of adventitious buds from enlarged apical domes and thickened leaf bases of shoot meristems cultured in MS medium supplemented with 0.5 mg/L 2,4-D and varying concentrations of BAP. Maheswari et al. (2006) reported plant regeneration through embryogenesis from shoot apices of sorghum cultured on MS medium supplemented with 2,4-D and Kinetin.

An intervening callus phase is disadvantageous for micropropagation because of the possible somaclonal variation (Makobe et al., 2006). The rate of shoot proliferation from cultured shoots of sorghum is low, and the regeneration is genotype-dependant (Baskaran & Jayabalan, 2005). Shoot meristems have been shown to be ideal explants for the rapid regeneration of maize and *Tripsacum*, irrespective of their genotypes as regeneration occurs rapidly and in large numbers at rates not previously observed (Sairam et al., 2002, 2003). The current study has demonstrated that shoot meristems of sorghum can proliferate in a fashion similar to maize and *Tripsacum*, resulting in rapid multiplication of shoot meristems suitable for transformation studies.

MS medium modified by the addition of specific growth regulators have been shown to be necessary for micropropagation of other cereals. Zhang et al. (1996) and Devi et al. (2000) reported multiple shoot production from cultured shoot apical meristems of oat and pearl millet [*Pennisetum glaucum* (L.) R. Br.]. Approximately 7-80 green shoots from each original shoot apex were obtained in 18 weeks after culture initiation on MS medium supplemented with BAP (4 mg/L) and 2,4-D (0.125 mg/L). Similarly, high frequency shoot proliferation from shoot meristems cultured on modified MS medium containing BAP was reported in maize (Sairam et al., 2003; Zhang et al., 1996), and *Tripsacum* (Sairam et al., 2002). Multiple shoot meristem clumps differentiated from single shoot meristems of wheat cultured in MS medium supplemented with BAP and 2,4-D (Anwaar et al., 2002).

The combination of BAP and TDZ in the medium apparently regulated the morphogenetic competence and shoot multiplication pathway of sorghum in this study. Multiple shoots developed primarily through axillary bud proliferation, although formation of adventitious shoots cannot be ruled out. Previous reports (Baskaran & Smith, 1988; Zhong et al., 1998; Phillips, 2004) indicated the possible production of large numbers of multiple shoots from the shoot meristem of sorghum through axillary and adventitious bud formation when serially subcultured. Thus, the composition of the tissue culture medium appears to circumvent apical dominance, resulting in a mass of shoots that, in turn, produce more shoots following the subcultures. Removal of elongated leaves at every subculture resulted in better shoot multiplication. Primary shoots produced several axillary shoots. Proliferating shoot clumps could be maintained indefinitely by subculturing them to fresh medium every two weeks.

All three genotypes responded to all the media combinations used in this study. A significant difference in the degree of response produced by the genotypes, however, was observed as shown in Figure 1. This data indicated that the ability of sorghum to produce multiple shoots in tissue culture, a seemingly genetically controlled trait, may in fact, be modified by manipulation of the culture media. Supporting this conclusion is the fact that shoot meristems excised from different

sorghum hybrids (genotypes) responded differently with respect to shoot multiplication in similar media. Similar reports of genotype-dependent variation in multiple shoot induction are on record for barley (Sharma et al., 2004), wheat (Anwar et al., 2002), and soybean (Barwale et al., 1986; Graybosch et al., 1987; Delzer et al., 1990). Genotype effect may be due to the difference in the number of morphogenetically competent cells present in the shoot meristem that have the capacity to respond to an altered signaling profile. In this case, the regeneration response appears to be a plant growth regulator-driven, genotype-dependent phenomenon. Since these genotypes showed a differential response (rate of shoot proliferation) to the treatments (media used), further studies will be carried out to see if the rate of shoot multiplication can be improved in all the genotypes.

These results indicate that an efficient protocol, independent of seasons, was developed for production and maintenance of shoot tips of sorghum hybrids. This protocol allows for the low cost production and maintenance of sorghum shoot tips for transformation studies during all seasons. Since maize (Shen et al., 1999) and many other members of the Gramineae, shoot meristems have been shown amenable to high frequency T-DNA transfer (Shrawat & Lorz, 2006). DNA delivery technology is likely to be linked to high frequency plant regeneration in sorghum providing for a robust protocol that would allow for the delivery of transgenic sorghum.

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