Plant Cell, Tissue and Organ Culture **61:** 169–173, 2000.

Research note

Regeneration of sorghum from shoot tip cultures and field performance of the progeny[1]

N. Seetharama, R.V. Sairam & T.S. Rani
ICRISAT PO, Patancheru, AP 502324, India

Received 27 April 1998; accepted in revised form 12 May 2000

Key words: embryogenic callus, friable embryogenic callus, shoot tip, *Sorghum bicolor*, tissue culture

Abstract

A system for rapid plant regeneration through somatic embryogenesis from shoot tip explants of sorghum [*Sorghum bicolor* (L.) Moench] is described. Somatic embryogenesis was observed after incubation of explants in dark for 6–7 weeks through a friable embryogenic callus phase. Linsmaier and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (2 mg l^{-1}) and kinetin (0.1 mg l^{-1}) was used for induction of friable embryogenic calli and somatic embryos. Germination of somatic embryos was achieved about 5 weeks after transfer onto Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (2 mg l^{-1}) and indole-3-acetic acid (0.5 mg l^{-1}) under light. Seeds from *in vitro*-regenerated plants produced a normal crop in a field trial, and were comparable to the crop grown with the seeds of the mother plant used to initiate tissue culture. The simplicity of the protocol and possible advantages of the system for transformation over other protocols using different explants are discussed.

Abbreviations: 2,4-D – 2,4 - dichlorophenoxy acetic acid; BA – 6-benzylaminopurine; FEC – friable embryogenic callus; IAA – indoleacetic acid; MS – Murashige and Skoog; LS – Linsmaier and Skoog

The progress on genetic transformation of sorghum (*Sorghum bicolor* (L.) Moench) is limited (Casas et al., 1993). Unlike in rice (Zhang et al., 1996), it is difficult to obtain embryogenic calli from mature seeds of cultivated sorghum genotypes. Use of immature embryos or isolated scutella can overcome the above problem to a significant extent. Though we have standardized a mesophyll protoplast regeneration system for sorghum (Sairam et al., 1999), it is tedious for use to routinely produce transgenics. Therefore, the most popular explant for genetic transformation of cereals is the immature embryo (Casas et al., 1993). Nevertheless, use of immature embryos for *in vitro* culture has some limitations owing to difficulties for maintenance until successful regeneration. Not all genotypes are capable of producing embryogenic calli, although this problem can be partially remedied by culturing scutella. Some genotypes producing good FEC are subsequently prone to the release of polyphenols or other inhibitory substances (Rao et al., 1995) which may reduce the final percentage of transformants. Successful maintenance and counteraction of such inhibitory substances may involve complex and laborious procedures such as use of series of amino acids in media to counter antioxidant production (Elkonin et al., 1995) and frequent sub-culturing and incubation under dark conditions (Elhag and Butler, 1992). In sorghum, successful regeneration is usually achieved from two types of embryogenic calli – friable embryogenic calli (FEC; Gendy et al., 1996) and compact nodular calli (Elhag and Butler, 1992). However, in all the above studies, the time taken from sowing donor mother plants in soil up to regeneration of explants into whole plants extended to nine months or more. An alternative is to use shoot tips as explants for transformation and plant regeneration (Bhaskaran and Smith, 1988; Nahdi et al., 1995). However, such shoot tips yield only few sites per explant for genetic manipulation.

[1] Published as ICRISAT Journal Article No. 2255

Zhong et al. (1992) developed a novel method to generate a large number of somatic embryos from maize shoot apices by allowing proliferation of meristems *in vitro*. Since it is convenient to obtain shoot apices from germinating seeds, we attempted to develop a similar *in vitro* system for sorghum using shoot tips from the germinating seeds. Here, we report a protocol for the production of numerous primary and secondary somatic embryos from shoot tips, and their subsequent regeneration into whole plants through FEC. Plants were regenerated by this method within six months and were found normal when tested in a field trial.

We have made some modifications to our protocol for shoot tip culture reported earlier (Sairam et al., 1996) and tested it with three popular sorghum genotypes, viz., 296B, BTx623 (popular seed parents of hybrids in India and the USA, respectively) and M35-1 (landrace cultivated during post-rainy season in India). Seeds were germinated after sterilization on a moist blotting paper in a Petri plate in the dark at 24± 2 °C. Three days after germination, 3–4 mm long segments (consisting of apex and part of the mesocotyl) were cut from the emerging shoot apex. On each plate, 12 such cut shoot tips were placed on a nutrient medium [LS medium (Linsmaier and Skoog, 1965) with 2 mg l^{-1} 2,4-D, 0.1 mg l^{-1} Kinetin and 0.8% agar (A-1296, Agar-Agar; type E; Cat. # A 6674; *Sigma* chemical company, St. Louis, MO 63178, USA) and incubated in the dark at 24±2 °C. Embryogenic calli were transferred to MS (Murashige and Skoog, 1962) regeneration medium supplemented with 2 mg l^{-1} BA and 0.5 mg l^{-1} IAA for further differentiation. Each experiment was performed for a minimum of 3 times with 4 replicates each. Altogether, there was a minimum of 144 explants for each treatment.

Panicles of 100 regenerated plants from the above 3 genotypes (296B and BTx623B, and 50 from M35-1) were selfed in the greenhouse. Seeds from 25 Ro plants (test entries) were sown in the field during the 1997 rainy season along with 4 plots of the same genotype using the original normal seed lots used to initiate tissue culture (repeated checks).

From the *in vitro* cultured shoot tips, calli started appearing within 10 days (Figure 1A), and they formed FEC (Figure 1B-1E) in about the next 4–5 weeks. The frequency of embryogenic calli within 5 weeks ranged from 84.3±2.3 (296B) to 90.6±0.9% (M35-1). Numerous somatic embryos (about 60–70 somatic embryos per explant) were formed within one week, and nearly half of them gave raise to secondary embryos. When transferred (each of the explant as a single unit, containing both primary and secondary embryos) to regeneration medium (MS medium with 2 mg l^{-1} BA and 0.5 mg l^{-1} IAA), these embryos differentiated (Figure 1F) and produced plantlets within 35–45 days (Figure 1G). The regeneration frequency ranged between 14.6±0.8 (296B) and 18.0±0.5% (M35-1). Thus, M35-1 is the best responding genotype for both production of embryogenic calli (90.6±0.9%) and regeneration (18.0±0.5%), but the other two genotypes also showed good response (>84% for embryogenic calli and >14% for regeneration). The regenerated plantlets were transferred into pots (Figure 1H) in greenhouse and grown to maturity.

Seeds (R_0) from the selfed panicles of the *in vitro* regenerated plants looked normal. R_1 plants (test entries) were compared with the control plants (check entries) in the field trial. All the test and check entries produced normal plants. In all cases, the plants in the two groups for each genotype were indistinguishable for morphological traits like grain color, leaf angle and shape, and culm thickness.

The differences in phenotype between the two sets of plots for each genotype were not significant in most cases (Table 1). However, a few exceptions in agronomic traits were detected due to crop management and environmental factors. In all the three genotypes, the differences between the means for the checks (n = 4) and those for the corresponding *in vitro* generated plants were within 1% of each other for the highly heritable phenological traits like time to flower and physiological maturity. The same was true for plant height in the cases of 296B and BTx623, and the difference in seed size was <3% for all genotypes. Only in the case of M35-1, and for other agronomic variables like grain and stover, yields significant difference between the two groups of entries (check and test entries) was detected. This was due to a difference in the plant density. The *in vitro* regenerated plants of M35-1 were placed outside the greenhouse after flowering during the later part of the 1996 rainy season and allowed to set seeds and mature as they were too tall to handle inside the greenhouse on benches, and needed support to prevent lodging (unlike the other two genotypes). Since the panicles were selfed just before flowering and the bags were retained until late maturity to prevent bird damage, some grain mold attack was unavoidable, as there were few frequent light showers. This resulted in poorer seed germination when planted in the field, causing reduction in plant stand (33% less than checks) and stover yield (34%). For the other two genotypes, differences in

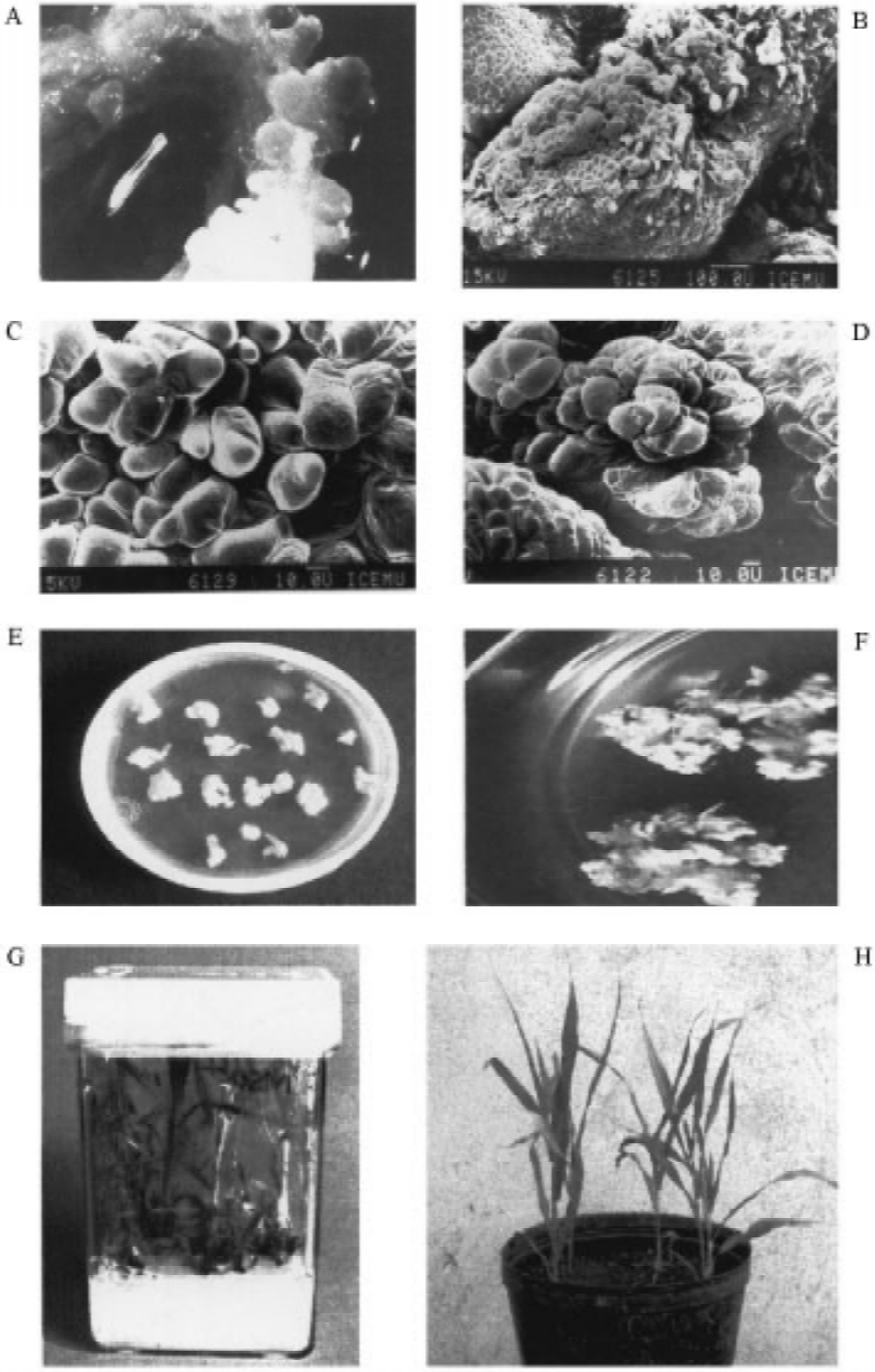

Figure 1. Shoot-tip cultures of sorghum. *(A)* Light micrograph showing callus initiation from *in vitro* cultured shoot tips of genotype 296B. *(B–D)* Electron micrograph showing FEC giving rise to somatic embryos. *(E)* Shoot tip derived embryogenic calli in a Petri dish. *(F)* Differentiation of somatic embryos. *(G)* Five week-old plants growing in Magenta box. *(H)* Complete plants transferred into pots and grown in the greenhouse.

Table 1. Comparison of field performance of checks (normal crop grown from seeds) and test entries (crop from seeds of *in vitro* regenerated plants of R1 generation)

	Time to flower (days)	Time to physiological maturity (days)	Plant height (cms)	Plant density (no. m^{-2})	Panicles (g m^{-2})	Panicle weight (g m^{-2})	Grain weight (g m^{-2})	Mass of 100 seeds (g)	Stover weight (g m^{-2})
M35-1									
Mean for 4 checks (C)	70.5	102.5	220.3	18.3	14.3	128.5	92.4	2.3	1347.5
Mean for 50 test entries (T)	70.4	101.6	229.1	12.8	10.3	133.9	100.7	2.4	889.8
Difference, (C-T)	0.1	0.9	−8.9	5.4	4.1	−5.4	−8.3	0.1	457.7
T prob.	0.87	0.13	0.41	<0.001**	<0.001**	0.86	0.74	0.66	<0.001**
296B									
Mean for 4 checks	73.2	105.7	119.5	11.5	8.8	117.2	73.6	2.1	420.0
Mean for 25 test entries	73.0	105.1	120.3	11.9	10.9	162.5	105.7	2.1	460.0
Difference	0.1	0.7	−0.8	−0.4	−2.1	−45.4	−32.1	0.1	−0.4
T prob.	0.69	0.37	0.74	0.74	0.16	0.10	0.11	0.96	0.44
BTx623									
Mean for 4 checks	76.8	109.8	132.3	10.8	8.5	141.5	105.3	2.2	400.0
Mean for 25 test entries	0.8	109.4	132.2	10.4	9.3	128.5	102.5	2.1	417.0
Difference	−0.3	0.4	0.1	0.4	−0.8	13.0	2.8	0.1	−17.0
T prob.	0.62	0.70	0.97	0.55	0.25	0.48	0.81	0.57	0.71

* Significant at 1% level of probability

stover weights between the two groups were less than 10%, and were not significant. Some of the differences in grain weight between groups can be attributed to severe damage caused by grain mold and bird attacks in the field trial. The yield levels were also low because of some water logging and delayed weeding – both caused by untimely rains during early vegetative growth. It is desirable to check agronomic traits during the dry season with full plant protection measures if grain yields are to be compared. However, we do not expect any significant differences between the two sets of materials.

With the immature embryos, successful transformation frequency (number of transgenic plants regenerated on selection medium/ number of somatic embryos bombarded) in sorghum was 1/380 (Casas et al., 1993). As compared to the limited somatic embryos produced in scutellar cultures, there is a many-fold increase in somatic embryo production and secondary embryo formation in the case of shoot tip cultures. Our present observations with shoot tip cultures are similar to the system described by Zhong et al. (1992). The sorghum FEC derived from shoot tips was bombarded with plasmid DNA pJS 108 (with *GUS* and bar genes provided by Jin Su and Ray Wu, Cornell University, USA). The calli showed extensive *gus* activity (numerous blue spots, or blue patches) after 3 days. In our present experiments with shoot tips as explants, friable embryogenic calli is the only type of embryogenic calli obtained, and no compact nodular calli often associated with inflorescence cultures were found. Our observation coincides with that of Kaeppler and Pedersen (1997), where FEC was rated to be highly favorable for regeneration. The shoot tip derived FEC may also serve as a good source system for initiating suspension cultures, which can provide cell lines for protoplast isolation and genetic transformation as found in other cereals.

A suitable *in vitro* regenerating system for transformation should fulfill two important criteria: (a) explants capable of regenerating into plants at high frequency through somatic embryogenesis or organogenesis, and (b) a minimum *in vitro* culture period from explant stage until whole plant development to minimize somaclonal variation. We conclude that the system described here satisfies both the above criteria. The above protocol is simple for plant regeneration from *in vitro* cultures.

Acknowledgements

We are grateful to Dr Mariam Sticklen for suggesting this idea to us, and to Dr K.V. Krishnamurthy for reviewing the manuscript. C.L. Narayana and M. Reddy rendered valuable technical assistance.

References

Bhaskaran S & Smith RH (1988) Enhanced somatic embryogenesis in *Sorghum bicolor* from shoot-tip culture. *In Vitro* Cell Dev. Bio. 24: 65–70

Casas A, Kononowicz AM, Zehr BU, Tomes DT, Axtell JD, Butler LG & Bressan RA (1993) Transgenic sorghum plants via microprojectile bombardment. Proc. Nat. Acad. Sci. USA 90: 11212–11216

Elhag H & Butler LG (1992) Effect of genotype, explant age and medium composition on callus production and plant regeneration from immature embryos of sorghum. Arab Gulf. J. Sci. Res. 10: 109–119

Elkonin LA, Lopushanskaya RF & Pakhomova NV (1995) Initiation and maintenance of friable embryogenic callus of Sorghum *Sorghum bicolor* (L.) Moench by amino acids. Maydica 40: 153–157

Kaeppler HF & Pedersen JF (1997) Evaluation of 41 elite and exotic inbred sorghum genotypes for high quality callus production. Plant Cell Tiss. Org. Cult. 48: 71–75

Linsmaier EM & Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100–127

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Nahdi S & de Wet JMJ (1995) *In vitro* regeneration of *Sorghum bicolor* lines from shoot apices. Int. Sorghum & Millets Newsletter 36: 88–90

Rao AM, Padmasree K & Kavikishore P B (1995) Enhanced plant regeneration in grain and sweet sorghum by aspargine, proline and cefotaxime. Plant Cell Rep. 15: 72–75

Sairam RV, Seetharama N, Murthy AK & Shyamala T (1996) Comparison of tissue culture systems for genetic transformation in sorghum. Page 150 in Abstracts of poster sessions. 2nd International Crop Science Congress: Crop Productivity and Sustainability – Shaping the Future, 17–24 Nov 1996, New Delhi, India. New Delhi: National Academy of Agricultural Sciences; Indian Council of Agricultural Research

Sairam RV, Seetharama N, Devi PS, Verma A, Murthy UR & Potrykus I (1999) Plant regeneration from mesophyll protoplasts in sorghum [*Sorghum bicolor* (L.) *Moench*]. Plant Cell Rep. 18: 972–977.

Zhong Z, Srinivasan C & Sticklen M (1992*) In vitro* morphogenesis of corn: Differentiation of multiple shoot clumps and somatic embryos from shoot tips. Planta 187: 483–489