Indian J. Plant Physiol., Vol. 14, No. 2, (N.S.) pp. 142-146 (April-June, 2009)

INDUCTION OF HIGH FREQUENCY OF SOMATIC EMBRYOIDS IN INDIAN COTTON CV. SURABHI THROUGH TEMPERATURE STRESS

A.H. PRAKASH* AND N. GOPALAKRISHNAN

Central Institute for Cotton Research, Regional Station, Coimbatore-641 003

Received on 27 May, 2009, Revised on 5 June, 2009

SUMMARY

An elite Indian cultivar of cotton (*Gossypium hirsutum* L.) Surabhi was tested for its embryogenic protential. Callus was induced from three explants viz. roots, cotyledons and hypocotyls on MS medium supplemented with auxins and cytokinins in different combinations. The hypocotyls were amenable to callus induction than roots or cotyledons. The ideal combination for callus induction was MS containing 0.5 NAA + 0.5 BA, 0.5 2, 4 D + 0.25/0.5 Kin and 0.5 2, 4-D + 0.25/ 0.5 BA. The friable callus was cultured on the same media combinations except that only KNO_3 was provided as nitrate source. The cultures were divided into two sets with one being maintained at optimum temperature (28± 2^0C) and other exposed to a higher temperature stress (33± 2^0C). Under controlled temperature, there was good callus production but no embryoids were formed even after forty day of culture. Irrespective of the hormonal combinations, the callus exposed to temperature stress of 33°C entered into embryogenesis. The callus maintained on 2, 4-D as auxin source formed embryoids which were watery and turned brown with time. A combination of 0.5 NAA + 0.5 BA was found to be the best which lead to production of embryoids in eighty per cent of the cultures. However, when these embryoids were transferred to maturation medium devoid of hormones, they failed to germinate.

INTRODUCTION

There is an increasing interest in cotton (*Gossypium* Spp.) biotechnology because cotton is a natural source of textile fibre and is widely grown in many countries. Genetic improvement of cotton through conventional breeding is limited by several factors including limited gene pool. Although plant biotechnology is an alternative means for improving cotton, the bottleneck is the availability of an effective regeneration protocol. Cotton somatic embryogenesis was first reported by Price and Smith (1979) in *Gossypium klotzchianum*, but no plantlet regeneration was reported. Davidonis and Hamilton (1983) regenerated plants from two-year-old cotton cv. Coker 310 via somatic embryogenesis. Since then, significant progress has been reported in cotton tissue culture (Han *et al.* 2009). *In vitro* cultured cotton cells have been induced to undergo somatic embryogenesis in numerous laboratories using different strategies (Shoemaker *et al.* 1986, Zhang and Wang 2001). Plantlets have been obtained from explants such as hypocotyls, roots and cotyledons (Zhang 1994a) and anther (Zhang 1994b) but with low frequency of reproducibility. Genotype dependence is one of the important factors that restricts somatic embryogenesis and plant regeneration. From 1971 to 1997, out of 100 cotton cultivars investigated for tissue culture, 54% exhibited different somatic embryogenesis capacity, while 46% could not proliferate to form somatic embryos (Feng *et al.* 1997). Since that time, about 150 additional cultivars were used in tissue culture within a decade, but only 20% of elite cotton cultivars could be

*Corresponding author, E-mail: prakashcicr@gmail.com

regenerated successfully. Furthermore, apart from model lines, cotton regeneration also takes a long time. Six to eight months has been reported as the shortest time for regeneration (Wang *et al.* 2006).

There is very little success in the regeneration of native Indian cotton cultivars (Rajasekaran *et al*. 1996, Kumar and Pental 1998, Prakash *et al.* 2004). Hence, the present work was undertaken to standardize callus induction, maintenance and induction of reproducible high frequency of embryogenic callus, ideal environment for induction of highly embryogenic callus from various explants of cotton (cv. Surabhi).

MATERIALS AND METHODS

Seeds of cotton (*Gossypium hirsutum* L. cv Surabhi) were obtained from Plant Breeding Section of Central Institute for Cotton Research, Regional Station, Coimbatore. The acid delinted seeds were surface-sterilized by treating then with Bavistin (0.1w/v) solution for 2 h, followed by mercuric chloride (0.1% w/v) treatment for 10 min. thorough washing with sterile double-distilled water 4-5 times. The seeds were then soaked in water overnight to soften the seed coat and these seeds were inoculated on half strength MS medium basal medium and were incubated in dark for germination.

Well established 7-10 days old seedlings were used for further experiments. Hypocotyl segments (5-7 mm long), cotyledon segments (10-16 mm^2 surface area) and root segments (5-7 mm long) were placed in semi-solid culture media. Ten explants per pertridish were maintained at 16/8 hours light/dark cycle at 28± 2°C for 30 days. The experiment was done in a randomized block design consisting of three explants, nine treatments and four replication. The Murashige and Skoog media (MS medium) was supplemented with 30 g l^{-1} of sucrose, gelrite (3g l^{-1}) and combination of auxins and cytokinins at a pH of 5.8. Subculturing was done at 30 days interval, wherein the callus was transferred to fresh medium of the same hormonal combinations.

After one sub-culture, friable callus developed on various media was cultured on modified MS medium by replacing ammonium nitrate with potassium nitrate. The cultures were divided into two sets. One set was maintained at 28 ± 2°C and the other at 33± 2 °C, with the 16/8 hours of light/dark cycle. The embryogenic callus was quantified 40 days after inoculation.

RESULTS AND DISCUSSION

All media except the basal MS medium, which is devoid of hormones produced callus with varying degree. The hypocotyl explants formed callus more readily than cotyledonary explants (Fig. 1). Similar observations have been reported by Sakhanokho *et al.* (2001). The root explants did not produce good callus. They either turned brown or continued to elongate and produce more roots. Of the nine treatments tested, nearly eight combinations led to production of callus with varying frequency from

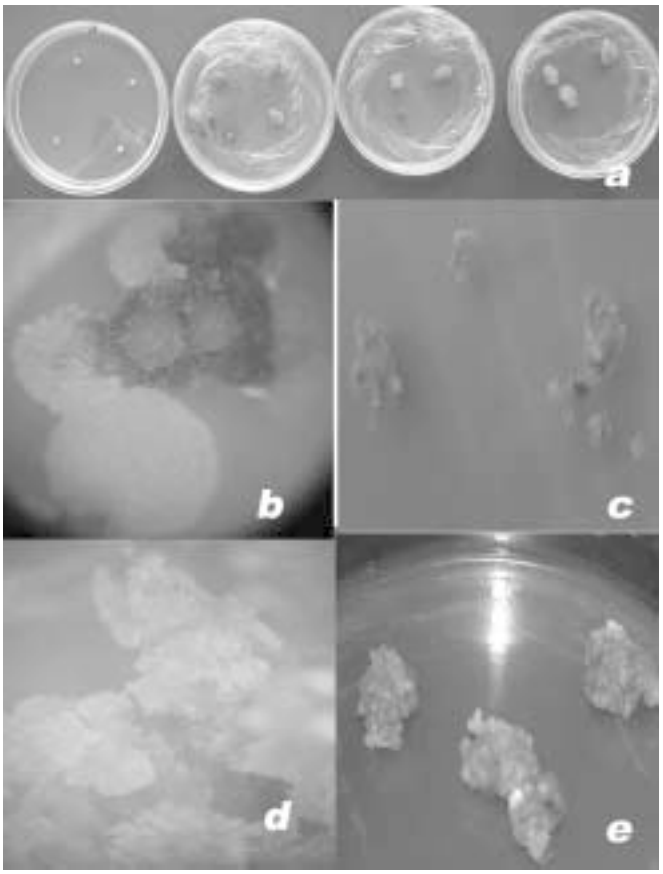

Fig. 1. Callus induction and embryogenesis from hypocotyl explants of cotton (cv. Surabhi)
(a) callus induction from hypocotyl explants, (b & c) embryoid formation, (d) embryoids reverting to callusion after developing into tulip-shaped structures and (e) watery embryoids formed on 2, 4-D embrygenesis medium

both cotyledonary and hypocotyl explants (Table 1). Among the treatments tested T3, T6, T7 and T8 led to production of good quantity of friable callus. The callus produced was sub-cultured on fresh medium to harvest good quantity of callus for further work. Good quality friable, yellowish green, granular pro-embryogenic callus was carefully isolated and used for embryogenesis according to Firoozabady and De Boer 1993.

The proembryonic callus was cultured as reported by Sakhanokho *et al*. (2004) wherein ammonium nitrate was replaced with potassium nitrate to standardize the hormonal combinations. Nitrogen source in the form of Ammonium nitrate inhibited embryogenic callus formation and nitrate form of nitrogen was recommended (Ikram and Zafar 2004). The set of cultures maintained at 28 ± 2°C showed good growth of callus but these were not embryogenic. The cultures maintained at 33 ± 2 °C started proliferating and produced good embryogenic cells (Table 2). Even though the hypocotyl tissues proliferated better in presence of 2, 4-D, the same was not reflected for embryogenesis. The frequency of embryoid formation was low and produced non-embryogenic watery callus (Fig. 1). The ideal hormonal

Table 1. Callus induction as influenced by explant and media constituents in cotton cv. Surabhi

Treatment	Explant	No. of explants	No. of explant showing callus	Callus induction (%)	Relative growth of callus
T1- Basal MS	Cotyledon	40	0	0	-
	Hypocotyl	40	5	12.5	+
	Root	40	0	0	-
T2- MS+0.5	Cotyledon	40	30	75	++
NAA + 0.25 BA	Hypocotyl	40	30	75	++
	Root	40	5	12.5	-
T3- MS+0.5	Cotyledon	40	40	100	++
NAA + 0.50 BA	Hypocotyl	40	40	100	++++
	Root	40	5	12.5	+
T4- MS+0.5	Cotyledon	40	25	62.7	++
NAA + 0.25 Kin	Hypocotyl	40	30	75	++
	Root	40	10	25	+
T5- MS+0.5	Cotyledon	40	35	87.5	++
2, 4-D + 0.50 Kin	Hypocotyl	40	35	87.5	+++
	Root	40	10	25	+
T6- MS+0.5	Cotyledon	40	38	95	++
2, 4-D + 0.25 BA	Hypocotyl	40	30	75	++++
	Root	40	5	12.5	+
T7- MS+0.5	Cotyledon	40	40	100	++
2, 4-D + 0.50 BA	Hypocotyl	40	40	100	++++
	Root	40	10	25	+
T8- MS+0.5	Cotyledon	40	40	100	++
2, 4-D + 0.25 Kin	Hypocotyl	40	40	100	++++
	Root	40	5	12.5	+
T9- MS+0.5	Cotyledon	40	40	100	++
2, 4-D + 0.50 Kin	Hypocotyl	40	40	100	++++
	Root	40	10	25	+

* Cultures were evaluated after one subculture (30 days). Number of + indicates the growth of induced callus; more +, more growth of induced callus, - indicates trace/no growth of callus.

combinations was observed to be 0.5 NAA + 0.5 BA (Table 2). A genotype's capability to produce large quantity of callus is not an indication of its regenerative capacity. Previous reports have recommended presence of 2, 4-D as a pre-requisite for conversion of proembryogenic callus to embryoids (Janagouder *et al*. 2004, Sakhanokho *et al*. 2004). Present results indicate that the temperature stress provided can mimic the 2, 4-D effect and there is a high frequency of embryogenic cells. Hundreds of tulip-shaped embryoids per petriplate (Fig. 1) was developed in all the combinations tested. These somatic embryos, when transferred to maturation media (MS basal medium without hormones), failed to mature and slowly degenerated (Fig. 1). Similar results of embryoids forming callus has been reported (Wilkins 2000, Mishra *et al.* 2003). Further efforts are under way to modify the media constituents and culture environment to favour embryo maturation.

Table 2. Effect of hormone and temperature stress on the frequency of somatic embryogenesis in cotton cv Surabhi

Treatment	Percentage of embryogenic callus at altered temperatures		Results/ inference
	28 ± 2 °C	33 ± 2 °C	
T3- MS+0.5 NAA + 0.50 BA	-	++++ (80 %)	Heart-shaped embryoids
T6- MS+0.5 2, 4-D + 0.25 BA	-	++ (20%)	Soft callus with few embryoids which degenerated over time
T7- MS+0.5 2, 4-D + 0.50 BA	-	++ (50%)	Soft callus with few embryoids which degenerated over time
T8- MS+0.5 2, 4-D + 0.25 Kin	-	++ (50%)	Soft callus with few embryoids which degenerated over time
T9- MS+0.5 2, 4-D + 0.50 Kin	-	++ (50%)	Soft callus with few embryoids which degenerated over time

* Number of + indicates the growth of embryoids; more +, better the embryoids, - indicates trace/no embryoids.

Hypocotyl was found to be an ideal explant for callus induction, with NAA and 2, 4-D as the auxin source and benzyl adenine and kinetin as cytokinin sources. Temperature stress was found to be an important switch to induce embryoids from callus. The ideal combination for callus induction was MS + 0.5 NAA + 0.5 BA, MS + 0.5 2, 4-D + 0.5 Kin and MS + 0.5 2, 4-D + 0.5 BA, while only MS + 0.5 NAA + 0.5 BA produced embryoids. This is also the first report wherein temperature stress has been used as a factor for somatic embryo induction in cotton. The high frequency of embryogenic callus produced can further be utilized for regeneration of plantlets by suitable modification of the culture conditions.

REFERENCES

Davidonis, G.H. and Hamilton, R.H. (1983). Plant regeneration from callus tissues of *G. hirustum* L. *Plant Sci. Lett.* **32:** 89-93.

Feng, R. and Zhang, B.H. (1994). Ovule culture and rescue of cotton hybrid embryos, *Shaanzi. J. Agric.* **2:** 46-48.

Firoozabada, E. and De Boer, D.L. (1993). Plant regeneration via somatic embryogenesis in many cultivars of cotton (*G. hirsutum* L.). *In vitro Cell Dev. Biol*. **299:** 166-173.

Han, G.Y., Wang, X.F., Zhang, G.Y. and Ma, Z.Y. (2009). Somatic embryogenesis and plant regeneration of recalcitrant cottons (*Gossypium hirsutum*). *African J. Biotech.* **8:** 432-437.

Ikram-ul-Haq and Zafar, Y. (2004). Effect of nitrates on embryo induction efficiency in cotton (*Gossypium hirsutum* L.), *African J. Biotech.* **3:** 319-323.

Janagoundar, B.S., Sheshagiri, R., Rajashekar, M., Kachapur and Shetti, M.S.K. (2004). Callus induction, proliferation and suspension culture initation in cotton (*Gossypium* Spp.). Presented in Int. Symposium on Strategies for Sustainable Cotton Production- A Global Vision Vol-I Crop Improvement, pp. 433-435. UAS, Dharwad.

Kumar, S. and Pental, D. (1998). Regeneration of Indian cotton variety MCU 5- through somatic embryogenesis. *Curr. Sci.* **74:** 538-540

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.* **15:** 473-497.

Prakash, A.H. Sudhagar, R., Manickam, S. and Thangapandian, R. (2004). Induction of high frequency of somatic embryos in interspecific hybrids and their parents in cotton. Paper presented in: International Symposium on Strategies for sustainable Cotton Production- A global vision Vol I, pp. 416-420. UAS, Dharwad.

Price, H.J. and Smith, R.H. (1979). Somatic embryogenesis in suspension culture of *Gossypium klotzschiaanum* Anderss. *Planta* **145:** 305-307.

Sakhanokho, H.F., Ozias-Akins, P., May, O.L. and Peng, W.C. (2004). Induction of somatic embryogenesis and plant regeneration in selected Georgia and Pee Dee cotton lines. *Crop Sci.* **44:** 2199-2205.

Shoemaker, R.C., Couche, L.J. and Galbraith, D.W. (1986). Characterization of somatic embryogenesis and plant regeneration in cotton (*G. hirsutum* L.). *Plant Cell Reports.* **3:** 178-181.

Wang Y.X., Wang, X.F., Ma, Z.Y., Zhang, G.Y. and Han, G.Y. (2006). Somatic embryogenesis and plant regeneration from two recalcitrant genotypes of Gossypium hirsutum L. *Sci. Agric. Sin.* **5:** 323-329.

Zhang, B.H. (1994a). A rapid induction method for cotton somatic embryos. *Chinese Sci. Bull.* **39:** 1340-1342.

Zhang, B.H. (1994b). List of cotton tissue culture (Continuous). *Plant Physiol. Commun.* **30:** 386-391.

Zhang, B.H. (1995). Advance of cotton protoplast culture. *J. Sichuan Agric. Univ.* **1:** 27-33.

Zhang, J.M., Sun, J.X., Liu, J.L. and Zhang, X.L. (1994). Studies on plant regeneration from somatic cell and transferring technique of plantlets in upland cotton. *Acta Agron. Sin.* **20:** 210-216.

Zhang, B.H., Feng, R., Liu, F., Zhou, D.Y. and Wang, Q.L (2001). Direct somatic embryogenesis and plant regeneration from cotton (*Gossypium hirsutum*) explants. *Isr. J. Plant Sci.* **49:** 193-196.