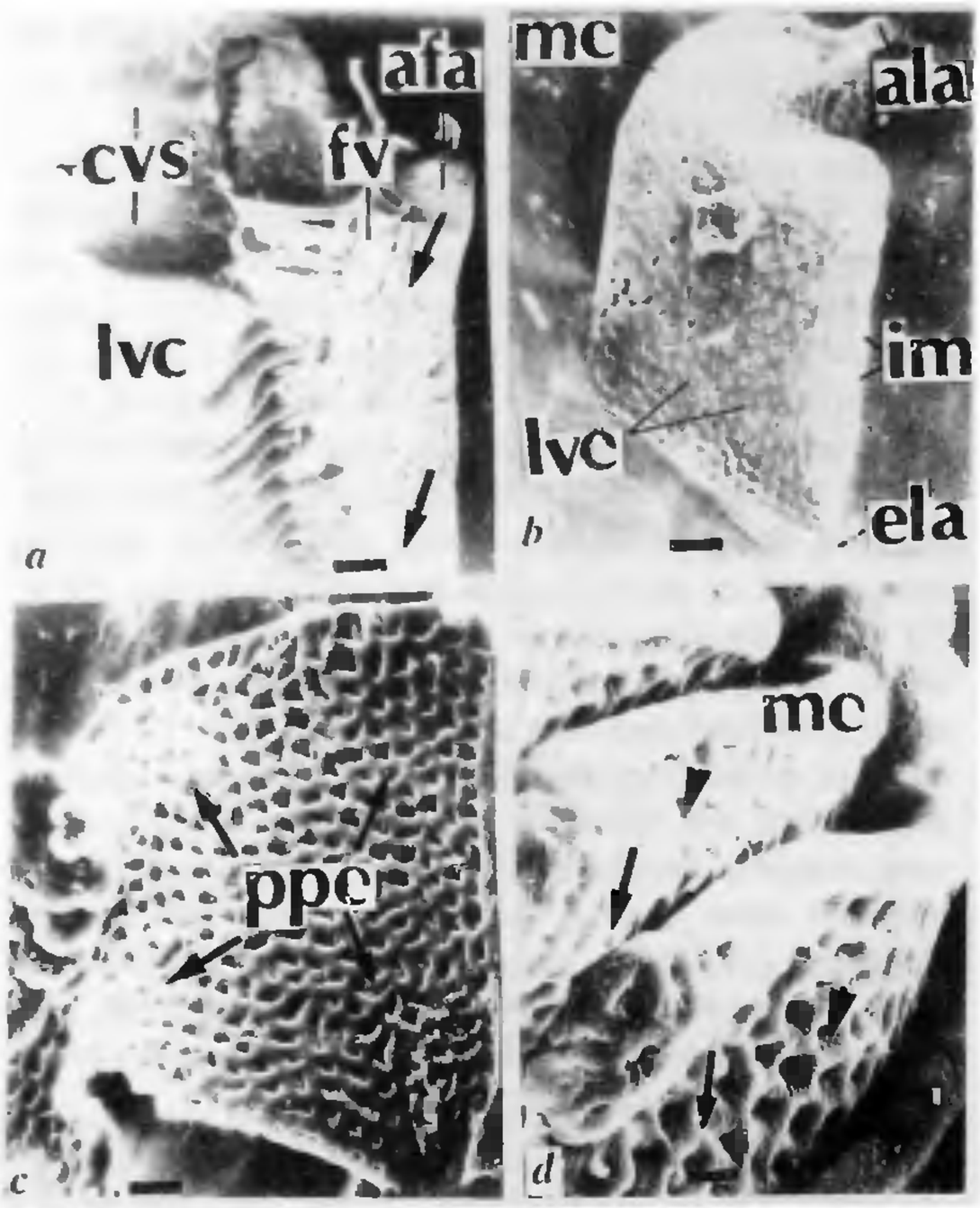

Figure 1. *a*, SEM of a part of gill filament vascular cast showing afferent filamentar artery (afa), central venous sinus (cvs), filamentar vein (fv) and lamellar vascular cast (lvc), bar = 90 µm. *b*, SEM of a lamellar vascular cast (lvc) showing afferent lamellar arteriole (ala), efferent lamellar arteriole (ela), inner margin (im) and marginal channel (mc) of a lamella, bar = 42 µm. *c*, SEM of a part of lamellar vascular cast showing position of pillar cells (ppc), bar = 21 µm. *d*, SEM of parts of three lamellar vascular cast showing marginal channels (mc), their ramification (arrows) and the position of pillar cells (arrowheads), bar = 12 µm.

value (0.06937 ± 0.01633 mm^2) obtained from Bouin's-fixed lamellae of about 62 ± 2 g *Chaca*[4]. Out of the total angio-lamellar replica area (0.11328 ± 0.01011 mm^2), about 39% (0.04458 ± 0.01011 mm^2) is occupied by pillar cell system (6721 mm^2) and the remainder (61%) (0.06937 ± 0.01633 mm^2) by blood channels. Out of the total blood channel area (0.06937 ± 0.01633), about 30.6% is occupied by the marginal channel and 69.4% by a network of central lamellar blood channels. 30.6% of the blood flow through the marginal channel is of great respiratory importance because of its smaller water–blood diffusion distance and greater diffusing capacity in comparison to the other parts of the gill lamellae[5]. The 39% pillar cell system does not participate in gaseous exchange and therefore should not be considered for the measurement of total respiratory area of a lamella. Measurement of total respiratory lamellar area of fresh- and fixed-gills includes the pillar cell system also and overestimates the respiratory surface. From the above findings it may be suggested that measurements of gill area should be made with the help of bilateral surface area of the lamellae sampled from corrosion vascular replica of the gill filaments.

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Regeneration of Indian cotton variety MCU-5 through somatic embryogenesis

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***In vitro* regeneration by somatic embryogenesis is highly varietal specific in cotton (*Gossypium hirsutum*). Highest frequency of regeneration has been reported in Coker varieties. However, even within Coker varieties, there is seed to seed variation for regeneration[1,2]. There is no report of *in vitro* regeneration of major Indian cultivars through the somatic embryogenesis pathway. We report here identification of genotypes within variety MCU-5 that regenerate profusely by somatic embryogenesis. This variety is extensively grown in the southern cotton zone of India. Regeneration by somatic embryogenesis has been achieved by a modification of the earlier *in vitro* culture protocols of Trolinder and Goodin[3] and Firoozabady and DeBoer[4]. Complete plants could be regenerated through somatic embryogenesis from hypocotyl explants in 6–7 months.**

SOMATIC embryogenesis was first observed by Price and Smith[5] in *Gossypium koltzchianum* but no plantlet regeneration was reported. Shoemaker *et al.*[6] described somatic embryogenesis and plant regeneration in *G. hirsutum* cvs Coker 201 and 315. Since then, significant progress has been reported in the regeneration of Coker

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genotypes by Trolinder and Goodin[3,7,8], Finer[9] and Firoozabady and DeBoer[4]. Cultivars T25 (ref. 2), GSA 78 (ref. 4) and Acala[10] have also been shown to regenerate but at frequencies lower than those reported for Coker cultivars.

We have earlier reported the development of a pure line for regeneration in Coker 310 (Kumar *et al.*, submitted). This pure line (Coker 310FR) was crossed with the varieties most extensively grown in India. The F1s of all the crosses except interspecific cross *G. hirsutum* Coker310FR × *G. barbadense*, regenerated by somatic embryogenesis. While it is now possible to introgress the trait of regeneration into Indian varieties, it would be more useful if these varieties could be regenerated without any input from Coker 310FR. Here we report a protocol for the *in vitro* regeneration of MCU-5, an important cotton variety of southern India.

Seeds of MCU-5 were delinted and sterilized by dipping in 70% ethanol for 2 min, followed by 8 min treatment with sodium hypochlorite solution containing approximately 4% available chlorine followed by treatment with 0.1% mercuric chloride solution (w/v) for 5 min. After surface-sterilization and 4–5 washes with sterile water, seeds were kept in sterile water for 4–5 h for softening the seed coat which was completely removed before placing the seeds on half-strength MS medium (1/2 MST) for germination.

Hypocotyl explants (3–4 mm long) of 5-day-old seedlings were placed vertically on different media for the induction of callus. Each seedling provided 10–12 explants. For the induction of somatic embryogenesis, protocols of Trolinder and Goodin[3,8] and Firoozabady and DeBoer[4] with some modifications were followed. Explants derived from a single seedling were equally distributed on the two media. Except 2iP (6-γ-γ-dimethylallyl aminopurine), all the other plant growth regulators (α-naphthaleneacetic acid; 2,4-dichlorophenoxyacetic acid and kinetin) were added prior to autoclaving. All media were prepared with MS salts[11]. MS media with suffix **T** contain vitamins used by Gamborg *et al.*[12], with suffix **F** contain 100 mg/l myo-inositol and 0.4 mg/l thiamine HCl and with suffix **G** contain 100 mg/l myo-inositol and 0.1 mg/l thiamine HCl. The pH of the media was adjusted to 5.8–5.9. Some of the media, i.e. 1/2 MST, MSG, MSG1 and MSG2 were solidified with 0.8% w/v agar (Hi Media). Rest of the media were solidified with 0.2% w/v phytagel (Sigma) with additional 0.8 g/l magnesium chloride. Somatic embryos that had germinated were placed in 500 ml jars containing 1/2 MST medium for 2–4 weeks to recover plantlets. All the *in vitro* experiments were conducted under controlled environmental conditions (temp 28 ± 2°C, 750 lux light at the culture level; 16 h light, 8 h dark period). Hardening of the plantlets was carried out in pots containing mixture of vermiculite, soilrite and garden soil in 1:1:1 ratio. During the 2-week hardening period, in the first week, plants were covered with a polythene bag for maintaining high humidity and plants were irrigated with progressively reduced concentrations of MS medium followed by irrigation with tap water in the second week.

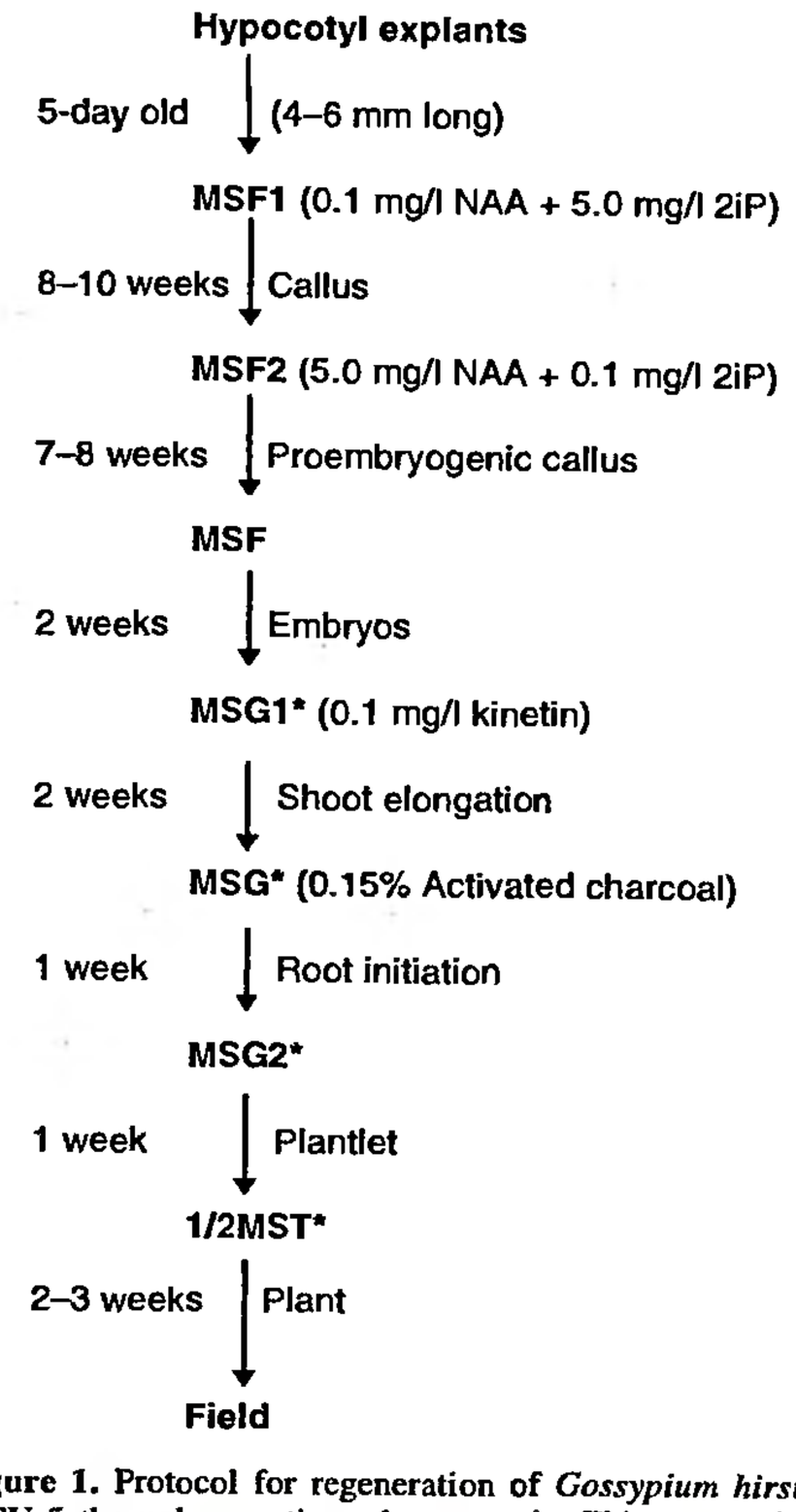

Figure 1. Protocol for regeneration of *Gossypium hirsutum* variety MCU-5 through somatic embryogenesis. This protocol is a modification of the protocol used by Firoozabady and DeBoer (FD)[4]. Media with asterisk are different from those used in the FD protocol.

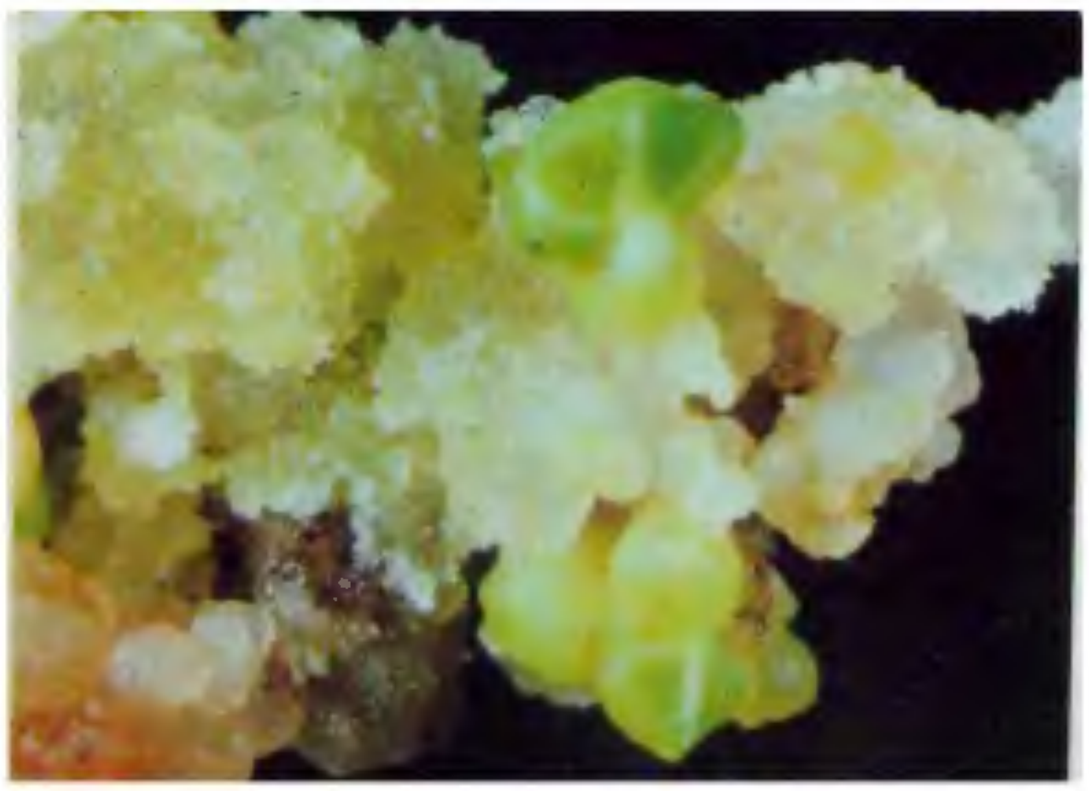

Figure 2. Somatic embryos differentiating from hypocotyl explant derived callus of *Gossypium hirsutum* variety MCU-5.

Table 1. *In vitro* somatic embryogenesis in the progeny plants produced from the selfed seed of *G. hirsutum* cv. MCU-5V plant with FD and MTG protocols

	FD protocol[a]			MTG protocol[b]		
Seedling number[c]	Number of explants per seedling	% explant response	Average number of embryos[d]	Number of explants per seedling	% explant response	Average number of embryos[d]
1	6	0	0	6	0	0
2	5	0	0	5	0	0
3	6	66.66	18.80 ± 3.91	6	0	0
4	6	0	0	6	0	0
5	7	85.71	27.33 ± 5.49	7	71.16	17.16 ± 2.60
6	2	0	0	2	0	0

[a]Modified protocol from ref. 4 as described in Figure 1.
[b]Modified protocol from ref. 3.
[c]Explants derived from each seedling (numbered as 1 to 6) were randomly distributed in equal numbers on the media of two protocols.
[d]Mean of somatic embryos per explant (mean ± SE).

In 1996, nine individual plants of MCU-5 (MCU-5 I-IX) were grown in the field. Four plants namely MCU-5 II, MCU-5 V, MCU-5 VI and MCU-5 IX were tested for *in vitro* regeneration following the FD (modified from ref. 4) and MTG protocols (modified from ref. 3; Kumar *et al.*, submitted). Hypocotyls derived from each seedling were equally distributed amongst the media of the two protocols. Only some of the progeny of plant MCU-5 V showed regeneration with modified FD protocol (Figure 1). Out of the 6 seedlings that were tested, explants derived from two seedlings regenerated through somatic embryogenesis (Figure 2 and Table 1). Explants from the same seedlings tested with MTG protocol showed poorer response as compared to response with FD protocol. Out of the 6 seedlings, only one showed somatic embryogenesis when cultured following the MTG protocol. Only progeny of MCU-5 V plant showed any regeneration response (Table 1), progeny of MCU-5 II, MCU-5 VI and MCU-5 IX plants did not show any morphogenesis with either of the two protocols.

Somatic embryos of MCU-5 showed abnormalities at the radicle pole. To regenerate normal plants from these embryos, the radicle end was excised and the embryos were placed on MSG1 medium containing 0.1 mg/l kinetin for 2 weeks. The elongated shoot apices, were placed on MSG2 medium containing 0.15% activated charcoal and MSG basal medium for root initiation. Out of the 43 somatic embryos excised at the radicle end and placed on 0.1 mg/l kinetin containing medium, 36.71% developed shoots out of which 88% formed roots. The plantlets, before hardening in pots, were placed in 500 ml glass jars for 2–3 weeks on 1/2MST medium at 2250 lux light intensity, for vigorous growth of plantlets. The complete protocol for regeneration of somatic embryos into plantlets is given in Figure 1.

Somatic embryogenesis from the embryogenic callus on MSF medium could be improved by putting embryogenic callus masses in MST liquid medium containing 0.3% activated charcoal (incubated on a shaker set at 120 rpm) for 1–2 weeks, wherein small embryos separated and elongated properly.

With the identification of genotypes in MCU-5 that show the ability to regenerate by somatic embryogenesis, it should be possible to develop a pure line in MCU-5 for the trait of somatic embryogenesis. This is being pursued by two strategies. In one of the approaches we have selfed the plants derived from the somatic embryos and the progeny will be tested for regeneration by somatic embryogenesis till the time, in the successive generations, a pure line is identified that will not be segregating for this character. In the other approach, embryo-derived plants have been crossed with the progeny of MCU-5V plant. The latter approach would allow production of lines which are not impaired by any culture-induced variation. A preliminary study shows that hypocotyl explants of MCU-5 can be readily transformed with *Agrobacterium*-based binary vectors, thereby opening the way for genetic engineering of a major cotton variety of India.

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