Multiplying embryos were mostly of globular and early heart-shaped during 6 weeks of culture. The subsequent growth of heart-shaped embryos to mature cotyledonary stage was observed in the medium containing combinations of GA_3. Embryo proliferation medium containing CH, L-glu and GA_3, not only supported multiplication but also resulted in embryo maturation. Thus asynchronous embryogenesis consisting of different stages of embryo development was recorded in the same medium (Figure 1 *c*). However, use of GA_3 in the embryo proliferation medium was very critical and effective only at very low concentrations (0.2–0.5 mg l^{-1}). Increase in concentration (> 1.0 mg l^{-1}) in the medium restricted embryo proliferation and resulted in embryo maturation leading to the typical dicotyledonary structure formation (Figure 1 *d*). Therefore, embryo multiplication and maturation could simultaneously be achieved with controlled GA_3 supplement in the embryo proliferation medium. The transition of late heart embryo to bipolar and cotyledonary phase showing the formation of root and shoot primordia was recorded in the basal medium containing GA_3 (2.0–5.0 mg l^{-1}) during three weeks of culture. The cotyledonary embryos were developed and germinated on fresh medium containing the combination of KN (2.0 mg l^{-1}), NH_4Cl (50 mg l^{-1}) and CM (10%, v/v) and allowed to grow for two to three weeks (Figure 1 *e*).

A plantlet thus obtained within six weeks in culture could successfully be transferred to soil (Figure 1 *f*). In most of the species, embryo germination to plantlet was reported in the original medium[16] whereas in *S. mukorossi* embryos never germinated simply in the basal medium even after prolonged culture. The above two-step procedure was conducive to obtain optimum results in embryo maturation and germination of the soapnut tree. The most striking features recorded in somatic embryogenesis of *S. mukorossi* clearly exhibit its embryogenic potential which was readily produced in the MS medium with elevated level of KNO_3 and CH. Nitrogenous supplements like CH and L-glu also enhanced embryogenesis and embryo proliferation. The present communication provides reliable and efficient system for the regeneration of *S. mukorossi* and can be utilized for micropropagation.

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Recovery of *in vitro* cotton shoots through micrografting

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An efficient and highly reproducible *in vitro* micrografting method was developed for microshoots of cotton (*Gossypium hirsutum* L.). Culture of grafted shoots in Murashige and Skoog's basal liquid medium devoid of growth regulators showed maximum rate of survival (95%), elongation of scions, number of nodes per plant and higher fresh weight compared to agar solidified medium. Rootstocks from *in vitro* grown decapitated etiolated seedlings were marginally better in survival percentage and growth parameters compared to light grown seedlings. *Ex vitro* micrografting though successful had low survival of grafted shoots (30%), minimum scion elongation and number of nodes per plant. The results demonstrate the compatibility of heterologous graft-unions (intervarietal or interspecies) in cotton. Grafted plants (70%) on transfer to greenhouse and field conditions grew to maturity and set seeds.

MICROGRAFTING consists of grafting of a shoot apex from a mother plant or a microshoot onto a young plant grown in the greenhouse/nursery or onto a decapitated

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seedling under aseptic growth conditions[1]. Micrografting has been successfully applied in the production of virus-free clones of citrus[2], tissue culture regenerants in citrus[3], tea[4], a grain legume[5], pistachio[6] and vegetative buds of 4–16-year-old Douglas fir[7]. In case of the kiwi fruit, micrografting shortened the time required for transfer of *in vitro*-derived kiwi fruit plants to the field[8]. Micrografting has been used to rejuvenate plants by grafting of shoot tips from mature plants onto juvenile rootstocks as in Sequoia[9], Hevea[10], Avocado[11] and citrus somatic embryos[12]. Micrografting has also facilitated the study of the histological nature of graft unions[6, 13].

In general, putative transformants are difficult or fail to root[5] as they are subjected to high concentrations of antibiotics for prolonged periods during selection. In our experiments on transformation with cotton plumular axes, the putative transformants maintained for prolonged periods under antibiotic stress similarly had extremely slow growth and were difficult to root. This prompted us to attempt micrografting of shoots. However, due to shortage of the available number of putative transformants, standardization of the micrografting technique for cotton was carried out with *in vitro* microshoots derived from plumular axes. Several aspects such as liquid vs semi-solid medium, rootstocks excised from etiolated vs non-etiolated seedlings, intervarietal vs interspecies graft unions, *ex vitro* grafting and subsequent hardening were studied during the course of this work. To our knowledge such studies for cotton have not been reported earlier.

Delinted seeds of Indian cultivars of cotton, NHH-44, DCH-32 (*Gossypium hirsutum* L.), and AKH-081 (*Gossypium arboreum*) were surface sterilized, disinfected and germinated for 2 days according to methods described earlier[14].

Plumular axes (2 mm) from the germinated seeds of cotton cultivar NHH-44 were dissected out discarding radicles[15]. These were cultured on a medium containing MS salts[16], Gamborg's vitamins (B5)[17], 3% glucose and 0.21% gelrite in 85 mm plastic petri dishes. The pH of the medium was adjusted to 5.8 before autoclaving. Explants were incubated at 30°C under 16 h photoperiod of light intensity of 27 $\mu E\ m^{-2}\ s^{-1}$. Each petri dish contained 20 explants. Tiny shoots (about 0.6–0.7 cm long) that developed from the plumular axes were used as scions. Their bases were obliquely cut from both the sides before grafting them onto rootstocks.

Surface sterilized and disinfected seeds of cotton cultivars DCH-32 and AKH-081 after emergence of radicles were transferred to test tubes with MS salts and vitamins, 2% sucrose and 0.65% agar. The germinated seeds of DCH-32 were also transferred to earthen pots containing a mixture of sand : soil : compost (1 : 1 : 1). The culture tubes were kept for 10 days in darkness (for etiolation) or in light conditions (non-etiolation) while earthen pots were kept at 25°C under 16 h photoperiod of light intensity of 16 $\mu E\ m^{-2}\ s^{-1}$. *In vitro* grown 10-day-old seedlings and seedlings grown in earthen pots were used as rootstocks for *in vitro* and *ex vitro* grafting experiments respectively.

In vitro and *ex vitro* micrografting was carried out in polypropylene Laxenta boxes and earthen pots respectively. Ten-day-old seedlings of DCH-32 (both etiolated and non-etiolated) grown under sterile conditions and decapitated about 3 cm above the root–shoot joint served as rootstocks. A longitudinal slit about 1 cm deep was made in the centre of the cut end of each rootstock and these were placed vertically in a Laxenta box containing 80 ml of liquid or semi-solid medium of MS salts, B5 vitamins, 2% sucrose and 0.65% agar. The rootstocks were supported in a vertical position in the liquid medium with the help of perforated filter paper. The scions were inserted into slits of the rootstocks. For *ex vitro* grafting, seedlings raised in earthen pots were decapitated about 3 cm above the soil and about 1 cm deep slit was made. Microshoots were inserted on the rootstocks and the pots were immediately enclosed in transparent polybags. Twenty grafts under *in vitro* and 30 grafts under *ex vitro* conditions were made for each set of experiments. All the grafts were incubated at 25°C under 16 h photoperiod of light intensity of 16 $\mu E\ m^{-2}\ s^{-1}$. Observations on growth parameters (scion length, fresh weight and number of nodes per plant) were recorded after 30 days of incubation. The data was analysed using Student's *t* test.

About 1 cm long segment of the graft-union of a 25-day-old graft was excised and fixed in formalin : acetic acid : ethanol (5 : 5 : 90) for 48 h. The tissue was dehydrated by passing through *t*-butanol series, followed by paraffin embedding[18]. Longitudinal sections of 10 µm thickness were cut, dewaxed and stained with haematoxylin-eosin, mounted with DPX-4 189-[2-chloro-*N*-(4-methoxy-6-methyl-1,3,5-triazin-2yl)-aminocarbonyl] benzene sulphonamide (Qualigens, Glaxo India Ltd.) and were observed under the microscope.

Grafted plants were carefully removed after 30 days from the Laxenta boxes; the roots were washed thoroughly to remove the adhering medium. Plants were transferred to earthen pots containing a mixture of autoclaved soil : sand : compost (1 : 1 : 1) and were immediately covered with transparent polybags. The pots were kept at 25°C under 16 h photoperiod of a high light intensity of 47 $\mu E\ m^{-2}\ s^{-1}$. These grafted plants were sprayed with 1% fungicide solution (Bavistin, BASF India Ltd.) and irrigated with sterile water every third day and were supplied with the nutrient solution (1/10th concentration of MS major salts + full minor salts) twice a week for 30 days. The plants were acclimatized by trimming the corners of the polybags. Thereafter these pots were kept in the greenhouse for 4 weeks and were later transferred to the field.

Scions which did not wilt and showed growth and elongation of the apical part within a few days of culture both in liquid and semi-solid media as well as under *ex vitro*

conditions were considered to have established graft-unions (Figure 1 *a*). Observations made 30 days after culture showed a marked difference in growth parameters in the grafts grown in liquid and semi-solid media (Figure 1 *b*). The difference was clearly reflected in scion length, fresh weight of grafted plants and the number of nodes per plant (Table 1). The maximum elongation of the scion (5.83 cm) and the highest number of nodes (6.3) per plant was observed in grafts made onto the rootstock derived from etiolated seedlings of the cultivar AKH-081 and cultured in liquid medium with a filter paper support. Grafts cultured in liquid medium developed a larger number of roots with greater elongation (Figure 1 *f*) than those on semi-solid medium (Figure 1 *e*). The maximum number of micrografts (95%) derived from liquid culture and grafted onto the etiolated rootstocks of the cultivar DCH-32 showed survival on hardening. Survival percentage of grafts on semi-solid medium decreased to 65% and 70%

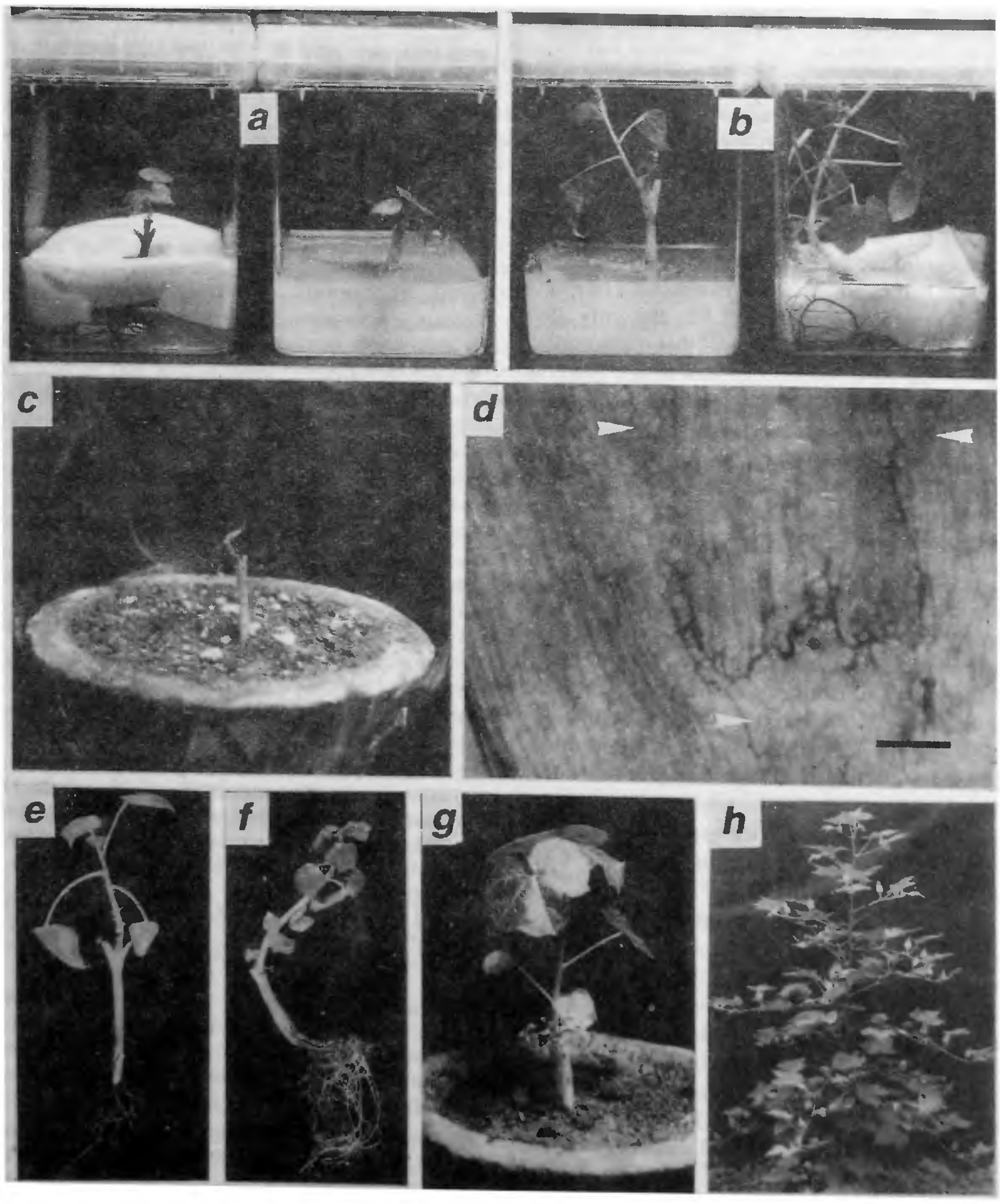

Figure 1. Micrografting in cotton. *a*, Micrografts after 10 days of culture in liquid and semi-solid media; *b*, Micrografts after 30 days of culture in semi-solid and liquid media; *c*, *Ex vitro* graft at initial stage covered with polybag; *d*, Longitudinal section of graft union (bar = 4 mm); *e*, *f*, Micrograft taken out after 30 days from semi-solid medium (*e*) and from liquid medium (*f*); *g*, Grafted plant of putative transformant; *h*, Mature grafted plant in field bearing flowers and bolls.

Table 1. Effect of culture medium, etiolation and *ex vitro* conditions on some growth parameters and the survival of micrografts in cotton

Growth conditions	Scion/ rootstock	No. of grafts	Survival of grafts after hardening		Scion length (cm)	Fresh weight (g)	No. of nodes/plant
			No	%			
Liquid medium/E	Intervarietal	20	19	95	5.59 ± (1.53)	0.78 ± (0.20)	5.6 ± (0.95)
Liquid medium/NE	Intervarietal	20	18	90	5.03 ± (1.01)	0.74 ± (0.19)	5.1 ± (0.84)
Semi-solid medium/E	Intervarietal	20	13	65	2.16 ± (0.46)	0.36 ± (0.07)	5.0 ± (0.83)
Semi-solid medium/NE	Intervarietal	20	14	70	2.44 ± (0.75)	0.46 ± (0.06)	4.72 ± (1.10)
Liquid medium/E	Interspecies	20	18	90	5.83 ± (1.48)	0.71 ± (0.21)	6.3 ± (1.50)
Semi-solid medium/E	Interspecies	20	14	70	2.03 ± (0.81)	0.27 ± (0.07)	4.18 ± (1.35)
Ex vitro grafts/NE	Intervarietal	30	10	30	1.41 ± (0.36)	–	2.52 ± (0.52)

E, rootstocks from etiolated seedlings; NE, rootstocks from non-etiolated seedlings; ± standard deviation (each value is mean of 10 replicates each of two experiments; –, data not recorded; growth parameters recorded after 30 days of incubation; differences between the liquid and semi-solid media conditions were significant at 95% level of confidence.

for etiolated and non-etiolated (intervarietal) rootstocks respectively. A similar trend was observed when scions were used on etiolated rootstocks (interspecies) of AKH-081 but with higher survival percentages of 90 and 70 for liquid and semi-solid media respectively.

Ex vitro grafts had minimum scion elongation (1.41 cm), lowest number of nodes per plant (2.52) and only 30% survival (Table 1). It has been reported[19] that during scion/rootstock union, the new callus tissue that arises from the cambial region is composed of thin-walled, turgid cells which easily desiccate and die. Therefore it is important that air moisture around the graft-union is kept high to promote proliferation of cells in the graft region (Figure 1 *c*).

In spite of the low success rate, *ex vitro* grafting has some advantages. It is less expensive and less time consuming as transplantation from *in vitro* to greenhouse is omitted. Our preliminary results demonstrate the feasibility of *ex vitro* intervarietal grafting in cotton. It should be possible to further enhance the survival percentage of *ex vitro* grafts by manipulating various factors such as the source, size of scion/rootstock and growth conditions.

Histological examination of graft-union showed multiplication of parenchymatous cells by scion and rootstocks (see arrows, Figure 1 *d*) and their integration to form a union. Hardened grafted plants transferred to greenhouse and field conditions grew to maturity and set seeds (Figure 1 *h*).

Simultaneously, in our preliminary attempts, putatively transformed microshoots of cotton cultivar NHH-44 (scion) could be successfully grafted on *in vitro* grown decapitated etiolated seedlings of cultivar DCH-32 (rootstock). Grafted plants survived after hardening (Figure 1 *g*).

Successful graft-unions established between two cultivars and between two species demonstrate a heterologous graft-union compatibility in cotton. The present micrografting method can be applied round the year and has several applications such as obtaining of a high percentage of viable plants from *in vitro* regenerants generally difficult to root, recovery of whole plants from putative transformants which are usually extremely slow growing due to antibiotic stress or fail to root, acceleration of shoot growth, and for understanding the histochemical basis of successful graft-unions and graft rejection.

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