RESEARCH PAPER

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Induction and establishment of somatic embryogenesis in elite Indian cotton cultivar (*Gossypium hirsutum* L. cv Khandwa-2)

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Keywords: *Gossypium hirsutum* L., Khandwa-2, Plant regeneration, Somatic embryogenesis, Recalcitrant

Abbreviations: 2, 4-D, 2, 4-Dichlorophenoxyacetic acid; 6-BAP, 6-Benzyl aminopurine; GA3, Gibberellic acid; IBA, Indole-3-butyric acid; MS, Murashige and Skoog's; NAA, Naphthalene acetic acid

Embryogenesis in cotton is a difficult task due its genome dependency. We used 3 cotton cultivars (Khandwa-2, G. Cot. 10, and BC-68–2) and Coker-312 as control for regeneration. Efficient somatic embryogenesis was induced in agronomically important Indian cotton cultivars, Khandwa-2 and G. Cot. 10. For callusing in all the cultivars, different media combinations were tried. Embryogenesis was initiated on a hormone-free MS medium (MSB). For embryo maturation and recovery excess of L-glutamine and L-asparagine were used. Khandwa-2 somatic embryos were successfully regenerated into plants. However, no plantlet was obtained in case of G. Cot. 10. Callus induction was also observed in BC-68–2 but there was no embryogenesis observed. The study indicated that the medium and genotype significantly effects embryogenesis. An efficient protocol is described here for regenerating plants via somatic embryogenesis in an elite Indian cotton cultivar Khandwa-2.

Introduction

Cotton is one of the most important fiber crops of the world. India ranks first in the world in respect of acreage under cotton cultivation (about 8 million hectares) and second in total cotton lint production (5 984 000 tons) (FAOSTAT 2011) after China. Many groups around the globe initiated cotton improvement program through which lot of high yielding varieties were developed. Drastic decline in crop yield due to biotic and abiotic stresses is well reported. Thus for any kind of genetic improvement and for development of transgenic plants with desired traits, an efficient regeneration system of the crop is a pre-requisite.

Somatic embryogenesis is one of the basic tools widely used in crop plant biotechnology research. It is useful for micro-propagation and for genetic manipulations of plants, which can be used for raising fully transformed plants after mutagenesis or gene transfer. The somatic embryos can be produced with high frequency, but maturation and conversion into plants are still a tedious task, requiring optimization of medium and environmental conditions.

However regeneration via somatic embryogenesis in cotton is a challenge due to: 1) genotype dependence, 2) somaclonal variation, 3) lack of knowledge about inheritance and gene action during in vitro embryogenesis, and 4) specific media requirements by different genotypes.[1] In cotton, somatic embryogenesis has been mostly restricted to *Gossypium hirsutum*, while there are only few reports of somatic embryogenesis mediated regeneration in other cotton species viz *G. arboreum*,[2] *G. barbadense*,[3] and in 2 wild cotton species *G. nelsonii* Fryx and *G. australe* F Muell.[4] For the genetic manipulation *G. hirsutum,* Coker variety has been maximally exploited with numerous reports of its regeneration through somatic embryogenesis.[5-11] Other varieties which have shown embryogenic potential are Acala, Lu, Deltapine, Paymaster, T25, T169, Lankart-175,[12] and GSA-78.[13] A few Chinese cultivars reported to regenerate via somatic embryogenesis are Simian-3,[14] Nazilli 143 and Nazilli M 503,[15] Yuzavo-1,[16] Zhongmian-35,[17] and few other cultivars of *G. hirsutum*.[18] Among Indian cultivars, successful somatic embryogenesis was reported only in MCU-5;[19] however attempts have also been performed in SVPR2,[20] SH-131, LH-900, Hybrid H-8 and Khandwa-2, MCU 5, MCU 12, MCU 13, and KC 3.[11] Thus only a limited number of Indian cultivars have been reported to produce somatic embryos and regenerate plants. Recently, people have also shown some direct methods for cotton regeneration.[21,22] Most responsive lines belong to Coker varieties that are no longer under cultivation, and for gene integration into cultivated varieties, it is mandatory for the seed companies to backcross the transgenic Coker cotton with local varieties. Genotype-dependent response restricts the application of cotton biotechnology to cotton breeding and production.

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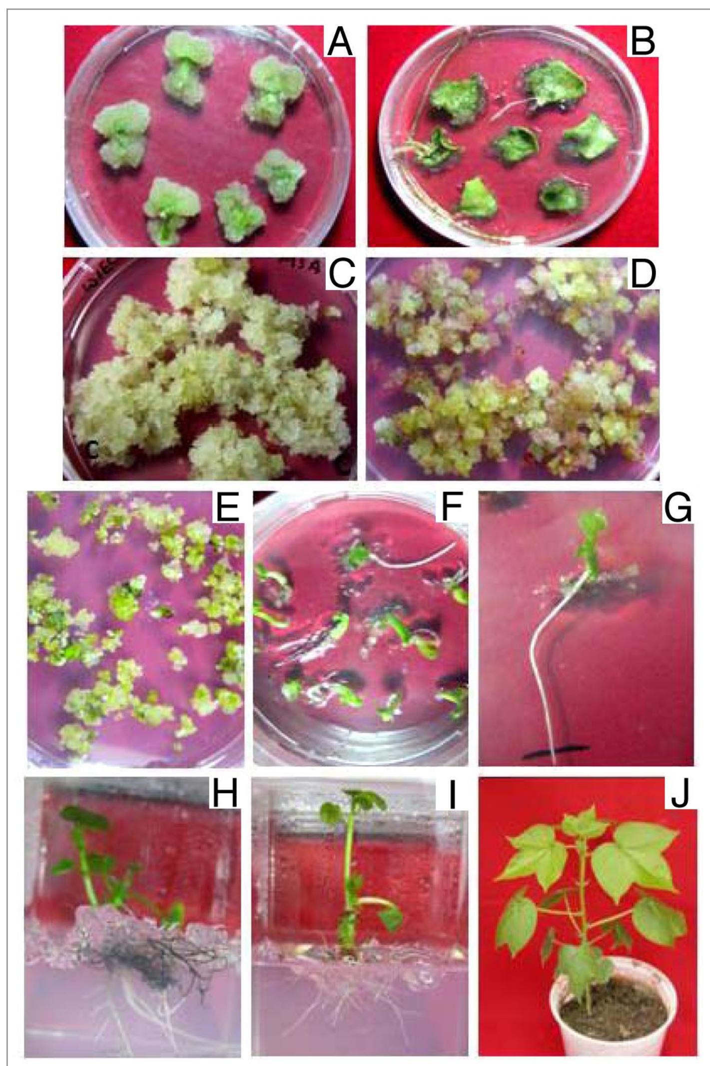

Figure 1. Somatic Embryogenesis in *Gossypium hirsutum* L. cultivar Khandwa-2. The explants hypocotyls (**A**) and cotyledonary pieces (**B**), embryogenic cultures (**C** and **D**), Immature and mature somatic embryos (**E**), Mature somatic embryos (**F** and **G**), Rooted plantlet (**H** and **I**), Acclimatized plant (**J**).

Table 1. Mean values of percent seedling response to callus and root formation for explant, cultivar, and media (Percent seedling response = Number of seedling with callus/root formation / Total number of seedlings × 100)

Character	Mean ± SE (%)	
	Callus formation	Root formation
I. Explants: a) Hypocotyls b) Cotyledons	 78.95 ± 2.4 66.9 ± 4.9	 9.79 ± 3.4 18.74 ± 4.6
II. Cultivars: a) Coker 312 b) Khandwa-2 c) G. Cot. 10 d) BC-68–2	 66.25 ± 7.0 80.41 ± 4.2 76.25 ± 5.9 72.0 ± 5.3	 14.99 ± 4.6 9.98 ± 4.4 20.0 ± 4.2 14.58 ± 6.6
III Media a) MSP-1 b) MSP-2 c) MSP-3 d) MSP-4	 76.25 ± 4.0 80.8 ± 5.1 62.5 ± 8.1 77.0 ± 3.4	 23.33 ± 2.2 21.66 ± 8.6 12.08 ± 5.1 0.00

Development of efficient regeneration system in cotton cultivars is required to widen the scope of biotechnological manipulation in cotton improvement. Our study successfully demonstrates somatic embryogenesis and conversion of embryos into plantlets in agronomically important cultivar Khandwa-2 and somatic embryogenesis in G. Cot. 10 by optimizing culture conditions that vary from genotype to genotype.

Results

Induction and proliferation of callus

Hypocotyl and cotyledon explants from individual seedlings of 4 cultivars of cotton (*G. hirsutum* L.) Coker 312, Khandwa-2, G. Cot. 10, and BC-68–2 were inoculated on each of the 4 media combinations (MSP-1, MSP-2, MSP-3, and MSP-4) (**Fig. 1A** and **B**). Initiation of callus formation was observed 10–12 d after inoculation of explants on callus induction media (MSP-1 to 4) (**Fig. 1C–D**). In hypocotyl explants, more calli originated from cut ends than from the surface, whereas in cotyledonary explants more calli proliferated from ventral surface as compared with the cut edges. Initially, the callus was white, translucent, and soft in all the 4 varieties. Callus growth continued further with slight change in color from white to yellow-green and cream. The response to callus formation differed significantly in different explants, cultivars, and media (**Table 1**). The hypocotyl explants had higher callus formation than the cotyledon explants. Among all the cultivars, highest response to callusing was seen in Khandwa-2 followed by G. Cot 10, BC-68-2, and Coker 312. Among the different media tested, MSP-2 was most successful followed by MSP-4, MSP-1, and MSP-3 for all cultivars (**Table 1**). The highest response to callusing was obtained in Khandwa-2 on MSP-2 in case of hypocotyl explants, and the lowest response was seen in cultivar Coker 312 on MSP-3 medium in case of cotyledon explants (**Table 2**).

Along with callusing, rooting was also observed from hypocotyl as well as cotyledon pieces of different cultivars on various media combinations (**Fig. 1B**). The response to root formation differed significantly in different explants, cultivars and media (**Table 1**). Higher response to rooting was observed in cotyledon explants than the hypocotyl explants. Among different cultivars, response to rooting was observed in G. Cot. 10 followed by Coker 312, BC-68-2, and Khandwa-2, whereas among different media it was highest in MSP-1, followed by MSP-2 and MSP-3. There was no root formation in MSP-4 medium (**Table 1**). Highest rooting was observed in the cultivar G. Cot. 10 on MSP-2 medium (**Table 3**).

Somatic embryogenesis

Actively growing calli obtained after 6 weeks of initial culture were transferred to the basal medium (MSB). Regular sub-culturing of callus was performed at an interval of 3 weeks. Embryogenesis was induced after 6–9 weeks in calli originated from hypocotyl explants (**Fig. 1E**). Response to embryogenesis differed significantly with respect to cultivars and media (**Table 4**). The embryogenesis was highest in Coker

312. Khandwa-2 and G. Cot. 10 also showed embryogenic response. In BC-68–2 embryogenesis was not observed. Embryogenic response was highest for MSP-2 medium followed by MSP-1, MSP-3, and MSP-4. In Khandwa-2, embryogenic callus was found to originate only from MSP-1 (**Fig. 2A**). Likewise, G. Cot. 10 showed embryogenesis only on MSP-4 originated callus. On the other hand, in Coker 312 embryogenesis was obtained in all the calli except from callus derived from MSP-4 medium. In this cultivar higher percent of seedlings (36.67%) gave embryogenesis in calli that originated on MSP-2 medium followed by MSP-1 and MSP-3, respectively. In almost all the cultivars, initial embryogenic callus was cream in color, and during subsequent sub-culturing most of them turned to golden yellow and green.

Embryo maturation and germination

For further development of somatic embryos, the embryogenic calli of Khandwa-2 was transferred to MSD medium for 2 cycles of 3 weeks each. From embryogenic calli globular, heart-shaped and torpedo-shaped embryos were obtained in sufficient number (**Figs. 1E** and **2B–D**). These embryos were transferred to MSM medium for further maturation (**Figs. 1F–G** and **2E**). Bipolar embryos measuring 5mm or longer were transferred to germination medium (MSG). They were maintained for 2 cycles on MSG for further elongation. About 34.8 ± 2% of the embryos were converted into plantlets (data not shown) (**Fig. 1H–J**). The plantlets obtained from the medium were transferred in pot (soilrite, vermiculite, and garden soil in 1:1:1 ratio) and kept in glass house under controlled conditions for their hardening. The complete protocol for regeneration of Khandwa-2 is given in the **Figure 3**.

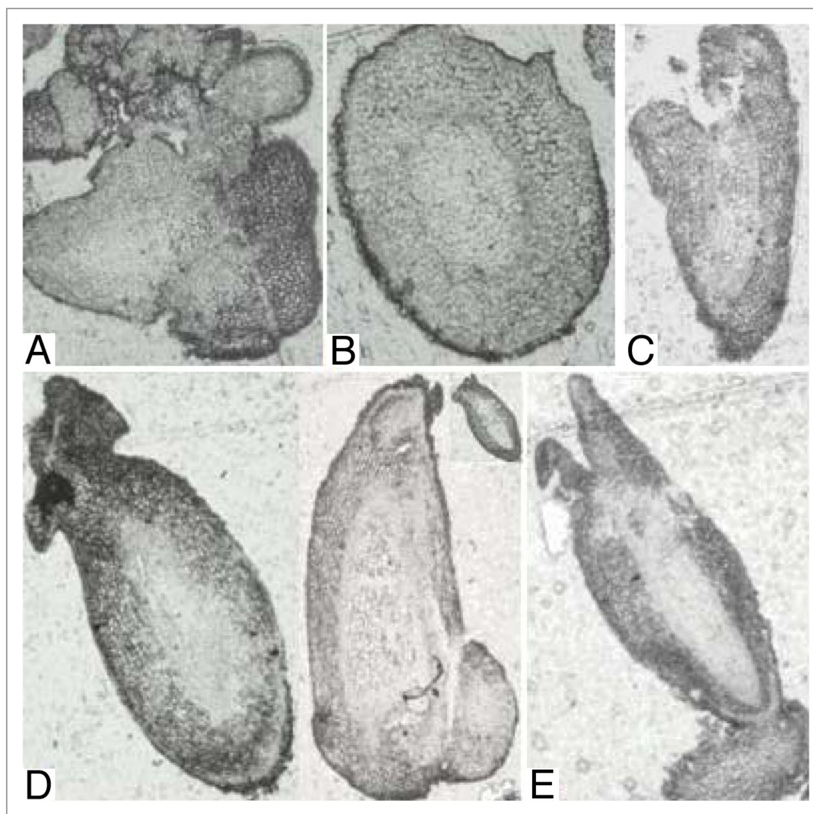

Figure 2. Somatic embryos in different embryological stages (**A**) Section showing embryogenic calli, (**B**) transverse section of globular embryo, (**C**) longitudinal section of heart shaped somatic embryo, (**D**) Torpedo shaped somatic embryos exhibiting closed vascular system (**E**) cotyledonary embryo linked to the callus mass.

Discussion

The genotype and growth regulators effect somatic embryogenesis in cotton. Kinetin is one of the most frequently used growth regulator in combination with: 1) 2, 4-D,[6,23-27] 2) NAA,[5,28] or 3) IBA.[8,16] Variations in embryogenic response in cotton due to culture medium and genotype have already been reported.[6,8,12,16,28] In the present investigation, similar results were obtained with Khandwa-2 when kinetin was used in combination with IBA. However, G. Cot. 10 derived callus gave embryogenic response on medium containing combination of 2, 4-D and IBA.

The response to callus formation differed significantly in the hypocotyl and cotyledon explants (**Table 1**). It was higher in hypocotyl than cotyledon explants. Superiority of hypocotyl over cotyledon for callus formation was also shown by Wilkins et al.[1] Callus formation was observed in all the varieties on all the growth regulator combinations (**Table 1**).

Table 2. Percent response of cotton cultivars to callus formation on different media combinations (Percent response = Number of seedlings with callus formation / Total number of seedlings × 100). Values are Mean ± SE (%)

	EXPLANTS							
Cultivars	Hypocotyl				Cotyledon			
	MSP-1[a]	MSP-2[b]	MSP-3[c]	MSP-4[d]	MSP-1	MSP-2	MSP-3	MSP-4
Coker 312	70 ± 5.77	80 ± 5.77	70 ± 5.77	73 ± 3.34	70 ± 5.77	53 ± 14.5	36 ± 16.7	76 ± 8.8
Khandwa-2	86 ± 3.34	86 ± 3.34	83 ± 3.34	86 ± 3.34	76 ± 8.8	76 ± 8.8	66 ± 12	80 ± 5.77
G. Cot. 10	80 ± 0	80 ± 10	86 ± 3.34	83 ± 3.34	80 ± 10	80 ± 10	43 ± 3.34	76 ± 3.34
BC-68–2	76 ± 8.8	76 ± 3.34	73 ± 3.34	66 ± 8.8	70 ± 5.8	73 ± 6.67	36 ± 3.34	73 ± 3.34

MSP-1 to MSP-4 are callus induction media with MS salts, B5 vitamins, 100 mg l^{-1} myoinositol, 30 g l^{-1} glucose, 0.75 g l^{-1} $MgCl_2$, and 2.2 g l^{-1} phytagel containing (**A**) 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} kinetin; (**B**) 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} GA_3, and 1.0 mg l^{-1} kinetin. (**C**) 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} kinetin. (**D**) 0.1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} 6-BAP.

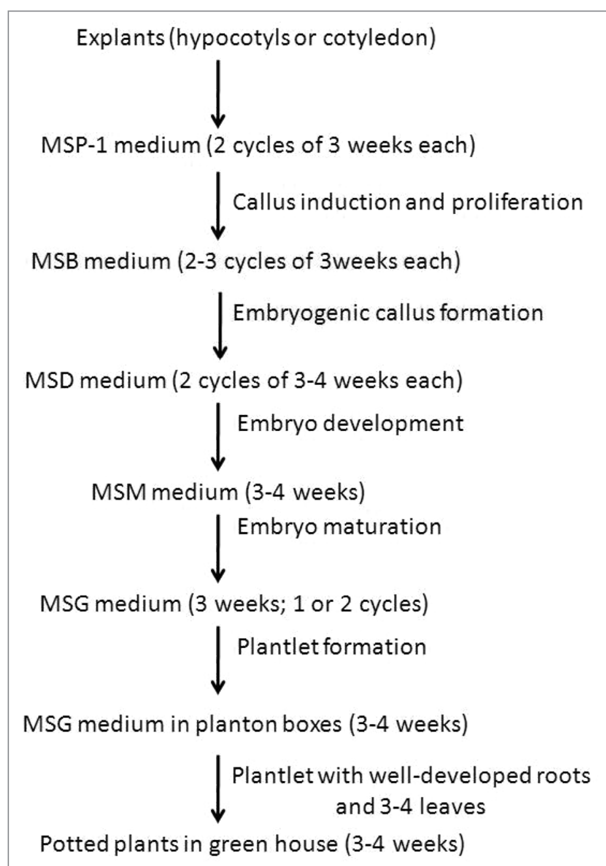

Figure 3. Protocol for somatic embryogenesis and plant regeneration in cultivar Khandwa-2

Table 3. Percent response of cotton cultivars to root formation on different media combinations (Percent response = Number of seedlings giving root / Total number of seedlings × 100). Values are Mean ± SE (%)

	Explants							
Cultivars	Hypocotyl				Cotyledon			
	MSP-1	MSP-2	MSP-3	MSP-4	MSP-1	MSP-2	MSP-3	MSP-4
Coker 312	23.3 ± 3.34	36.6 ± 3.34	23.3 ± 3.34	0	23.3 ± 3.34	13 ± 3.34	0	0
Khandwa-2	20 ± 0	0	0	0	13.3 ± 3.34	13 ± 3.34	33 ± 3.34	0
G. Cot. 10	26.6 ± 3.34	0	0	0	26.6 ± 3.34	60 ± 5.77	26 ± 3.34	0
BC-68–2	26.6 ± 3.34	0	0	0	26.6 ± 3.34	50 ± 5.77	13 ± 3.34	0

Along with callusing, root formation was also observed in both (hypocotyl and cotyledon) the explants (**Tables 1** and **3**) on all the media combinations, except in case of MSP-4 containing 2, 4-D and BA. These results suggest that media containing growth regulator combination with 2, 4-D prevents rooting in explants. Formation of roots in explants did not interfere with embryogenesis.

In our study, the growth regulator combinations of IBA and kinetin (MSP-1) and 2, 4-D and BA (MSP-4) favored somatic embryogenesis in specific varieties. Wu et al.[8] and Jin et al.[16] also reported the induction of somatic embryogenesis by the combination of IBA and kinetin in cotton varieties. Kumar and Tuli[9] showed 2, 4-D and BA as the best combination for embryogenesis in cotton. On the basis of growth regulators, we can categorize cotton regeneration into: 1) highly responsive variety, which includes Coker 312, 2) moderately responsive including Khandwa-2 and G. Cot. 10, and 3) non-responsive BC-68-2.

The embryogenic calli continued to give rise to somatic embryos on the hormone-free (MSB) medium. KNO_3 was added to the media, and NH_4^+ was reduced for the maturation of embryos. The addition of the extra KNO_3 and the reduction of NH_4^+ have been reported to be essential for maturation of somatic embryos in cotton.[5]

Fertile plants of Khandwa-2 and embryos of G. Cot. 10 were successfully obtained. However, we could not obtain plants from the somatic embryos of G. Cot. 10. The study emphasizes the need for examining the non-responsive cultivars of cotton like BC-68–2 for embryogenesis on a wider range of growth regulator combinations. Such studies are desirable to widen the scope of applying biotechnological approaches to the recalcitrant cotton cultivars.

Materials and Methods

Sterilization and germination of seeds

Seeds of 4 different cotton varieties Coker 312, Khandwa-2, G. Cot. 10, and BC-68–2 were de-linted with concentrated H_2SO_4, followed by neutralization with sodium bicarbonate, and then rinsed 4 times with tap water. De-linted seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 6 min followed by 5–6 times rinsing with sterile distilled water. Further seeds were dipped in 95% ethanol for 10 s, scorched with flame, and germinated in Growtek growth chamber (Tarsons India, Catalog No. 020090). After 2 d, the seedlings were transferred on paper bridges in test-tubes (25 × 150 mm) having medium containing half strength MS salts, B5 vitamins, 100 mg l^{-1} myo-inositol, and 20 g/l sucrose (pH 5.6). These seeds were further grown at 28 ± 2 °C under 16/8h light/ dark photoperiod in white fluorescent tube lights (60 µmol/m^2/s). All the biochemicals and medium constituents used were of Molecular Biology or Cell Culture grade from Sigma (USA).

Induction of callus

The explants used for callus induction were 6–8 mm long hypocotyls and 1 cm^2 cotyledon pieces taken from 7–8 d old healthy germinated seedlings. The explants were transferred to callus induction medium containing MS salts, B5 vitamins, 100 mg l^{-1} myo-inositol, 30 g l^{-1} glucose, 0.75 g l^{-1} $MgCl_2$, and 2.2 g l^{-1} phytagel with different combinations of growth regulators viz: 1) MSP-1, containing 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} Kinetin, 2) MSP-2, containing 0.5 mg l^{-1} NAA, 0.1 mg l^{-1} GA_3, and 1.0 mg l^{-1} Kinetin, 3) MSP-3, containing 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kinetin, and 4) MSP-4, containing 0.1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} 6-BAP. Except GA_3, which was filter sterilized, all

Table 4. Percent response to embryogenesis on MSB medium from hypocotyl callusoriginated on MSP-1 to MSP-4 media. (Percent response = Number of seedlings showing embryogenesis / Total number of seedlings × 100). Values are Mean ± SE (%)

	Callus forming medium			
Cultivars	MSP-1	MSP-2	MSP-3	MSP-4
Coker-312	13.3 ± 3.34	36.6 ± 3.34	10.0 ± 0	0
Khandwa-2	10.0 ± 0	0	0	0
G.Cot.10	0	0	0	6.67 ± 0
BC-68–2	0	0	0	0

growth regulators were added before autoclaving at 121 °C for 15 min. The pH of the medium was adjusted to 5.8 prior to autoclaving. Hypocotyl and cotyledon leaf explants of 4 varieties were inoculated on aforesaid 4 different media combinations. For each treatment, 10 individual seedlings were taken, and for each seedling, 6 pieces of explants (hypocotyl / cotyledon) were cultured in 1 petri-dish each to maintain seedling identity. During subsequent cycles of sub-culturing, identity of the seedlings was maintained and seedling-wise data were recorded. Entire experiment was repeated thrice. Statistical analysis was done by ANOVA through factorial CRD.

Induction of somatic embryogenesis

After 2 cycles of sub-culturing of 3 weeks each, actively growing calli were transferred to medium containing MS salts, B5 vitamins, 100 mg l^{-1} myo-inositol, 30 g l^{-1} glucose, 8 g l^{-1} agar at pH 5.8 (MSB). Embryogenic calli were then maintained by regular sub-culturing at an interval of 3 weeks on MSB medium. For further development of somatic embryos, embryogenic calli were transferred to MSB medium without NH_4NO_3 but doubled the amount of KNO_3 (MSD) for 2 cycles of 3 weeks each. Embryos obtained and those longer than 5mm were transferred to germination medium containing 0.5 strength MS salts, 0.5 strength B5 vitamins, 20 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 0.75 g l^{-1} $MgCl_2$, and 2.2 g l^{-1} phytagel (MSG). Cultures were kept under 60 µmol/m^2/s light intensity, 16/8 h light/dark photoperiod in petri-plates sealed with Parafilm (Sigma, cat. no. P7793). Rooted embryos with shoot-apex were transferred to 7.5 × 7.5 × 10 cm (l × b × h) Magenta boxes (Tarsons India, cat. no. 020080) containing 100 ml of MSG medium. The plantlets were transferred to pots for hardening.

Hardening of plants

The acclimatization of the regenerated plantlets was performed in plastic pots containing sterile potting mixture comprising of garden (sandy loam) soil, soilrite and vermiculite in 1:1:1 ratio. During the 14–18 d of hardening period, the plants were covered with polythene bags to maintain high humidity and irrigated with sterile water. For the next 2 d, the polythene bags were raised partially, before removing them completely. During this period, the plants were incubated at 28 ± 2 °C, 60 µmol $m^{-2}s^{-1}$ light intensity, and 16 h photoperiod. After 20 d, the plants were transferred to a glass house for further growth in earthen pots. The glass house-grown plants were brought to a net-house after 4–6 weeks and planted in soil.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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