Simple and reproducible protocol for direct somatic embryogenesis from cultured immature inflorescence segments of sugarcane (*Saccharum* spp.)

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A protocol for direct somatic embryogenesis without an intervening callus phase was developed for sugarcane (*Saccharum* spp. hybrids) using immature inflorescence segments. Murashige and Skoog (MS) medium supplemented with 0.5 mg/l naphthaleneacetic acid, 2.5 mg/l kinetin, 100 mg/l L-glutamine and 4% sucrose showed high frequency of somatic embryo development (54.09 ± 2.7%), with an average 7.72 ± 0.89 plants per explant. Embryo development was seen all over the cultured explants within four weeks of culture and the embryos germinated within a week upon transfer to basal MS medium without any growth regulators and all the plants grew normally in the greenhouse. This is a report on the induction of direct embryogenesis from inflorescence segments in Indian sugarcane. This method could be useful for regenerating large number of plants as well as provide a target tissue for genetic transformation studies.

SUGARCANE (*Saccharum* spp. hybrids) is a highly polyploid plant ($2n$ = 36–170) grown in different parts of the world from the tropics to subtropics, and accounts for around 60% of the world's sugar. It is also one of the important cash crops in many developing/developed countries, with a high trade value. The importance of sugarcane has increased in recent years because cane is an important industrial raw material for sugar industries and allied industries producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes and animal feed[1]. Considering its importance in the agricultural industry, concerted efforts are being made for its improvement using biotechnological approaches. Somatic embryogenesis is an efficient and high volume propagation system for the production of large number of plants within a short period[2]. Plant regeneration through somatic embryogenesis has been reported in sugarcane using young leaf rolls and immature inflorescences[3–6]. In these and other studies, embryogenic callus was induced in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram and regeneration was obtained by reducing the concentration of the auxin or deleting it from the medium[7], or by media supplementation with thidiazuron[8].

Successful genetic transformation attempts have mostly employed embryogenic callus/cell cultures as the target tissue in several crop plants, including sugarcane[9]. However, a major limitation of this callus system is the repeated subculture to select embryogenic callus portions among highly proliferating non-embryogenic tissue. This process is not highly reproducible and furthermore increases the chances of somaclonal variation[10]. As these limitations have become unavoidable, strategies to improve plant regeneration must necessarily include manipulation of the explant material to embark upon new morphogenetic pathways.

Direct somatic embryogenesis offers several advantages in crop improvement, as cost-effective and large-scale clonal propagation is possible using bioreactors, ultimately leading to automation of somatic seed production and development of artificial seeds. Besides, such a system could also provide a new source for use in genetic transformation. The plants derived from direct somatic embryogenesis usually are unicellular in origin and hence genetically uniform[11]. The immature inflorescences of graminaceous plants have become an excellent source of young meristematic tissue for the induction of direct or indirect somatic embryogenesis[12–14]. Factors controlling the process of direct somatic embryogenesis[15] have been studied extensively in many plant species. Recently, Eudes *et al.*[16] reported induction of direct somatic embryogenesis and regeneration of fertile plants in cereals. In sugarcane, direct somatic embryogenesis has only been reported using young leaf rolls and the culture system has been employed for genetic transformation[17]. To our knowledge, there has been no study demonstrating direct somatic embryogenesis in Indian sugarcane using inflorescence. Our primary objective was to develop a simple and reproducible protocol for plant regeneration through direct embryogenesis, which can be used in studies aimed at genetic transformation. Herein, we report a protocol for the induction of direct somatic embryogenesis without

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any callus formation and high frequency plant regeneration from immature inflorescence segments of an elite Indian sugarcane var. CoC-671.

Materials and methods

Plant material and establishment of cultures

Sugarcane variety *Saccharum officinarum*, CoC-671 was used in all the experiments of this study. Fresh plant material of immature inflorescence segments was collected from 10-month-old field-grown plants (kindly provided by the Sugarcane and Gur Research Center, Kolhapur, India). The field-collected material was washed several times with tap water with a few drops of liquid soap. The outer old leaf-base coverings were removed carefully without damaging the inner young and delicate tissue, followed by the immersion of portion of inflorescence in absolute alcohol for 5 min for surface sterilization. After removing the outer sheaths, the innermost inflorescence segments were cut into 3–6 mm long pieces and inoculated on Murashige and Skoog (MS)[18] medium supplemented with different plant growth regulators and additives, either singly or in combination (Table 1). The pH of the medium was adjusted to 5.8 and autoclaved at 15 lb for 15 min. Cultures were maintained initially in the dark for 3–6 weeks and thereafter all the cultures were incubated in the culture room at 26 ± 1°C under cool, white fluorescent light (1000 lux) for 12 h/day, with relative humidity of 70–80%.

Embryogenic response was observed by the presence of somatic embryos on the cultured explant after four-week culture period and the number of regenerated plants was recorded per explant after eight-week culture period. For each treatment, 70–80 explants were employed and the experiments were repeated at least thrice and the data were statistically analysed.

Histology

The different developmental stages of somatic embryos were monitored by observing the cultures under stereomicroscope. To study the different developmental stages of the explants for direct somatic embryogenesis, thin sections were cut at 15 µm thickness using disposable blade, carried out in a cryotome (Lica). Good and intact sections were mounted in drops of aqueous safranine (2%) and observed under the microscope. The photomicrography was carried out using Zeiss Axioplan compound microscope.

Acclimatization of regenerated plants

Well-developed somatic embryos were cultured on MS basal media for germination. Agar was washed-off carefully under running tap water and germinated embryos with good root and shoot system (emblings) were allowed to grow in small plastic pots containing autoclaved soil, covered with polythene sheets and maintained under 25°C temperature and 70% relative humidity in the greenhouse.

Results

The immature inflorescence segments of 3–6 mm size showed swelling of the explant and initiation of small embryo-like structures in the first week of culture (Figure 1 *a* and *b*). In the next two weeks, embryogenic clumps were visible at the cut end of the explants (Figure 1 *c*). Well-developed embryos were observed all over the cultured explants within four weeks of culture. Absence of any callus formation indicated that the process of embryo development was direct, with the appearance of globular-stage embryos (Figure 1 *d* and *e*). These embryos exhibited compactly arranged cells with thick cytoplasm (Figure 1 *e*). Further, the initiation of direct somatic embryos was observed from the lower layer of epidermis, with initiation of procambium (Figure 1 *h*). The globular embryos developed into club-shaped embryos (Figure 1 *f*, *g* and *i*), characteristic of monocot embryos, with development of scutellum followed by well-differentiated shoot and root primordia (Figure 1 *j* and *k*).

Maximum embryogenic response (54.09 ± 2.7%) with well-developed plants (7.72 ± 0.89) per explant was observed on S1 medium with naphthaleneacetic acid (NAA), kinetin (KN) and L-glutamine (Figure 2). Omission of L-glutamine and addition of coconut water while lowering

Table 1. Composition of different media used for induction of direct somatic embryogenesis in sugarcane var. CoC-671

Media: growth regulators/additives (mg/l)	S1	S2	S3	S4	S5	S6	S7
Naphthaleneacetic acid	0.5	0.5	0.5	–	0.5	0.5	–
Kinetin	2.5	2.5	–	–	–	–	–
Thidiazuron	–	–	–	1	1	2	–
Benzylaminopurine	–	–	2.0	–	–	–	–
L-glutamine	100	–	–	–	–	–	–
Sucrose (%)	4	3	3	3	3	3	3
Coconut water (%)	–	5	5	–	–	–	–

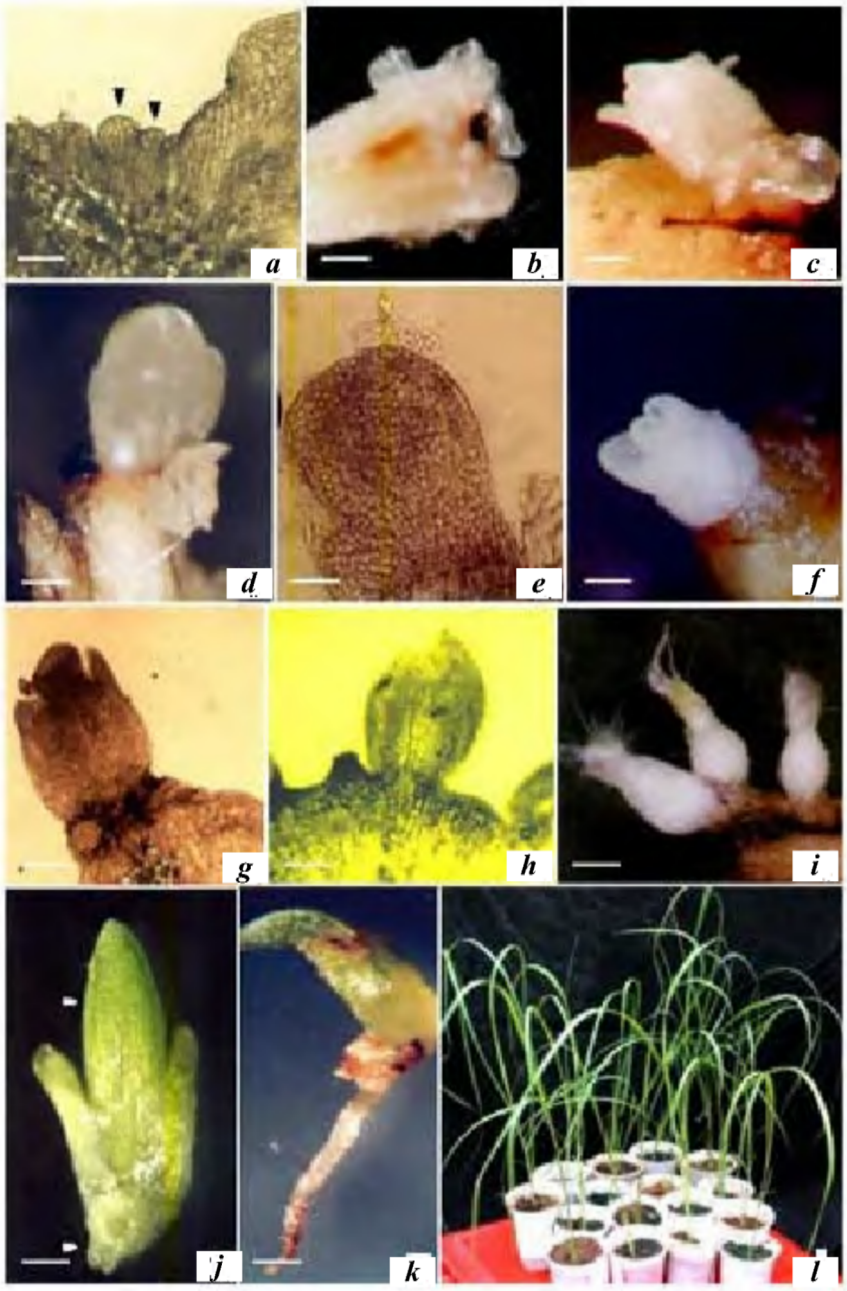

Figure 1. Different developmental stages in the induction of direct somatic embryogenesis in sugarcane cv. CoC-671. ***a***, Longitudinal section showing embryo initiation (shows early stage embryos, bar = 0.2 mm); ***b***, Embryo initiation (bar = 0.8 mm); ***c***, Single developing embryo from cut end of inflorescence segment (bar = 1 mm); ***d***, Globular embryo (bar = 1 mm); ***e***, Longitudinal section of globular-stage embryo (bar = 0.5 mm); ***f***, Club-shaped embryo showing initiation of leaf (bar = 0.8 mm); ***g***, ***h***, Longitudinal sections showing initiation of embryo from the explant (bar = 0.8 mm); ***i***, Developed embryos (bar = 1 mm); ***j***, Embryo showing initiation of coleoptile (➨ root primordia, bar = 1 mm); ***k***, Fully developed embryo with root and shoot development; ***l***, Hardened plants regenerated from embryos.

the concentration of sucrose (S2) resulted in $46.82 \pm 2.35\%$ explant response with 2.78 ± 0.13 plants per explant (Figure 2).

Among the media combinations tested (Table 1, Figure 2), replacement of KN with benzylaminopurine (BA; S3) did not induce any change in the frequency of response of

the explant (35.29 ± 1.8) and plant number per explant (2.08 ± 0.14), while the explants on S4 medium did not exhibit any embryogenic response. The explants on S4 medium initially showed slight swelling, but subsequently these turned brown resulting in the death of the explant. However, S4 medium further enriched with addition of NAA (S5) showed better response (17.64 ± 0.9), with an average of two plants per explant compared to S4 medium (Figure 2). Further increase in thidiazuron concentration in S6 medium resulted in better response (44.73 ± 1.76).

Somatic embryos that formed on S1 media supplemented with KN (2.5 mg/l), NAA (0.5 mg/l), L-glutamine (100 mg/l) with 4% sucrose showed vigorous growth, while those on S4 medium were smaller. The embryos germinated into plantlets with good rooting and vigorous growth on basal media (S7) without growth regulators. The process of direct somatic embryo induction followed by the formation of well-rooted plants took 6–8 weeks. All the regenerants grew normally under greenhouse conditions with 95% survival (Figure 1).

Discussion

In graminaceous species, plant regeneration has been facilitated by the identification of appropriate explant and *in vitro* culture conditions[11]. In this study, we identified explants and proper combinations of growth regulators for the induction of direct somatic embryogenesis in an Indian cultivar of sugarcane (CoC-671). The method is routinely employed with CoC-671 and other sugarcane cultivars (data not shown). The embryogenic culture response (ECR) of cultured immature inflorescence segments was directly related to the presence of suitable growth regulators (NAA and KN) and L-glutamine (S1 medium), since other growth regulator combinations did not yield comparable response in terms of plant regeneration. In rice, ECR was related to the growth regulator composition (2,4-D and BAP) of the medium in a two-step protocol developed for manipulating embryogenic response from immature inflorescence explants[13].

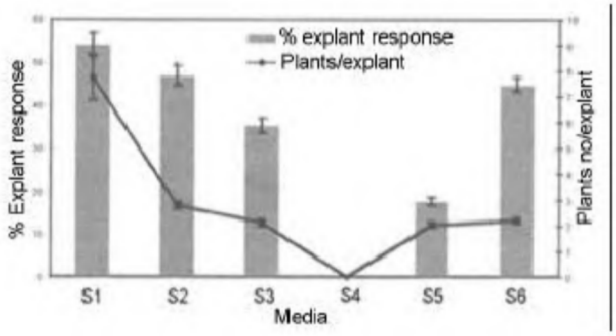

Figure 2. Effect of different media composition on direct somatic embryogenesis in sugarcane. See Table 1 for medium composition S1 to S6.

The immature inflorescence of sugarcane consists of main rachis and many sub-rachii and is delicate and fragile, light to dark pink in colour. Segments from the rachii were cultured on various combinations of media for the induction of direct somatic embryogenesis in sugarcane[19]. The use of immature inflorescence explants is advantageous, as these are discarded and hence sacrificing the plants for culture initiation is unnecessary. Moreover, the inflorescence material is abundantly available and free from disease. The inflorescence culture system does not need surface sterilization with toxic surface sterilants and there was no phenolic secretion during culture initiation. Hence the method is free from the problem of phenolics, often faced during culture establishment.

Direct somatic embryogenesis without intervening callus phase has also been reported in several other monocots like orchard grass[20], garlic[21], minor millet[22] and rice[14]. Such a callus-free development and regeneration pattern through the induction of direct somatic embryogenesis can be advantageous since callus culture is associated with problems of embryo formation, maturity and plantlet regeneration[23]. In barley[24], it has been reported that the regeneration system through callus cultures produced plants with albinism, phenotypic abnormalities and reduced fertility, suggesting that the callus culture system is at the realm of the origin of genome instability and regeneration problems. In this study, the initiation of embryos was observed from the wounded regions of the explant and histological examination of the stages clearly indicated embryo initiation, globular embryo formation and club-shaped embryos (Figure 1 *b–h*). Further histological studies are necessary to identify the cell types involved in the formation of somatic embryos. In SEM studies with embryogenic callus formation in sugarcane, the embryos were found with structural bipolar embryonic orientation showing distinctively clear embryo axis and scutellum[25]. Direct somatic embryo formation on sugarcane leaf discs was earlier reported in selections through rapid regeneration for sugarcane mosaic virus resistance[26] and in a transformation protocol using leaf discs[17].

In the present study, induction of direct somatic embryogenesis using immature inflorescence segment yielded a large number of plants in a short time. The healthy and well-grown rooted plants were obtained within seven weeks. The number of plants regenerated was 7.72 ± 0.89 per explant, and assuming an average of 24 segments per inflorescence, the total number of plants that can be generated is around 185–200. Using rice immature inflorescences, a large number of plants were regenerated in the range of 800–1400 per plant[14]. For an efficient application of the somatic embryogenesis system for cloning and propagation, it is essential to ensure the rapid development of embryos from cultured explants with subsequent regeneration into complete plants. Clonal propagation in sugarcane has been reported by the use of apical meristems and there has been no report to utilize a propaga-

tion system based on immature inflorescence segments. In this context the protocol developed in this study could be useful commercially for large-scale plant production. The regenerated plants appeared normal and uniform in their growth in the greenhouse and RAPD analysis of plants did not reveal any variation[27].

In conclusion, the induction of direct somatic embryogenesis using inflorescence segments as described in the present study, could be useful in rapid propagation of elite sugarcane varieties. Further, such a system should allow transformation methods like *Agrobacterium* or particle bombardment to be more successful by giving rise to more transformed plants in a short time, avoiding somaclonal variation.

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