Micropropagation and Somatic Embryogenesis in Sugarcane

Ajinder Kaur, Pawan K. Malhotra, Pooja Manchanda, and Satbir Singh Gosal

Abstract  Sugarcane propagation through conventional means does not provide sufficient planting material of a variety, particularly desirable in case of newly released varieties to achieve large-scale dissemination; this is attributed to slow rate of seed multiplication by conventional sett planting. On the other hand, micropropagation technique of tissue culture ensures production of disease-free and true-to-type planting material of popular (new as well as old) varieties in an abundant quantity in a short period of time. The cultures of meristematic buds or spindle leaves, collected from healthy plants, are established aseptically under controlled nutritional and environmental conditions in vitro, followed by multiplication of shoots and induction of roots; the plantlets are hardened and supplied to growers. Somatic embryogenesis is the process of embryo formation and development from somatic cells of an explant under in vitro conditions. The somatic cells in culture can follow two pathways for somatic embryogenesis, either direct or indirect. The plants regenerated through direct somatic embryogenesis are often uniform; thus, the pathway finds use in clonal propagation and genetic transformation of sugarcane genotypes. In indirect somatic embryogenesis pathway, first callus is induced from cultured explants under the influence of an auxin (mostly 2, 4-D) which is then regenerated into plants; such plants may exhibit somaclonal variation.

Keywords  Micropropagation · Organogenesis · Plant regeneration · Root formation · Shoot multiplication · Somatic embryogenesis · Sugarcane
1 Micropropagation: An Introduction

Sugarcane (*Saccharum* spp.) is a perennial monocot grass grown in the tropical and subtropical regions of the world (Wekesa et al. 2015) for its sweet stalk and is a commercially important sugar crop worldwide. India ranks second in the world for the production of sugarcane (Salokhe 2016). Besides sugar, sugarcane is a source of useful byproducts such as bagasse, molasses, bioethanol, press mud, biofertilizer and green tops for livestock feed (Jalaja et al. 2008; Salokhe 2016). The crop is conventionally propagated vegetatively through cane cuttings called ‘setts or billets’ containing one to three buds. The major drawback of conventional propagation is that it cannot provide enough planting material required for large-scale cultivation, due to low rate of seed multiplication (1:6 to 1:8 in a year) leading to slow dissemination of new high-yielding cultivars [2–3 years for small-scale spread, 7–8 years for large-scale spread (Geijskes et al. 2003)]. Further, during conventional propagation, there is build-up of pathogens causing diseases, such as red rot, smut, wilt, grassy shoot, ratoon stunting, yellow leaf and leaf scald, in seed canes over a period of time, leading to the deterioration of newly released varieties. Hence, non-availability of good quality, disease-free planting material is a main problem in sugarcane production and improvement. Besides this, a proper record about the different categories of sugarcane seed, viz. breeder seed, foundation seed and certified seed, is not maintained by the various sugarcane-growing states in India, leading to reduced seed replacement rate in the different states (Directorate of Sugarcane Development, Lucknow, India 2013) that affects the productivity potential of the varieties (Salokhe 2016). It has been reported that the planting material of a sugarcane variety should be replaced every 4 years to maintain its productivity potential and vigour (Sundara 2000; Sawant et al. 2014).

The micropropagation technique helps in the sustained production of quality planting material in an abundant quantity and is thus one of the finest and most successful examples of the commercial application of tissue culture technology. Although the first definition of micropropagation reads as ‘any aseptic procedure involving the manipulation of plant organs, tissues or cells that produces a population of plantlets thereby making it possible to bypass conventional sexual or vegetative propagation’ (Krikorian 1982), now it is widely defined as clonal propagation of plants from very small plant parts (0.2–10.0 mm) under in vitro conditions in a tissue culture laboratory. The plants raised through micropropagation are usually disease-free, so the vigour of a newly developed variety is maintained and that of an old variety is restored leading to its rejuvenation (Lal et al. 2015). This is due to the fact that healthy meristematic regions are cultured aseptically and shoot number is increased under controlled chemical and physical conditions resulting in quick bulking of the planting material of new varieties and enabling rapid coverage of a region with rejuvenated material, in contrast to 2–3 years using conventional means of propagation. In the field, the micropropagated sugarcane plants have been reported to exhibit better tillering, cane yield, juice content and quality in contrast to conventionally propagated plants (Gosal et al. 1998; Jalaja et al. 2008). The micropropagated plants are largely identical, apart from some rare off-type plants.
showing abnormal morphology, which should be uprooted and discarded in the first
generation itself (Sreenivasan and Jalaja 1981). The tissue culture-raised sugarcane
plants are used as breeder seed, and seed obtained from tissue culture regenerated
plants is used as foundation seed (Nerkar 2006; Tawar 2006; Sawant et al. 2014).

2 Micropropagation of Sugarcane: Historical Perspective

The technique of micropropagation was developed by Ball (1946) who obtained
complete plants of Tropaeolum majus and Lupinus albus by in vitro culture of shoot
tips and is regarded as the father of micropropagation (Gautheret 1985). Rotor
(1949) was the first to develop in vitro method for clonal propagation of orchids by
culturing nodal cuttings (bearing buds) of Phalaenopsis inflorescences. Micropropagation of sugarcane has been obtained through the culture of shoot tips
et al. 2008), apical/axillary buds (Sauvaire and Galzy 1978; Taylor and Duke 1993;
Taylor 1994; Chattha et al. 2001) and leaf segments (Gosal et al. 1998; Gill et al.
2006; Lakshmanan et al. 2006; Kaur and Sandhu 2014). The regeneration of adven-
titious shoots from leaf segments can occur either through direct somatic embryo-
genesis (Gill et al. 2006; Lakshmanan et al. 2006) or indirect somatic embryogenesis
through callus (Ho and Vasil 1983a; Lee 1987; Chowdhury and Vasil 1993; de
Alcantara et al. 2014) or organogenesis (Gill et al. 2006; Lakshmanan et al. 2006).
Leva et al. (2012) reported that plants regenerated from shoot tips or buds or adven-
titious shoots possess high genetic fidelity, whereas plants regenerated from callus
cultures exhibit somaclonal variation (Lee 1987).

2.1 Micropropagation Through Shoot-Tip Culture

Sugarcane can be successfully freed from pathogens especially viruses using shoot-
tip culture, and the plants so produced are identical to the mother plant (Lee 1987).
The shoot tips should be excised from actively growing tops (Fig. 1a) of primary
shoots or secondary shoots (used if a plant is disease-free) as these possess high
regeneration capacity and contain no or very low virus concentration. Micropropagation through shoot-tip culture can be of two types, viz. (a) micro-
propagation through apical meristem culture and (b) micropropagation through
shoot apex culture. The apical meristem in sugarcane is present in the apical bud,
observed after removing the leaf whorls from the apical top. The apical meristem
measures approximately 0.1 mm in diameter and 0.25–0.30 mm in length (Chawla
2009) and is obtained by cautiously taking off the leaf sheaths from the apical bud.
The meristem cells are genetically stable, thus producing plants alike the mother
plant (Hendre et al. 1983; Sreenivasan and Jalaja 1992). Coleman (1970) and
Hendre et al. (1975) cultured apical meristem to develop sugarcane mosaic virus-free plants. Hendre et al. (1983) optimized an apical meristem culture procedure for regeneration and multiplication of sugarcane mosaic virus-free plants of variety Co 740. A shoot apex measures 0.1 mm in diameter and 0.5 mm in length since it comprises a couple of young leaf primordia also (Chawla 2009) and is easy to excise as compared to apical meristem coupled with high frequency of organogenesis (Jalaja et al. 2008). Sreenivasan and Jalaja (1981) used shoot apex for sugarcane micropropagation, and Jalaja et al. (2008) reported that a large number of plantlets (1,80,000) could be produced from one shoot apex in a time period of 372 days. The shoot tips measuring 2–3 mm in size give higher frequency of shoot regeneration (Dhumle et al. 1994) as compared to shoot apex. Hendre et al. (1983) documented that nearly 2 lakh plants can be regenerated in a period of 6 months from one shoot tip. Anita et al. (2000) reported that a single shoot tip can produce about 15 lakh plants in 6 months through micropropagation. Ali and Afghan (2001) observed that a shoot tip measuring 4 mm in size was best for sugarcane micropropagation producing plants morphologically similar to the mother plants. The Philippine Sugar Research Institute Foundation, Inc., Philippines, has a commercial tissue culture
Micropropagation and Somatic Embryogenesis in Sugarcane

laboratory that generates 40,000 plantlets per week using shoot tips as explants. MS (Murashige and Skoog 1962) medium supplemented with cytokinins such as BAP and kinetin has been found to be suitable for shoot multiplication from shoot-tip explants (Naritoom et al. 1993; Ali and Afghan 2001; Baksha et al. 2003).

2.2 Micropropagation Through Apical/Axillary Bud Culture

The axillary buds also contain meristems and can be used for initiating tissue culture of sugarcane. The outer scale leaves of dormant axillary buds are first wiped with 70% ethanol before removal; thereafter, the buds are surface sterilized, excised and cultured (Jalaja et al. 2008). Sauvaire and Galzy (1978) produced genetically similar clones in a number of sugarcane varieties through axillary bud culture. Cheema and Hussain (2004) reported sugarcane micropropagation through the use of both apical and axillary buds and observed that the different genotypes responded differently on different MS media compositions with respect to establishment of explants, shoot differentiation, shoot multiplication rate, shoot elongation and root length. Thus, an efficient micropropagation method needs to be worked out for every variety or elite clone. Wagih et al. (1995) developed axillary bud culture technique for sugarcane cultivar NCo 310 on half strength MS medium containing naphthalene acetic acid, NAA (2 mg/l) and malt extract (500 mg/l). Mulleegadoo and Dookun (1999) found that axillary buds showed poor growth response as compared to apical buds. Chattha et al. (2001) and Khan and Rashid (2003) micropropagated sugarcane by culturing apical and axillary buds on MS medium containing cytokinin and gibberellic acid. Biradar et al. (2009) documented that axillary bud is the most appropriate explant for culture initiation on MS medium supplemented with 6-benzylaminopurine, BAP (2 mg/l). Wagih et al. (2009) established a tissue culture method for overcoming contamination from mature axillary buds of sugarcane. Godheja et al. (2014) standardized sugarcane micropropagation protocol from apical buds for large-scale production of shoots. In general, the apical/axillary buds have an edge over apical meristems since these produce shoots on a wide range of media, besides shoot development and multiplication from apical/axillary buds is also faster than apical meristems (Sauvaire and Galzy 1978; Hendre et al. 1983; Taylor 1994).

2.3 Micropropagation Through Spindle Leaf Segment Culture

The young leaf segments of sugarcane have an immense regeneration potential and are widely used for commercial plant production. The primary cultures of young leaf segments exhibit regeneration of multiple shoots in contrast to regeneration of one to two shoots in the primary cultures of apical/axillary buds. Gosal et al. (1998)
developed an effective protocol for large-scale establishment of shoot cultures from spindle explants (0.5–1.0 cm) on semi-solid MS + 0.5 mg/l IAA (indole-3-acetic acid) + 0.5 mg/l BAP + 0.5 mg/l kinetin medium. Geijskes et al. (2003) developed SmartSett™ micropropagation technology for quick clonal propagation of sugarcane (with up to 35-fold shoot multiplication rate) from immature leaf whorl explants, resulting in plant production in 3–4 weeks with up to 95% survival and a production cost of US $ 0.50 per plant. The cane yield (101 t/ha) and cane sugar (15.17%) of SmartSett™ seedlings were parallel to cane yield (104 t/ha) and cane sugar (15–15.5%) of plants raised conventionally from one-eye setts (Mordocco 2006). The SmartSett™ process takes 12–14 weeks for producing plants, making it possible to produce thousands of plants in a year. Gill et al. (2006) developed a distinctive one-step method for direct plant regeneration (without callus interphase) from immature leaf roll segments of sugarcane through culturing on semi-solid MS + NAA (5 mg/l) + Kin (0.5 mg/l) medium, where the shoots regenerated either through organogenesis or direct somatic embryogenesis or both; the method is highly sought-after for mass cloning of newly bred varieties/elite planting material of sugarcane. Lakshmanan et al. (2006) established developmental and hormonal basis of high-frequency in vitro regeneration (≥ 20 shoots per explant) through adventitious shoot production and somatic embryogenesis from transverse thin layer sections (1–2 mm thick) of sugarcane spindle leaf rolls producing a large number of plantlets directly and rapidly. Kaur and Sandhu (2014) developed a cost-effective high-throughput in vitro micropropagation protocol in sugarcane for use in agribusiness industry. The protocol comprising of five stages led to production of complete plants at a high frequency in a period of 157 days with 97% survival rate. The shoots were generated through direct adventitious shoot regeneration without an intervening callus phase and comprised six subculture passages with up to 25-fold shoot multiplication rate. The practicability of the protocol lied in its cheap per plant production cost (US$ 0.13) effected through incorporation of low-cost options. A detailed review on sugarcane micropropagation is presented in Table 1.

2.4 Micropropagation of Sugarcane for Commercial Plant Production

The methodology involves four stages:

2.4.1 Establishment of Aseptic Cultures of Explants In Vitro (Stage 1)

This is a stage in which field-grown plants are brought in the laboratory to grow under in vitro conditions, thus requiring additional concern and skillfulness. It comprises of the following steps:
<table>
<thead>
<tr>
<th>Plant material/cultivar</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>Plant differentiation from callus induced on shoot apices, leaves and inflorescences</td>
<td>Heinz and Mee (1969)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>Sugarcane mosaic virus-free plants through apical meristem culture</td>
<td>Coleman (1970), Hendre et al. (1975)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>Root and shoot development from callus</td>
<td>Nadar and Heinz (1977)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>Micropropagation from callus culture within 9½ months</td>
<td>Barba et al. (1978)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>Production of disease-free plants through meristem culture</td>
<td>Leu (1978)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>Micropropagation using axillary buds</td>
<td>Sauvaire and Galzy (1978)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>Standardized micropropagation using meristem tip culture</td>
<td>Sreenivasan and Jalaja (1981)</td>
</tr>
<tr>
<td>IJ76-316</td>
<td>Plant regeneration from embryogenic callus cultures established from primordial leaves and apical meristems</td>
<td>Ahloowalia and Maretzki (1983)</td>
</tr>
<tr>
<td>Co 740</td>
<td>Standardized apical meristem culture technique for quick multiplication of mosaic virus-free plants</td>
<td>Hendre et al. (1983)</td>
</tr>
<tr>
<td>NA56-79</td>
<td>Mass propagation through shoot-tip culture</td>
<td>Lee (1986)</td>
</tr>
<tr>
<td>H75-8776</td>
<td>Micropropagation through apical meristem culture</td>
<td>Nagai (1986)</td>
</tr>
<tr>
<td>RB735275</td>
<td>Micropropagation through shoot-tip culture is better than from indirect somatic embryogenesis</td>
<td>Lee (1987)</td>
</tr>
<tr>
<td>NC0310, NC0376, Nl2</td>
<td>Compared tissue culture-derived seed cane with conventional seed cane</td>
<td>Bailey and Bechet (1989)</td>
</tr>
<tr>
<td>CP 65-357, CP 70-321</td>
<td>Comparison of in vitro propagation efficiency by direct regeneration from leaf tissue and shoot-tip culture</td>
<td>Grisham and Bourg (1989)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>Shoot vigour and multiplication rate was maximum at 4% sucrose</td>
<td>Lal (1993)</td>
</tr>
<tr>
<td>Breeding lines, viz. 87-588, 87-693, 87-696</td>
<td>Plant regeneration from callus induced from immature inflorescences</td>
<td>Liu (1993)</td>
</tr>
<tr>
<td>CP 74-383</td>
<td>Shoot-tip culture induced phenotypic variation</td>
<td>Burner and Grisham (1994)</td>
</tr>
<tr>
<td>CoC 671</td>
<td>In vitro regeneration using 2- to 3-mm-long shoot tips</td>
<td>Dhumle et al. (1994)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>In vitro clonal propagation on modified MS media</td>
<td>Shukla et al. (1994)</td>
</tr>
<tr>
<td>BL4, AEC81-8415</td>
<td>Rapid multiplication of plantlets using apical meristem</td>
<td>Siddiqui et al. (1994)</td>
</tr>
<tr>
<td><em>Saccharum</em> sp.</td>
<td>High-frequency in vitro plant regeneration</td>
<td>Alam et al. (1995)</td>
</tr>
</tbody>
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### Table 1 (continued)

<table>
<thead>
<tr>
<th>Plant material/cultivar</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCo 310</td>
<td>Elimination of Fiji disease virus by thermotherapy and axillary bud culture</td>
<td>Wagih et al. (1995)</td>
</tr>
<tr>
<td>Co. Se. 92423, U.P.22</td>
<td>Crop raised from mericlone-derived seed cane gave higher yield than conventionally derived seed cane</td>
<td>Lai and Krishna (1997)</td>
</tr>
<tr>
<td>CoJ 64, CoJ 83, CoP 84-211</td>
<td>Protocol for commercial plant production from spindle explants</td>
<td>Gosal et al. (1998)</td>
</tr>
<tr>
<td>CoS 91269, CoS 687</td>
<td>Plantlet regeneration from callus cultures raised from young leaf explants</td>
<td>Lal and Singh (1999)</td>
</tr>
<tr>
<td>CoLK 8001</td>
<td>Effect of medium composition on in vitro establishment and growth of sugarcane meristem</td>
<td>Patel et al. (1999)</td>
</tr>
<tr>
<td>Twelve cultivars</td>
<td>Plantlets (80–100%) derived from meristem culture were free from sugarcane mosaic virus</td>
<td>Visessuwan et al. (1999)</td>
</tr>
<tr>
<td>Saccharum sp.</td>
<td>Addition of BAP and coconut water to MS media resulted in multiple shoot formation</td>
<td>Geetha et al. (2000)</td>
</tr>
<tr>
<td>Quarantine material</td>
<td>Regeneration of sugarcane yellow leaf virus-free plants through apical meristem culture of infected plants</td>
<td>Chatenet et al. (2001)</td>
</tr>
<tr>
<td>C-1051-73</td>
<td>Close relationship exists between shoot formation and phenolic excretion during micropropagation</td>
<td>Lorenzo et al. (2001a)</td>
</tr>
<tr>
<td>Saccharum sp.</td>
<td>Field performance of temporary immersion bioreactor-derived sugarcane plants</td>
<td>Lorenzo et al. (2001b)</td>
</tr>
<tr>
<td>Isd 28</td>
<td>In vitro shoot-tip culture of sugarcane</td>
<td>Baksha et al. (2002)</td>
</tr>
<tr>
<td>Isd 31</td>
<td>In vitro clonal propagation from callus induced on leaf sheath explants</td>
<td>Karim et al. (2002a)</td>
</tr>
<tr>
<td>Isd 16, Isd 28</td>
<td>Micropropagation from callus culture</td>
<td>Karim et al. (2002b)</td>
</tr>
<tr>
<td>SPF-213, CPF-237</td>
<td>Plantlet production from callus induced on young leaves</td>
<td>Niaz and Quraishi (2002)</td>
</tr>
<tr>
<td>CC 8527, CC 8215, R 830288, R 831592, R 830395, R 832065, R 840653, R 832276, G 75368, N 27, Q 159, Q 135, Q 155, Q 127, SP 80185, ROC 14, ROC 13, SP 803390, SP 792233</td>
<td>Production of disease-free plants from callus induced on young leaf rolls</td>
<td>Parmessur et al. (2002)</td>
</tr>
<tr>
<td>Saccharum sp.</td>
<td>Half MS media supplemented with elevated sucrose is better than full MS medium for rooting</td>
<td>Pawar et al. (2002)</td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Plant material/cultivar</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB83-5486, SP80-185</td>
<td>Sugarcane plants derived from meristem cultures exhibit somaclonal variation detected using molecular markers</td>
<td>Zucchi et al. (2002)</td>
</tr>
<tr>
<td>Isd 31</td>
<td>Effect of auxin, sucrose and pH on in vitro rooting of shoots induced from callus</td>
<td>Baksha et al. (2003)</td>
</tr>
<tr>
<td>Co 740, CoC 671</td>
<td>Elimination of sugarcane mosaic virus using chemotherapy and meristem culture</td>
<td>Balamuralikrishnan et al. (2003)</td>
</tr>
<tr>
<td>Q196^A, Q205^A, 85 N1205, 87A1413</td>
<td>Production of SmartSet^TM seedlings using immature leaf whorl explants</td>
<td>Geijskes et al. (2003)</td>
</tr>
<tr>
<td>CP 70-321, LCP85-384, HoCP 85-845</td>
<td>Yield components were similar for micropropagated plants derived from apical meristem and plants derived from conventional bud propagation</td>
<td>Hoy et al. (2003)</td>
</tr>
<tr>
<td>N32</td>
<td>Production of virus-free plants from immature leaf roll discs</td>
<td>Pillay et al. (2003)</td>
</tr>
<tr>
<td>HSF-240, SPF-213, SPF-234, CP43/33, CP77/400, CPF237</td>
<td>Micropropagation through apical and axillary buds</td>
<td>Cheema and Hussain (2004)</td>
</tr>
<tr>
<td>Erianthus 3854, SES 089</td>
<td>Micropropagation through bud culture</td>
<td>Razi-ud-din Shah et al. (2004)</td>
</tr>
<tr>
<td>CP 84-1198</td>
<td>In vitro plant regeneration from sugarcane seed-derived callus</td>
<td>Chengalrayan et al. (2005)</td>
</tr>
<tr>
<td>CoJ 64, CoJ 83</td>
<td>Tissue culture-raised plants were superior in agro-morphological and quality traits as compared to conventionally raised plants</td>
<td>Lal and Singh (2005)</td>
</tr>
<tr>
<td>N14, N27, N30, N32, NCo376</td>
<td>Simultaneous removal of viruses, bacteria from diseased plants and large-scale micropropagation through NOVACANE®</td>
<td>Snyman et al. (2005)</td>
</tr>
<tr>
<td>CoJ 83</td>
<td>Direct plant regeneration from young leaf segments</td>
<td>Gill et al. (2006)</td>
</tr>
<tr>
<td>NIA-2004, BL4, NIA-98, AEC82-223</td>
<td>Effect of sucrose and growth regulators on micropropagation</td>
<td>Khan et al. (2006)</td>
</tr>
<tr>
<td>Plant material/cultivar</td>
<td>Remarks</td>
<td>References</td>
</tr>
<tr>
<td>------------------------</td>
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<tr>
<td><em>Saccharum</em> sp.</td>
<td>Transverse thin cell layer culture system from young leaf spindle rolls for mass propagation of commercial varieties</td>
<td>Lakshmanan et al. (2006)</td>
</tr>
<tr>
<td>S-3807/99</td>
<td>Plantlet regeneration through callus culture induced on young meristematic leaf sheath explants</td>
<td>Ramanand et al. (2006)</td>
</tr>
<tr>
<td>Co 89003, Co 91010, Co 96258, Co 97017, CoP 84211, CoP 84212, CoP 90223, CoS 767</td>
<td>Effect of genotype on micropropagation using shoot-tip explants</td>
<td>Singh et al. (2006)</td>
</tr>
<tr>
<td>CoJ 64</td>
<td>The tissue culture-raised plants were better in cane height, number of buds, cane yield and sugar recovery as compared to conventionally raised plants</td>
<td>Sood et al. (2006)</td>
</tr>
<tr>
<td>CoS 99259</td>
<td>Spacing of 90 cm × 60 cm was most suitable for transplanting tissue-cultured plantlets</td>
<td>Ramanand et al. (2007)</td>
</tr>
<tr>
<td>Isd 32</td>
<td>In vitro mass propagation through shoot tips and folded leaves culture</td>
<td>Roy and Kabir (2007)</td>
</tr>
<tr>
<td><em>S. officinarum</em> L.</td>
<td>Micropropagation through axillary buds</td>
<td>Warakagoda et al. (2007)</td>
</tr>
<tr>
<td>Co 419, Co 740, Co 6907, Co 7219, Co 7717, Co 8014, Co 8021, Co 8122, Co 8208, Co 85007, Co 85019, Co 86010, Co 86032, Co 86249, Co 87025, CoC 671, CoC 86062, CoC 90063, 85 R 186, CoJ 64</td>
<td>Micropropagation through meristem tip culture for seed production</td>
<td>Jalaja et al. (2008)</td>
</tr>
<tr>
<td>HSF-240, CP-77- 400, CPF-237</td>
<td>Rapid micropropagation by shoot-tip culture</td>
<td>Khan et al. (2008)</td>
</tr>
<tr>
<td><em>S. officinarum</em> L.</td>
<td>The regenerated plants did not exhibit any variation from each other as well as from the mother plant on the basis of molecular markers</td>
<td>Lal et al. (2008)</td>
</tr>
<tr>
<td>HSF-243, HSF-245</td>
<td>Somatic embryogenesis and plant formation in sugarcane</td>
<td>Naz et al. (2008)</td>
</tr>
<tr>
<td>N19, N23, N25, N32, N40, N41</td>
<td>Micropropagation of sugarcane via NOVACANE® using apical leaf rolls</td>
<td>Snyman et al. (2008)</td>
</tr>
<tr>
<td>SP-241</td>
<td>Efficient regeneration from meristematic explant callus through inclusion of amino acids in regeneration medium</td>
<td>Asad et al. (2009)</td>
</tr>
<tr>
<td>Thatta-10</td>
<td>Optimized protocol for callus induction, regeneration and acclimatization</td>
<td>Ather et al. (2009)</td>
</tr>
<tr>
<td>Nayana</td>
<td>Rapid in vitro micropropagation through callus culture from young meristem explants</td>
<td>Behera and Sahoo (2009)</td>
</tr>
</tbody>
</table>
### Table 1 (continued)

<table>
<thead>
<tr>
<th>Plant material/cultivar</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoC-671</td>
<td>Direct shoot regeneration without intervening callus phase by using shoot-tip culture</td>
<td>Biradar et al. (2009)</td>
</tr>
<tr>
<td>Q117, Q165, Q205</td>
<td>Development of a temporary immersion system (RITA®) for mass production of sugarcane</td>
<td>Mordocco et al. (2009)</td>
</tr>
<tr>
<td>NCo 310</td>
<td>Plant regeneration from in vitro decontaminated mature axillary bud culture</td>
<td>Wagih et al. (2009)</td>
</tr>
<tr>
<td>CoC 671</td>
<td>Effect of the different auxins and cytokinins on callus induction, shoot regeneration and root regeneration induced from innermost leaf whorls</td>
<td>Gopitha et al. (2010)</td>
</tr>
<tr>
<td>Saccharum sp.</td>
<td>Influence of hormonal supplementations on callus induction, somatic embryoid induction and plantlet regeneration</td>
<td>Jahangir et al. (2010)</td>
</tr>
<tr>
<td>NCo 376</td>
<td>Elimination of <em>sugarcane mosaic virus</em> and sugarcane yellow leaf virus using thermotherapy and apical meristem culture</td>
<td>Ramgareeb et al. (2010)</td>
</tr>
<tr>
<td>Saccharum sp.</td>
<td>Mass propagation via shoot-tip culture</td>
<td>Dash et al. (2011)</td>
</tr>
<tr>
<td>GT54-9 (C9)</td>
<td>Plant regeneration through direct organogenesis and indirect somatic embryogenesis using young leaf segments</td>
<td>Eldessoky et al. (2011)</td>
</tr>
<tr>
<td>Co 94032, CoC 671, Co 86032, SNK 754, SNK 61, SNK 44</td>
<td>Thidiazuron-induced callus formation, somatic embryogenesis and plant regeneration using leaf explants of different varieties</td>
<td>Malabadi et al. (2011)</td>
</tr>
<tr>
<td>CoS 96268</td>
<td>Cost reduction in sugarcane micropropagation through direct adventitious regeneration and ex vitro rooting</td>
<td>Pandey et al. (2011)</td>
</tr>
<tr>
<td>Saccharum sp. hybrid</td>
<td>Development of sugarcane streak mosaic virus-free plants from infected plants using meristem-tip culture</td>
<td>Reddy and Sreenivasulu (2011)</td>
</tr>
<tr>
<td>CoS 96258, CoS 99259</td>
<td>Effect of in vitro environmental conditions on micropropagation</td>
<td>Sengar et al. (2011)</td>
</tr>
<tr>
<td>Saccharum sp.</td>
<td>Elimination of sugarcane grassy shoot disease through apical meristem culture</td>
<td>Tiwari et al. (2011)</td>
</tr>
</tbody>
</table>

(continued)
The actively growing sugarcane tops are excised from 3-month-old field-grown healthy mother plants. The tops (Fig. 1a) can be collected from primary shoots or secondary axillary shoots, and the outer leaf sheaths of tops are removed one after the other to obtain spindles of about 6 cm length (Fig. 1b) by giving cuts at both ends.
Sterilization of Spindles and Preparation of Explants

The spindles (six in number) are rinsed in Teepol detergent solution contained in a culture vessel (500 ml) for 2 min for eliminating wax from leaf sheaths and then washed under running tap water. Thereafter, the surface sterilization of spindles is done in a laminar air flow cabinet by treating with 5% (v/v) sodium hypochlorite solution for 30 min with vigorous manual shaking followed by washing thrice in sterile distilled water. The spindle ends and an outer leaf whorl are removed using sterilized forceps and scalpel by giving gentle cuts in a longitudinal fashion so that the inner leaf layers of spindle are not damaged. The procedure is repeated till tender spindles are obtained, which are transversely cut to obtain 1.0-cm-long spindle leaf segments referred to as explants. The forceps and scalpel are made red hot and cooled in ethanol after culturing each spindle.

Inoculation and Incubation of Spindle Leaf Segments

The explants are cultured on semi-solid MS medium supplemented with growth regulators and incubated under aseptic laboratory conditions at a temperature of 25 ± 2 °C, relative humidity of 60–80% and light intensity of 5000 lux till the appearance of new shoots or callus. Initially, the growth is slow and it takes about 3–4 weeks for new shoots to appear (Fig. 1c). The explants are slightly embedded into the medium during inoculation to ensure accessibility of the nutrients to the explants. In some sugarcane varieties, the cut ends of leaf segments secrete phenols, which are oxidized by plant phenol oxidases; the oxidation products hinder the uptake of nutrients by the explants resulting in their death. Incorporation of activated charcoal in the medium avoids the browning problem due to release of phenols. Otherwise, the explants are shifted on fresh medium after 1 week of culturing. In case browning reoccurs, another shifting on fresh medium is carried out. The lowermost nodal portion of the spindle is retained and cultured on a different medium to obtain shoot formation from apical buds; axillary buds can also be used for shoot regeneration in vitro; however, it is difficult to excise them.

2.4.2 Shoot Multiplication (Stage 2)

This is the actual multiplication stage in which developing shoot cultures from stage 1 are excised, divided and subcultured aseptically in separate culture vessels containing fresh medium for shoot multiplication (Fig. 1d). This step indeed is the game of cytokinins, and there is about ten times shoot multiplication (Fig. 1e) per cycle of 2 weeks depending on the variety. A variety showing high rate of shoot multiplication may be required to be subcultured once a week. Likewise, frequent subculturing is done if medium gets exhausted or there is secretion of phenols or drying of leaves. A total of 26 shoot multiplication cycles can be carried out in 1 year resulting into production of lacs of propagules. However, according to tissue
culture standards of Department of Biotechnology, Government of India, New Delhi, one should not carry out more than seven shoot multiplication cycles in sugarcane to avoid somaclonal variation. Some shoot cultures may show vitrified (ball-like) growth due to intense shoot multiplication; this can be prevented by reducing the number of subculture cycles and lowering concentration of cytokinin in the medium. In this stage, due to high cytokinin level in the culture medium, roots are not formed on the shoots.

2.4.3 Induction of Roots and Hardening of Plantlets In Vitro (Stage 3)

The well-elongated shoots/shoot clumps from stage 2 are taken and cultured on semi-solid or liquid rooting medium to obtain root formation (Fig. 1f). Prior to transfer on rooting medium, the dry leaves are taken off from the shoot clumps, and green clumps are divided so that each clump being transferred contains five to seven shoots. Care is taken not to injure the base of shoots while removing the dry leaves or dividing the shoot clumps. Roots emerge in a period of 10–15 days; once these are 5–10 mm in length, the plantlets are taken out from the culture vessels and washed thoroughly under slow-running tap water to remove the culture medium adhered to the roots. The plantlets are then hardened by keeping on water-moist cotton in plastic trays that are placed in the incubation room with daily change of water for 3–4 days (Fig. 1g).

2.4.4 Transfer of Plantlets to Soil (Stage 4)

The hardened plantlet clumps are separated, and individual plantlets are transferred to potting mixture (field soil + farm yard manure in 3:1 ratio) in polythene bags kept in the greenhouse (Fig. 2a). The humidity is maintained in the greenhouse for the first 2 weeks during which new leaves emerge. The initial growth of plants is facilitated by spraying urea (0.05% w/v) once a week and mixture of FeSO₄ and ZnSO₄ (0.1% w/v) once in 2 weeks. The plants are kept in the greenhouse for 45 days before delivery to farmers or transfer to field (Fig. 2b). The soil-grown plants branch profusely with thin tillers indicating the residual effect of cytokinin in the shoot multiplication medium. It is for this reason that the tissue-cultured plants are used for seed multiplication and not for commercial use. We have so far developed three micropropagation protocols for mass multiplication of elite planting material (Table 2).

2.5 Transplanting of Micropropagated Plants in the Field

The micropropagated plants are grown at a plant-to-plant distance of 60 cm and row-to-row distance of 75 cm in a disease-free field without taking off the root-soil mass. The irrigation is applied immediately after transplantation, and the subsequent irrigations are applied at regular intervals. There is more than 95% survival of
the hardened plants under field conditions if proper care is taken. The formation of new leaves on the transplanted plants gives an indication of their successful establishment in the field. First dose of nitrogen (20 kg/acre) is applied after 3 weeks of transplantation along with irrigation, and remaining amount of nitrogen is applied in two doses at monthly intervals (Sandhu et al. 2009). Care is taken to rogue out the off-type plants that are observed only during the first year. The plants grow uniformly, possess thin cane stalks and are free from pathogens and insect pests (Fig. 2c). Gosal et al. (1998) observed that the micropropagated plants displayed better cane number and mean cane yield in comparison with the conventionally propagated crop. All intercultural operations are performed on time, and canes obtained from tissue-cultured plantlets are harvested within a period of 10 months.

### 2.6 Production of Commercial Seed

The stalk canes obtained from the micropropagated plants growing in the field form the breeder seed. The breeder seed canes are cut into two-budded or three-budded setts and sown to obtain foundation seed; the canes are again cut into setts which are
Table 2  Efficient protocols developed for micropropagation of sugarcane in our laboratories

<table>
<thead>
<tr>
<th>Explant</th>
<th>Media used</th>
<th>% Efficiency</th>
<th>No. of plants formed</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spindle leaf explants (0.5–1.0 cm length)</td>
<td>MS salts +0.5 mg/l IAA + 0.5 mg/l BAP + 0.5 mg/l kin +3% (w/v) sucrose +0.8% (w/v) agar for establishment of shoot cultures; liquid MS + 0.5 mg/l BAP + 0.5 mg/l kin +3% (w/v) sucrose for shoot multiplication; liquid half strength MS for shoot elongation; liquid MS + 5 mg/l NAA + 7% (w/v) sucrose for root induction; tap water for hardening in vitro; field soil in polythene bags for transfer of plants</td>
<td>Culture establishment, 75%; root induction, 95%; survival in soil, 90%</td>
<td>More than 15,000 involving one shoot multiplication cycle</td>
<td>Gosal et al. (1998)</td>
</tr>
<tr>
<td>Spindle leaf explants (1.0–1.5 cm length)</td>
<td>MS salts +5 mg/l NAA + 0.5 mg/l kin +3% (w/v) sucrose +0.8% (w/v) agar for establishment and proliferation of shoot cultures and root induction; tap water for hardening in vitro; field soil and fly ash (1:1) in polythene bags for transfer of plants</td>
<td>Culture establishment, 74.37–83.12%; survival in soil, 95%</td>
<td>–</td>
<td>Gill et al. (2006)</td>
</tr>
<tr>
<td>Spindle leaf explants (1.0 cm length and 0.5 cm diameter)</td>
<td>MS salts +5 mg/l NAA + 0.5 mg/l kin +100 mg/l myo-inositol +3% (w/v) sucrose +0.8% (w/v) agar for culture initiation; MS salts +0.5 mg/l BAP + 0.5 mg/l kin +0.5 mg/l IAA + 100 mg/l myo-inositol +3% (w/v) sucrose +0.8% (w/v) agar for shoot multiplication; liquid MS + 0.5 mg/l BAP + 0.5 mg/l GA3 + 100 mg/l myo-inositol +3% (w/v) sucrose for shoot multiplication and elongation; liquid MS + 3 mg/l IBA + 3 mg/l NAA + 200 mg/l myo-inositol +3% (w/v) sucrose for root induction; tap water for hardening in vitro; farmyard manure + field soil (1:3) in polythene bags for transfer of plants</td>
<td>Culture establishment, 97%; root induction, 100%; survival during hardening, 94%; survival in soil, 97%</td>
<td>1,01,434 involving five shoot multiplication cycles</td>
<td>Kaur and Sandhu (2014)</td>
</tr>
</tbody>
</table>
then used to raise commercial crop (Sinha 2006). The breeder seed grown on an area of 0.125 acre provides planting material for 1–1.5 acres in the second year and for 15–20 acres in the third year (Kaur et al. 2014). The breeder seed should be changed after every 4 years of propagation (Jalaja et al. 2008).

2.7 Precautions to Be Taken During Micropropagation

The following points should be kept in mind to maintain the quality of micropropagated plants:

• In order to raise genetically pure stock plants, the plant material should be procured from the breeder or the research institute that has developed the variety.
• The tops should be collected only from healthy stock plants.
• The tissue culture laboratory should be accredited by a competent authority to ensure that the infrastructure is according to the guidelines.
• The protocol to be followed for micropropagation should not result in somaclonal variation in the regenerated plants.
• Only well-established plants with a good shoot and root system should be handed over to the end users.
• The tissue-cultured plants should be tested for genetic purity through molecular markers and indexed to be virus-free through ELISA.

2.8 Scenario of Sugarcane Micropropagation

Production of superelite planting material of sugarcane through micropropagation is now done in India, Pakistan, Australia, the Philippines, Bangladesh, Indonesia, Thailand and Sri Lanka. India has more than 100 commercial micropropagation units with a yearly production capacity of 0.2–5.0 million plants (Singh and Shetty 2011). The Department of Biotechnology (DBT), Government of India, has recognized a total of 95 commercial tissue culture units under the National Certification System for Tissue Culture Raised Plants (NCS-TCP) located in states of Assam, Andhra Pradesh, Bihar, Chattisgarh, Gujarat, Haryana, Himachal Pradesh, Karnataka, Maharashtra, Madhya Pradesh and Orissa that are involved in the micropropagation of potato, apple, bamboo, sugarcane, vanilla, banana, black pepper and citrus (www.dbtncstcp.nic.in). DBT along with Biotech Consortium India Limited, New Delhi, has made available handy information on norms and procedure of sugarcane micropropagation (www.dbtncstcp.nic.in). The University of Agricultural Sciences, Bangalore, has been accredited for virus indexing and genetic fidelity of tissue culture-raised plants including sugarcane under NCS-TCP. The micropropagation-based seed production technology is also widely accepted by the farmers (Kaur and Sandhu 2014). In general, it is the responsibility of the regional
sugar mills to establish and popularize this technology among the farmers so that the superior planting material is available in abundant amount contributing to enhanced sugarcane productivity and income of the farmers. The micropropagation-raised crop can be used for multiratooning due to freedom from sett-borne diseases (Jalaja et al. 2008).

2.9 Significance of Micropropagation

The micropropagation of sugarcane offers the following advantages:

- A new variety can be quickly multiplied in large numbers in a short period of time through micropropagation (due to 1:10 shoot multiplication rate per cycle of 2 weeks each), thus making it possible to disseminate the seed material of the new variety to the farmers’ fields and diversify the cropping pattern. On the contrary, it takes several years to produce the same number of plants through conventional vegetative propagation method.
- Only a small number of starting plant material (apical tops) is required for generating a large number of clonal plants.
- Disease-free plants can be obtained by culturing the shoot apices/apical buds of diseased plants as the apices/buds are devoid of pathogens. Hence, old varieties/clones can be rejuvenated for improving their yield and quality. Thus, micropropagation helps to increase sugarcane production potential in a sustainable manner.
- The micropropagated plants obtained through axillary bud proliferation approach and direct adventitious shoot regeneration approach have high genetic fidelity and are true to type, i.e. identical to mother plant.
- The in vitro-maintained mother stocks can be quickly proliferated at any time of the year, thus providing year-round nursery for different varieties and exhibiting independence from seasonal and raw material availability constraints.
- The micropropagated plants are easy to transport, in contrast to setts which are voluminous posing difficulty in transportation.
- The field-grown micropropagated plants exhibit better agronomic and biochemical characters such as cane yield, sucrose, juice content and quality as compared to conventionally propagated plants. Gosal et al. (1998) reported that the micropropagated plants revealed up to 22.9% increase in mean cane yield per plot in comparison with conventionally propagated sugarcane using three-budded setts.
- The micropropagated plants grown in the field display better tillering (an after-effect of growth regulators added in the culture medium) and hence provide more seed material (setts) for planting next clonal generation as compared to that provided by conventionally propagated plants. Gosal et al. (1998) reported an increase up to 44.96% in the number of canes per plot of micropropagated plants and 22.9% increase in mean cane yield per plot as compared to conventionally propagated sugarcane using three-budded setts. Further, the setts obtained from
micropropagated plants exhibit high germination frequency since these are disease-free.

- The commercial crop raised from tissue culture-derived seed display synchronous cane maturity, thus enabling harvesting at a particular time.
- There is fast interstate/international exchange of plant material as it is free from pathogens and insects; thus, the period of quarantine is bypassed and time is saved.
- Artificial seeds can be produced by encapsulating the somatic embryos, buds, etc., in a hydrogel for supply to the farmers.
- The elite transgenic events/plants obtained through genetic transformation can be multiplied through micropropagation, thus providing enough clonal plants for precise characterization and transfer to greenhouse.

### 2.10 Problems Associated with Micropropagation

Various problems are encountered while carrying out in vitro micropropagation, an account of which is given below:

- For carrying out micropropagation, proper infrastructure, costly equipments and skilled workers are required; consequently, the initial investment cost is very high.
- The tissue culture regenerated plants may contain somaclonal variants (Rani and Raina 2000; Zucchi et al. 2002) at a low frequency. The somaclones have abnormal morphology affecting crop uniformity and productivity; thus, in vitro shoot multiplication through callus cultures should be avoided. The axillary branching approach and direct adventitious shoot regeneration from young leaf segments ensures the production of genetically stable and true-to-type plants. The number of shoot multiplication cycles should be kept to a minimum so that there is no problem of hyperhydration (morphological, physiological and metabolic defects) in the regenerated plants.
- The field-grown micropropagated plants exhibit epigenetic changes such as excessive tillering, slender canes and short internodes due to exogenous application of cytokinin in the culture medium. As a result, the breeder seed so produced is not morphologically identical to the mother plant; however, these changes are temporary, and plants may relapse to the regular phenotype quite easily (Smulders and de Klerk 2011). Lourens and Martin (1987) observed a few transient morphological changes in the sugarcane plants raised from 1.5-month-old callus due to epigenetic effects, which disappeared in the second year of their vegetative propagation.
- Contamination in the cultures appearing soon after culturing due to latent bacteria and fungi could result in considerable losses in a short duration. To avoid systemic infection, explants are treated with an antibiotic solution before culturing, or the culture medium is supplemented with an antibiotic.
• Many genotypes of sugarcane, e.g. CoJ 85, are loaded with polyphenols (e.g. gallic acid, p-coumaric acid, etc.); these are released from the cut ends of explants and oxidized by polyphenol oxidases (Chawla 2009). The oxidation products are responsible for browning of the culture medium and obstructing the uptake of nutrients by the explants resulting in their death. The majority of phenolic excretion occurs after 11–20 days of culturing and prior to shoot formation period of 21–30 days (Lorenzo et al. 2001a). Incorporation of antioxidants such as activated charcoal or ascorbic acid in the medium avoids the browning problem due to release of phenols. Otherwise, the explants are shifted onto fresh medium as and when the phenols are excreted in the medium. In case browning reoccurs, another shifting of explants on fresh medium is carried out. Incubation of the explants under partial light or dark conditions also prevents browning problem as the formation of oxidation products takes place under light conditions (Chawla 2009).

• Per unit plant production cost is high that should be reduced by the incorporation of low-cost options during micropropagation (Kaur and Sandhu 2014). Such low-cost options are incorporated during sugarcane micropropagation in Cuba (Ahloowalia 2004). Automation of micropropagation using bioreactors can also help in lowering per plant production cost by cutting the labour costs; however, asepsis is necessary for making bioreactor-based micropropagation a success. Further, the farmers should be encouraged to buy planting material for a small area and produce their own seed.

In conclusion, micropropagation has proven a practical and victorious technology for quick production of quality and true-to-type sugarcane seed in large numbers. The newly bred varieties, old varieties popular among farmers and diseased varieties – all can be benefitted from micropropagation in terms of quick multiplication, rejuvenation and freedom from pathogen, respectively. The use of micropropagated plantlets by farmers helps in enhancing crop productivity in a sustainable manner. The Government of India has identified micropropagation as the main concern area for further research, development and commercialization.

3 Somatic Embryogenesis

Somatic embryogenesis is defined as the process of embryo initiation and development from somatic cells or plant tissues grown under in vitro conditions, by undergoing some changes in the developmental pathways under the influence of plant growth regulators. During this developmental process, the differentiated cells undergo dedifferentiation by active cell division, reprogram their physiological and metabolic pathways by changing gene expression (Yang and Zhang 2010; Bajpai et al. 2016) and develop into proembryonic cell mass, leading to formation of somatic embryos, their maturation and regeneration (Hussein et al. 2006; Widuri
et al. 2016). Somatic embryo is a bipolar structure that has no vascular connection with the parental tissue and undergoes different stages typical for zygotic embryo development (Sharp et al. 1980). The underlying principle of somatic embryogenesis is cellular totipotency, which demonstrates how somatic cells undergo genetic changes for complete plant development without fertilization (Zimmerman 1993). Besides the occurrence of somatic embryos, new embryos emerge from primary somatic embryos called as secondary somatic embryos that have several applications in plant biotechnology including rapid and mass in vitro clonal propagation of plants, genetic transformation, induction of mutations and cryopreservation (Litz and Gray 1995; Raemakers et al. 1995). The phenomenon of somatic embryogenesis has been reported in some plant species of angiosperms and gymnosperms including sugarcane (Raza et al. 2012; Widuri et al. 2016). In tissue culture, the initiation and development of somatic embryos were first reported by Steward et al. (1958) in Daucus carota. Sharp et al. (1980) illustrated two routes to somatic embryogenesis, viz. direct somatic embryogenesis and indirect somatic embryogenesis. In direct somatic embryogenesis, the somatic embryos initiate directly from an explant in the absence of callus phase under the influence of a stimulator or removal of an inhibitor; such explant cells are referred to as ‘pre-embryogenic determined cells’ (PEDC) as these are committed to embryonic development and need only to be released. PEDC are present in embryonic tissues, e.g. scutellum of cereals. In indirect somatic embryogenesis, explant cells first proliferate to form callus, e.g. leaf of coffee; this is followed by embryo development near the callus surface or inside the callus (Widuri et al. 2016). Such explant cells are called as ‘induced embryogenic determined cells’ (IEDC). In majority of instances, somatic embryogenesis occurs through indirect method.

### 3.1 Regulation of Somatic Embryogenesis

For a somatic cell to develop into a complete plant through tissue culture, there is a prerequisite to convert the highly differentiated cell into an undifferentiated stage, i.e. callus, which can be achieved by using different growth regulators in the culture medium. Generally, an auxin is used for callus induction, e.g. in members of Poaceae family to which sugarcane belongs; 2,4-dichlorophenoxyacetic acid (2,4-D) is the most potent auxin used for callus induction. Several studies have reported high concentration of 2,4-D (3–4 mg/l) to be the best for callus initiation in sugarcane, whereas low concentration of 2,4-D or a combination of 2,4-D and cytokinin helps in the development of somatic embryos in the callus (Naz et al. 2008; Jahangir et al. 2010). Ho and Vasil (1983a) used 0.5 mg/l of 2,4-D for obtaining maximum frequency of embryogenic callus; besides nitrogen in ammonia form was observed to be better than the nitrate form for embryogenic callus development. Brisibe et al. (1994) observed that dicamba was better than 2,4-D and NAA in maintaining embryogenic potential of callus cultures for a long period. They reported that
somatic embryogenesis can also be prolonged by supplementing the culture medium with high concentration of maltose (6%) or corn syrup (6–9%). Some other culture medium factors like source of carbohydrate, proline, activated charcoal, abscisic acid (ABA) and antibiotic (cefotaxime) have been found to be related to somatic embryo development in sugarcane. Replacement of sucrose (3%) with maltose (3%) in MS medium resulted in high percentage of embryogenic calli (Gill et al. 2004) with increased callus mass (Kaur and Kapoor 2016); this is because maltose is slowly metabolized in the cultures and remains available to the cells for a longer period as compared to other carbohydrate sources (Orshinsky et al. 1990). Similar observations on increased frequency of embryogenic calli have been noted in separate experiments with proline (560 mg/l) alone (Gill et al. 2004) and proline in combination with maltose (Kaur and Kapoor 2016). Proline provides buffering to the medium and resists any change in the pH of medium during culture. Addition of activated charcoal (2.0 g/l) in the culture medium has also been reported to increase the embryogenic response in callus cultures (Kaur and Kapoor 2016) by reducing the effect of phenolic substances released from the cut ends of explants into the medium. ABA along with some other growth adjuvants like corn syrup and casein hydrolysate affect both embryogenic callus formation and frequency of embryogenesis in long-term callus cultures of sugarcane (Brisibe et al. 1994; Gill et al. 2004). Although ABA is a growth retardant, but its effect on somatic embryogenesis is profound; it was observed that sugarcane callus grew as well as differentiated in presence of ABA in the culture medium (Kaur and Kapoor 2016). Desiccation conditions caused by addition of high concentration of agar (10 g/l) in the culture medium induced compact and whitish embryogenic callus in sugarcane (Himanshu et al. 2000). Liu (1993) observed that cold treatment of sugarcane callus cultures induced from inflorescence explants at 13 °C for a few days improved their embryogenic potential. Age of explant is also an important factor determining the embryogenic potential of callus cultures.

3.2 Effect of Source of Explant on Somatic Embryogenesis

Generally, callus can be produced from any part of the plant. In sugarcane, practically all plant parts produce callus; however, immature leaves (Ho and Vasil 1983a) and young inflorescences produce embryogenic callus (Table 3). Ho and Vasil (1983a) divided callus into two types: (i) hard, compact and embryogenic and (ii) soft, friable, translucent and non-embryogenic. The embryogenic callus induced from leaf sheath was found to be better than that induced from leaf blade. Further, the leaf explants taken from field-grown and in vitro plants showed different percentages of non-embryogenic and embryogenic calli. In the leaf explants prepared from field-grown plants, callus initiation was observed after 7 days of culturing; the non-embryogenic calli turned into 70% non-embryogenic callus and 30% embryogenic callus after 45 days of culturing. In case of leaf explants taken from
in vitro-raised plants, origin of callus was observed after 10 days of culturing, and 60-day-old cultures consisted of 60% non-embryogenic callus and 40% embryogenic callus. Further, the cell suspension cultures were established from the two types of callus cultures. The embryogenic cells were small (30 μm diameter), rich in cytoplasm, actively dividing, had prominent nucleus and conspicuous starch grains. In contrast, the non-embryogenic cells were large, elongated (70 μm × 30 μm) and vacuolated with scanty cytoplasm and a few starch grains.

### 3.3 Evidence for Somatic Embryogenesis and Proteins Produced from Callus

The experimental evidence for occurrence of somatic embryogenesis in sugarcane was first given by Ahloowalia and Maretzki (1983) and Ho and Vasil (1983a, 1983b). Ho and Vasil (1983a) reported somatic embryogenesis in callus cultures, cell suspension cultures and protoplasts. Histologically, callus induction in sugarcane can be divided into two types, viz. mucilaginous and nodular (de Alcantara et al. 2014). The mucilaginous callus is soft and watery, does not undergo embryogenesis or develop into shoots and turns necrotic. On the other hand, cells of whitish nodular callus have dense cytoplasm with high nuclear to cytoplasmic ratio revealing their meristematic nature. These cells develop into somatic embryos followed by regeneration of shoots. Different stages of somatic embryogenesis in sugarcane include the development of proembryos, which divide and turn into globular-stage embryos.

<table>
<thead>
<tr>
<th>Morphogenic pathway</th>
<th>Explant</th>
<th>Plant regeneration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf roll</td>
<td>R</td>
<td>Lee (1987)</td>
</tr>
<tr>
<td></td>
<td>Leaf roll</td>
<td>R</td>
<td>Meyer et al. (2009)</td>
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<td>Apical meristem</td>
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<td>Ramgareeb et al. (2010)</td>
</tr>
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<td></td>
<td>Shoot apices from in vitro plants</td>
<td>R</td>
<td>Garcia et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Immature inflorescence</td>
<td>R</td>
<td>Liu (1993), Desai et al. (2004), Snyman et al. (2006)</td>
</tr>
<tr>
<td>Direct somatic embryogenesis</td>
<td>Immature inflorescence</td>
<td>R</td>
<td>Desai et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>Leaf roll with pre-emergent inflorescence</td>
<td>R</td>
<td>Snyman et al. (2006)</td>
</tr>
</tbody>
</table>

*a R Plant regeneration, T field transfer, A phenotypic or genotypic analysis
enclosed by protoderm. The globular embryos either separate themselves from the rest of the callus cells or remain attached to the surface of callus by suspensor. The globular embryos develop into scutellum by formation of scutellar node. The scutellar cells are rich in cytoplasm, irregular in shape, and divide further to form coleoptiles, apical shoot and root vascular system. The embryogenic callus of sugarcane has more protein than non-embryogenic callus; this could be related to their cytological characters. The cells of embryogenic calli are small and actively dividing and have dense cytoplasm with prominent nuclei, so their metabolic activity is higher than the non-embryogenic cells; consequently, these have higher level of proteins, mRNAs and other cytoplasmic components as compared to non-embryogenic cells. The electrophoretic analysis of protein pattern showed polypeptides of 38–44 kDa and another polypeptide of 23 kDa in embryogenic calli; however, these polypeptides were absent, and another polypeptide of 35 kD was present in non-embryogenic calli. Thus, sugarcane has a complex protein pattern and could be due to the effect of 2,4-D on embryogenesis (Oropeza et al. 2001). In another study, activated charcoal was used in the culture medium at different concentrations (0.0, 0.75, 1.5 and 2.0 g/l). The data on somatic embryo development and protein expression was recorded at 0 and 21 days using shotgun proteomic analyses. Use of activated charcoal (1.5 g/l) resulted in faster maturation of somatic embryos in embryogenic callus and showed no effect on non-embryogenic callus. Embryogenic callus showed 65 exclusive proteins on day 0 and 14 exclusive proteins on maturation at day 21. Non-embryogenic callus expressed 23 exclusive proteins on day 0 and 10 exclusive proteins after 21 days (Heringer et al. 2015).

3.4 Somatic Embryogenesis from Cell Suspension Cultures

Cell suspension cultures are prepared by growing callus cultures in liquid medium under constant agitation and maintained by selection of embryogenic cells; this is accomplished by allowing the cell suspension cultures to settle for some time, removing the supernatant followed by replacing it with fresh culture medium every 7 days. The cell suspension growth is measured by the number of embryogenic cells, packed cell volume and cell fresh and dry weight (Falco et al. 1996). Ho and Vasil (1983b) were the first to report plant regeneration from embryogenic cell suspension cultures of sugarcane. The small, actively dividing cells of suspension cultures became embryogenic upon supplementing the medium with high concentration of sucrose (6–10%). The embryogenic potential of the suspension cultures was maintained by addition of coconut water and casein hydrolysate in the culture medium. Such suspension cultures produced embryogenic callus when plated on solidified MS medium supplemented with coconut water, casein hydrolysate and 2,4-D; the callus subsequently showed plant regeneration when cultured on a suitable medium. Falco et al. (1996) observed plant regeneration only in juvenile cell suspension cultures and in culture media containing low or no 2,4-D.
3.5 **Somatic Embryogenesis and Cryopreservation**

Cryopreservation is the storage and preservation of cells, tissues and organs by immersion in liquid nitrogen (−196 °C). It is used for the conservation of plant genetic resources and as a technique for preserving the regeneration capacity of somatic embryos in cell suspension or callus cultures. In vitro conservation has several advantages over in vivo conservation, e.g. it saves space and time and can be used to conserve endangered species, sterile plants and in vitro material produced using tissue culture. Slow cooling (0.5–1 °C/min) up to 40 °C followed by immersion of samples in liquid nitrogen is usually employed for conservation of cell suspensions and embryogenic calli (Kartha and Engelmann 1994). During cryopreservation, there is no cell division, so the chances of somaclonal variation are limited, which otherwise increase with culture duration. With the establishment of tissue culture in sugarcane, considerable efforts were made to develop cryopreservation protocols for germplasm conservation using various explants like apeces of in vitro plantlets, cell suspensions and calli (Eksomtramage et al. 1992). During initial attempts, the success was limited to obtaining viable cultures but not in regenerating plants from the frozen suspension and callus cultures (Finkle and Ulrich 1982; Ulrich et al. 1984). Gnanapragasam and Vasil (1990) developed a procedure for successful regeneration of sugarcane plants with 92% efficiency from cryopreserved cells and reported that enrichment of cell suspension culture with embryogenic cells is important to get high plant regeneration during re-culture. Martinez-Montero et al. (1998) compared three vitrification techniques to preserve somatic embryos of sugarcane and concluded that there is a need to develop different cryopreservation protocols for different genotypes.

3.6 **Somatic Embryogenesis and Synthetic Seeds**

Somatic embryos encapsulated in hydrated or desiccated gel coating which helps in their protection and germination are called synthetic or artificial seeds. Besides somatic embryos, shoot tips and axillary buds can also be used for encapsulation. There is a great scope for the production of synthetic seeds in asexually propagated crops like sugarcane, where these can be used for large-scale production of better quality plants (Aamir et al. 2013), and thus synthetic seed technology is an advancement in micropropagation (Naik and Chand 2006). In sugarcane, the survival rate of synthetic seeds is controlled by the concentration of gelling matrix components, i.e. sodium alginate and calcium chloride, which affects the type, colour and quality of encapsulation. Treatment of somatic embryos with 3% sodium alginate and 100 mM calcium chloride for 15 min resulted in the formation of isodiametric and compact beads. Decrease in treatment time (10 min or less) formed fragile and soft beads, whereas increase in treatment time (20 min or more) resulted in hard and whitish
82

3.7 Somatic Embryogenesis and Plant Regeneration

Direct plant regeneration in sugarcane from spindle leaf culture occurs either through direct somatic embryogenesis or organogenesis (Gill et al. 2006; Lakshmanan 2006) and has decreased chance of somaclonal variation (Chowdhury and Vasil 1993) due to minimum culture duration and callus formation. The direct somatic embryogenesis or organogenesis system (Fig. 3) has become routine protocol for mass propagation (Kaur and Sandhu 2014) and genetic transformation (Taparia et al. 2012). Nickell (1964) was the first to report plant regeneration from callus cultures of sugarcane. The plants regenerated through callus formation

Fig. 3 Plant regeneration in sugarcane via direct somatic embryogenesis or organogenesis. (a) Cultured young leaf segments showing swelling and unwhorling during first week of incubation. (b, c). Stereo micrographs of direct shoot regeneration from cut ends of cultured segments. (d) Profuse direct shoot regeneration. (e) Profuse rooting from base of shoots during fifth week of incubation. (f) Tissue culture-derived hardened plantlets transferred to soil in the greenhouse. (g) Micropropagated plants in the field exhibiting normal growth and better tillering.

coloured beads, which hampered germination potential of somatic embryos (Aamir et al. 2013). Concerted efforts are required to develop synthetic seeds in sugarcane for propagation.
(Fig. 4) possess significant somaclonal variation (Vickers et al. 2005). However, the callus cells can be screened in vitro for various biotic and abiotic stresses to develop new resistant cultivars of sugarcane (Geijskes et al. 2003).

Thus, somatic embryogenesis reveals the totipotency of plant cells (Raghavan 1997) and has significant importance in combining efficient multiplication of desirable genotypes with genetic modification (Sharp et al. 1980) for sugarcane improvement.
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Micropropagation and Somatic Embryogenesis in Sugarcane

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