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RESEARCH ARTICLE

Effect of Activated Charcoal, Carbon Sources and Gelling Agents on Direct Somatic Embryogenesis and Regeneration in Sugarcane via Leaf Roll Segments

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Abstract Efficient plant regeneration is an essential prerequisite for a complete genetic transformation protocol. Direct somatic embryogenic cultures were established in commercial sugarcane varieties viz. CoJ 83 and CoH 119. Problem of browning of media was observed mainly in cultures of CoJ 83 as compared to CoH119. To study the effect of adsorption of phenolics by activated charcoal, two treatments were assayed in *cv*. CoJ 83 i.e. on Murashige and Skoog (MS) medium supplemented with α-Naphthalene acetic acid (NAA; 5 mgl^{-1}) and Kinetin (Kin; 0.5 mgl^{-1}) (control) and MS + NAA (5 mgl^{-1}) + Kin (0.5 mgl^{-1}) + charcoal (0.2% w/v) medium (treatment). In addition to control of browning of medium by phenolics, per cent somatic embryogenesis (SE) increased from 80.21 to 84.88 and per cent regeneration was also enhanced from 75.15 to 81.22 in sugarcane variety CoJ 83, when the medium was supplemented with 2,000 mgl^{-1} of activated charcoal (AC). Further, the effect of addition of different carbon sources i.e. sucrose and maltose in four different concentrations 20, 25, 30 and 35 gl^{-1} was studied and maximum per cent SE (98.35% in CoJ 83 and 93.12% in CoH 119; respectively) and maximum per cent regeneration (94.17% in CoJ 83 and 89.67% in CoH 119; respectively) was observed on MS + NAA (5 mgl^{-1}) and Kin (0.5 mgl^{-1}) medium supplemented with 25 gl^{-1} maltose. Direct SE and regeneration was also affected considerably by varying the gelling agent (agar or gelrite) and its concentration. Best results were observed with increases in per cent SE and regeneration on medium solidified with agar (1.2%) compared to gelrite, when used as a gelling agent. Thus, this fairly simple activated charcoal (2% w/v), maltose (25 gl^{-1}) and agar (1.2%) containing direct SE medium provided an alternative method for improving SE and hence, regeneration frequency of sugarcane.

Keywords *Saccharum* spp. · Somatic embryogenesis · Activated charcoal · Regeneration · Carbon sources · Gelling agent

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Introduction

Sugar industry is the second largest agro-based industry in India next to cotton and India is the second biggest producer next to Brazil in sugarcane with production of about 345 million metric tons (FAO Sugar Statistics 2010). Commercially, sugarcane is propagated from stem cuttings with each cutting or set having two or three buds. After the development of clone/variety, major bottleneck in spreading of clone or variety is slow propagation rate through conventional method, which takes years. After the concept of totipotency given by Haberlandt in 1902, it became clear that induction of cellular differentiation in vitro depends upon medium formulation, incubation conditions and genotype of the plant. Nickell (1964) first started the research on sugarcane tissue and cell culture in Hawaii. The use of indirect somatic embryogenesis (SE) i.e. through callus phase is common in sugarcane (Kaur 2004) but it leads to somaclonal variations, and hence the genetic fidelity of plants is changed. Therefore, an intervening callus phase is not desirable during micro propagation and genetic transformation process. Keeping in view the above facts, an attempt was made in the present investigation to study the different factors affecting direct

SE (without callus phase) and regeneration i.e. a single step process for a commercially grown high yielding sugarcane varieties, CoJ 83 and CoH 119, using leaf roll segments as explants.

During in vitro culture process in sugarcane, undesirable or inhibitory compounds such as excess phenolic metabolites are produced and it often influences the result. To get rid of browning of the media due to release of phenolics during sugarcane tissue culture, activated charcoal (AC) was added into the medium [MS + NAA (5 mgl^{-1}) + Kin (0.5 mgl^{-1})] and their effect on per cent SE and regeneration was observed. AC is composed of carbon arranged in a quasi graphitic form in small particle size. It is a porous and tasteless material and is distinguished from elementary carbon by removal of all non-carbon impurities and the oxidation of carbon surface (Mattson and Mark 1971). AC is often been used in plant tissue culture to improve cell growth and development (Pan and van Staden 1998). AC has been used for adsorption of phenolics in a number of studies (Fridborg et al. 1978; Horner et al. 1977; Theander and Nelson 1988; Weatherhead et al. 1978, Carlberg et al. 1983; Liu 1993 and Teixeria et al. 1994) and provides advantages like enhancing the embryogenic potential (Chee and Tricoli 1988) and enhancing rooting (Buter et al. 1993 and Dumas and Monteuuis 1995).

Carbohydrates are the sole energy source for in vitro plants. During in vitro conditions, cells and plants do not perform their normal photosynthesis in artificial light. Tissues that are green initially gradually lose their green pigments in culture and depend on an external source of carbon. The most common source of carbon in plant tissue culture medium is the sugar. It was observed in the previous studies conducted by Orchinsky et al. (1990) and Navarro et al. (1994) that the concentration and the type of carbon source affects the SE potential in wheat anther cultures. Thus, an investigation was carried out to study the effect of different carbon sources i.e. sucrose and maltose in the culture medium on direct SE and regeneration in sugarcane cvs. CoJ 83 and CoH 119. Agar and gelrite, extracted from red algae and bacteria, respectively, are commonly used gelling agents. Ideally, a gelling agent would be an inert constituent of the plant growth medium (Jain et al. 2009). Studies show, however, that the agent itself causes variations in plant growth responses on otherwise identical nutrient media (Beruto et al. 1999). Differences in the performance of gelling agents have been attributed to their variable physiochemical characteristics, such as nutrient diffusion rate, elemental and organic impurities and gel strength (Debergh 1983; Nairn et al. 1995). Thus, the aim of this study was to find out the effect of gelrite and agar and their different concentrations on in vitro direct SE and regeneration of sugarcane *cvs*. CoJ 83 and CoH 119.

Materials and Methods

Plant Material

The experiment was conducted on high yielding commercial sugarcane (*Saccharum* spp.) varieties CoJ 83 and CoH 119 obtained from the Sugarcane Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India. The research work was carried out in the Tissue Culture and Transformation Laboratories, School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India during 2011.

Explant Preparation

The apical stem portions of 6-month-old healthy cane stalks of sugarcane cultivar 'CoJ 83' and 'CoH 119' were collected from the field. These were unwhorled (up to about 1 cm diameter), trimmed to 6.0 cm length, washed in commercial detergent 'teepol' (2%) for 5 min, sterilized with 1% bavistin (BASF, India) on a rotary shaker for 30 min followed by its sterilization with 0.1% mercuric chloride for 8–10 min under aseptic conditions in the laminar flow cabinet and finally washed thrice with sterile distilled water.

Establishment for Direct SE

The sterilized spindles were used for the preparation of explants suitable for culturing. The outer 1–2 whorls of spindles (apical portions) were removed and rolls of inner leaf whorls were cut transversely into 1–1.5 cm long segments. For direct SE, the leaf roll segments were cultured on direct SE medium, MS + NAA (5 mgl^{-1}) + Kin (0.5 mgl^{-1}) (Gill et al. 2006). A single explant was inoculated per test tube. All the culture tubes were incubated at 25 ± 2°C in 16:8 h light:dark condition.

Regeneration, Multiplication and Rooting

Shoot buds regenerated on regeneration medium and were transferred to 1/2 MS medium for shoot elongation. The established shoot cultures were multiplied in the liquid MS medium supplemented with IAA (3.0 mgl^{-1}) + BAP (0.3 mgl^{-1}) + Kin (0.3 mgl^{-1}). The black portion of the spindle was removed with the help of scalpel and forceps so as to minimize the problem of death of cultures due to mal-nutrient absorption. Plants obtained from embryogenic tissue and young leaf rolls were transferred on rooting medium MS + NAA (3.0 mgl^{-1}) + IBA (2.0 mgl^{-1}) + sucrose (7%).

Hardening and Transfer to Soil

Hardening of the plantlets was carried out in test tubes having presoaked cotton, under good light intensity (5,000

lux) for 4–5 days in the incubation room for elongation of roots and hardening of plant. The hardened and fully grown in vitro rooted plantlets were transferred to polythene bags containing field soil with farmyard manure and kept in the glasshouse for 6–8 weeks under a temperature of 30 ± 2°C and humidity of 70–80%. The experiments on SE and regeneration were replicated twice and the data were analysed using completely randomized design.

Results and Discussion

Effect of Activated Charcoal

In the present investigation, browning of the media was observed in majority of the cultures of variety CoJ 83 as compared to CoH 119 (Fig. 1a). This browning of media might be due to injuring of the plant as during preparation of explants, the phenolic compounds that are largely located in the vacuoles are mixed with the contents of plastids and other organelles and consequently, dark pigmentation appears. These are highly reactive compounds which polymerize rapidly and form bonds with proteins and also inhibit enzyme activity, and thus, may result in lethal browning of the explant and medium as well (Gill et al. 2004). AC is frequently added to culture medium formulations to either reduce or eliminate undesirable compounds and thus improve anticipated morphogenic responses of explants. When AC (2,000 mgl^{-1}) was added into the medium, it was observed that per cent SE enhanced from 80.21 to 84.88% (Fig. 1b) and further per cent regeneration increased from 75.15 to 81.22% (Figs. 2 and 1d). This might be due to adsorption by AC of some inhibitory chemicals like 5-hydroxymethyl-furfural produced during autoclaving from sucrose by dehydration. These results were consistent with the experiments conducted by Blanc et al. (1999), Lambe et al. (1999), Zhang et al. (2000), Taylor et al. (2001), Aderkas et al. (2002), Gallo-Meagher and Green (2002), Catarina et al. (2004), Andrade and Merkle (2005), Steinmacher et al. (2007), Wu et al. (2007), Raharjo and Litz (2007) and Gambino et al. (2007) in which, SE increased with the addition of AC in rubber, pearl millet, cotton, cassava, hybrid larch, ginseng, saw palmetto (*Seronoa repens*), ocotea, European chestnut,

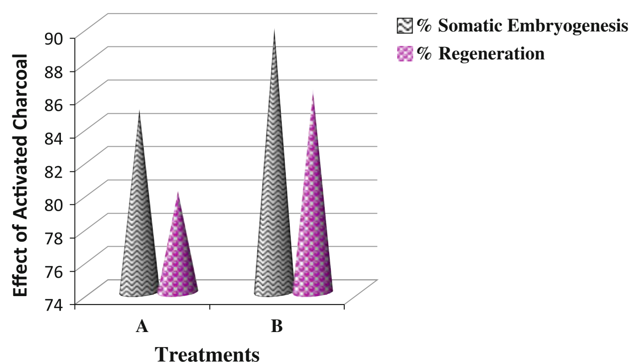

Fig. 2 Effect of activated charcoal on direct somatic embryogenesis and regeneration in sugarcane *cv.* CoJ 83 (*A*) MS + NAA (5 mgl^{-1}) + Kin (0.5 mgl^{-1}) (NAC) (*B*) MS + NAA (5 mgl^{-1}) + Kin (0.5 mgl^{-1}) + 2% (w/v) charcoal (AC)

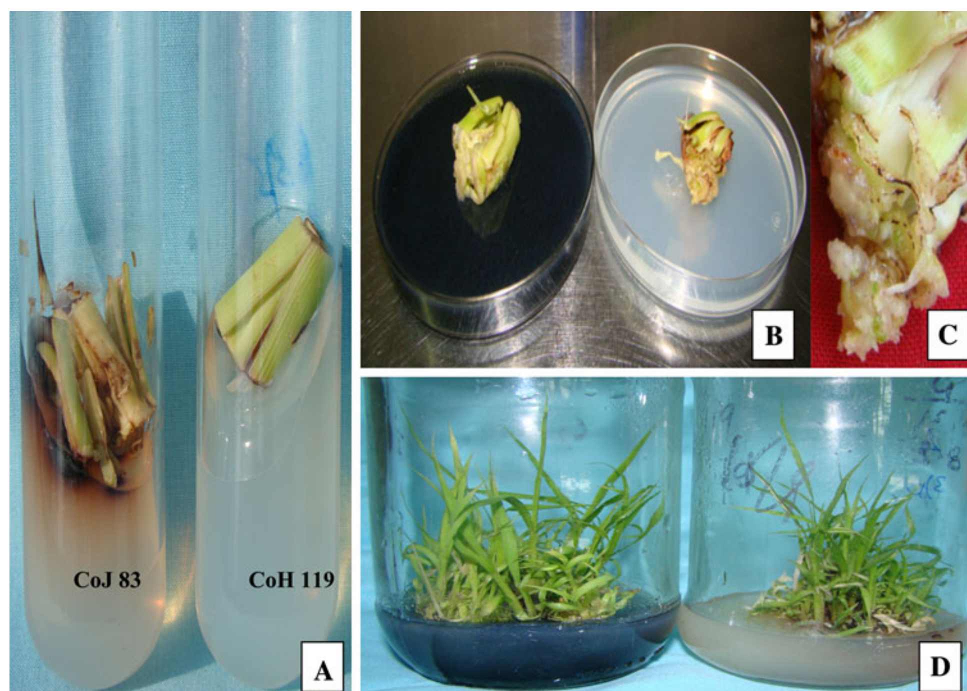

Fig. 1 Effect of activated charcoal on direct somatic embryogenesis and regeneration in sugarcane variety CoJ 83. **a** Browning of the medium due to release of phenolics from the cut ends of the explant is more in CoJ 83 as compared to CoH 119. **b** Showing enhanced somatic embryogenesis on the leaf roll segment on charcoal containing medium. **c** Closer view of direct somatic embryos formed on the cut ends of leaf roll segment in CoJ 83. **d** More number of plantlets produced i.e. enhanced per cent regeneration on charcoal containing medium in variety CoJ 83

peach palm, mango (*Mangifera indica*), litchi (*Litchi chinensis*) and grape vine (*Vitis* spp.), respectively. AC has been included in the formulation of in vitro culture protocols primarily to avoid or retard browning.

Effect of Carbon Sources

During the present study, effect of two different carbon sources (sucrose and maltose) in four different concentrations ((20, 25, 30 and 35 gl^{-1}) was studied and the perusal of data from Fig. 3(a, b) revealed that replacing sucrose with maltose as a carbon source in the SE induction medium resulted in higher SE percentage i.e. 98.35 and 93.12% in CoJ 83 and CoH 119, respectively over control i.e. 89.95 and 85.02% (Fig. 3a) and higher percentage of regeneration i.e. 94.17 and 89.67% in CoJ 83 and CoH 119 (Fig. 3b), respectively over control viz. 82.36% and 80.12%. Thus, the M_6 [MS + NAA (5 mgl^{-1}) + Kin (0.5 mgl^{-1}) + Agar (0.8%) + maltose (25 gl^{-1})] was found to be significantly better than M_3 medium i.e. control (containing 30 gl^{-1} sucrose). In some earlier studies conducted by Jain (1997) and Kaur (2004), maltose was found to be better as a carbon source. This might be because of the fact that maltose stimulates cell differentiation and metabolism and due to its slow metabolization, it is a long duration energy source than the other carbohydrates (Orchinsky et al. 1990). Sucrose is rapidly hydrolysed to glucose and fructose which further increases the osmolality

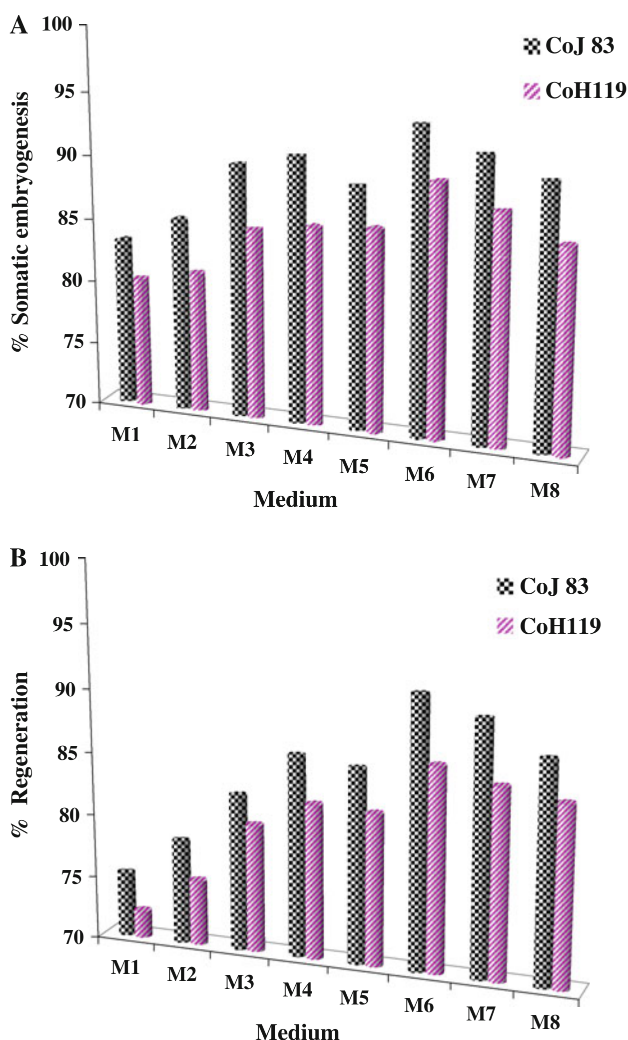

Fig. 3 Effect of different carbon sources i.e. sucrose and maltose in MS + 5 mgl^{-1} NAA + 0.5 mgl^{-1} Kin + 0.8% agar on- **a** Direct somatic embryogenesis and **b** Regeneration in sugarcane *cvs.* CoJ 83 and CoH 119. (M1) 20 gl^{-1} sucrose; (M2) 25 gl^{-1} sucrose; (M3-Control) 30 gl^{-1} sucrose; (M4) 35 gl^{-1} sucrose; (M5) 20 gl^{-1} maltose; (M6) 25 gl^{-1} maltose; (M7) 30 gl^{-1} maltose; (M8) 35 gl^{-1} maltose

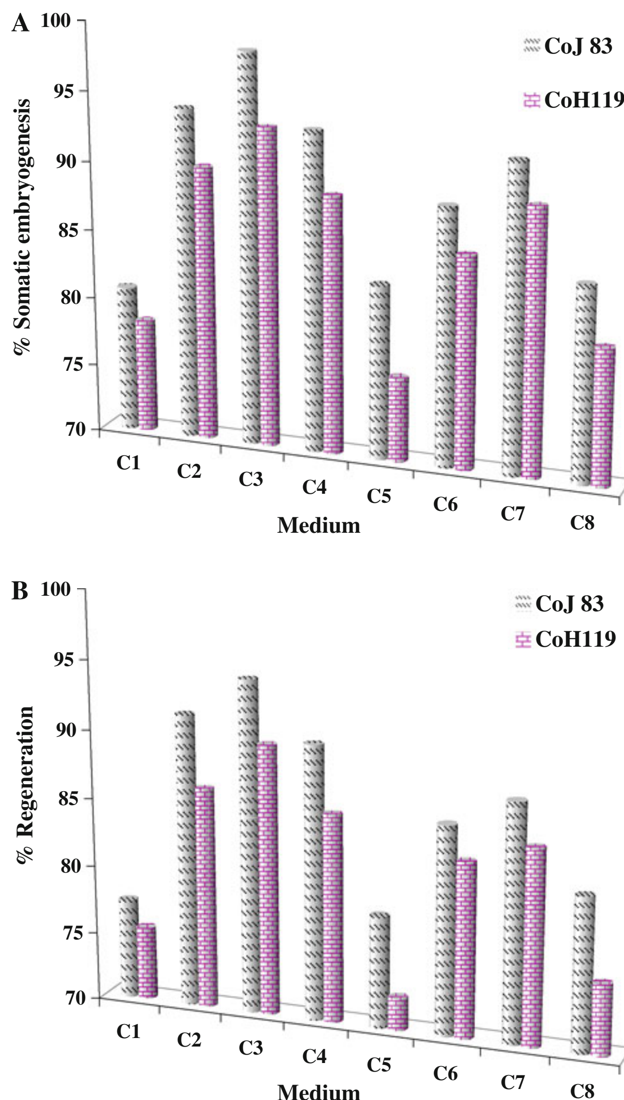

Fig. 4 Effect of different gelling agents i.e. agar and gelrite in MS + 5 mgl^{-1} NAA + 0.5 mgl^{-1} Kin + 25 gl^{-1} maltose on- **a** Direct somatic embryogenesis and **b** Regeneration in sugarcane *cvs.* CoJ 83 and CoH 119. (C1) 4 gl^{-1} agar; (C2-control) 8 gl^{-1} agar; (C3) 12 gl^{-1} agar; (C4) 16 gl^{-1} agar; (C5) 1 gl^{-1} gelrite; (C6) 3 gl^{-1} gelrite; (C7) 5 gl^{-1} gelrite; (C8) 7 gl^{-1} gelrite

Fig. 5 Figure showing transfer of plantlets from in vitro to in vivo conditions. **a** Plantlets having well developed shoot and root systems. **b** The in vitro rooted plantlets transferred to polythene bags containing field soil with farmyard manure and kept in the glasshouse

of the medium whereas the catalysis of maltose is about 20 fold less than that of sucrose (Oehlschlager et al.1990).

Effect of Gelling Agents

The aim of this study was to evaluate the potential of various commercial gelling agents namely; agar and gelrite, in culture media. Four different of agar (4, 8, 12 and 16 gl^{-1}) and gelrite (1, 3, 5 and 7 gl^{-1}) were tested. It was observed that SE occurred in all treatments (agar and gelrite) in both the cultivars (CoJ 83 and CoH 119) however it started earlier on medium containing agar than gelrite. Our results when combined with two in vitro manipulations in culture medium [addition of AC in CoJ 83 and addition of maltose (25 gl^{-1}) in both *cvs*.] led to an efficient direct SE and regeneration in sugarcane. But maximum SE (98.35%) was observed in *cv*. CoJ 83 on medium containing 12 gl^{-1} agar as compared to control (94.01%) (Fig. 4a) and per cent regeneration was also enhanced to 94.17 from 91.36% on medium containing 8 gl^{-1} agar which served as control (Fig. 4b). Per cent SE was also enhanced to 93.12% in comparison with control (89.92%) and respective per cent regeneration was also increased to 94.17 from 91.36% in CoH 119 on medium supplemented with 12 gl^{-1} agar. This might be because when agar concentration is increased, some amount of water is lost from the explant which provided a trigger for SE but by increasing the concentration further, excessive dehydration may occur which, may have a negative impact on SE and regeneration potential. Although gelrite containing provided many advantages like it sets a clear gel which assisted easy observation of cultures and their possible contamination but our results recommended use of 12 gl^{-1} agar in direct SE and regeneration medium in sugarcane *cvs.* CoJ 83 and CoH 119. Kyte (1987) has recommended the use of a mixture of gelrite and agar in a ratio of 3:1. Malik (2010) has recommended the use of 16 gl^{-1} of agar in CoJ 64 variety of sugarcane.

Plantlets regenerated via tissue culture are usually very fragile and most of these die if directly transferred to the field conditions because of the transplantation shock. Rooting was done on half strength MS medium without growth regulators. Regenerated plantlets with well developed root and shoot systems (Fig. 5a) were hardened and transferred to the soil in the earthen pots and were kept in glass house. The transferred plants got established in the soil and exhibited normal growth (Fig. 5b). Therefore, protocol reported here is suitable for genetic transformation due to its high efficiency and reproducibility.

In vitro tissue culture is an important means to improve crop tolerance and yield through genetic transformation as well as induction of somaclonal variation (Wani et al. 2010). So it is important to devise an efficient protocol for SE and regeneration to start in vitro selection for salt and drought stress tolerance.

The results of this present study indicated that sugarcane *cvs.* CoJ 83 and CoH 119 have good SE and regeneration ability. The experiment gave us an idea about the role of optimum level of AC, carbon source and gelling agent to be used for SE studies in sugarcane.

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