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GENETICS AND PLANT BREEDING

Influence of ethylene inhibitor on shoot organogenesis and regeneration in sugarcane (*Saccharum* spp. hybrid)

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Abstract

Ethylene is a gaseous plant hormone (phytohormone) that play an important role on *in vitro* shoot morphogenesis in many plant species. The aim of the present investigation was to critically analyse the role of an ethylene inhibitor *i.e.* silver nitrate ($AgNO_3$) on shoot organogenesis in sugarcane*cvs*. CoJ 83 and CoH 119. Different concentrations of silver nitrate were tested in the medium: 1 mgl^{-1}, 3 mgl^{-1}, 5 mgl^{-1} and 10 mgl^{-1}. Genotypic dependence was observed between cvs. CoJ 83 and CoH 119 with different concentrations of $AgNO_3$ tested in the medium. Among the varying concentrations (1–10 mgl^{-1}) of silver nitrate tested, per centage of leaf roll segments responding were improved giving highest number of shoots per explant on MS medium + Naphthalene Acetic Acid (NAA; 5 mgl^{-1}) + Kinetin (Kin; 0.5 mgl^{-1}) + $AgNO_3$ (3 mgl^{-1}) in *cv.* CoJ 83 and on MS medium + NAA (5.5 mgl^{-1}) + Kin (0.5 mgl^{-1}) + $AgNO_3$ (5 mgl^{-1}). The number of shoots formed per explant increased to two-fold and the shoot length was also increased as compared to control. Data recorded on the number of roots and root length also showed a significant increase over the control in both the varieties.

Highlights

- A ubiquitous hormone, ethylene (C_2H_4), is produced which is associated with poor regeneration and recalcitrant behaviour of culture material during plant tissue culture experiments
- In order to study its effect, ethylene production was inhibited in the culture bottles using silver nitrate ($AgNO_3$)
- In two commercial cultivars of sugarcane, a significant enhancement in the per cent establishment of cultures, number of shoots formed per explant, length of shoots, number of roots and root length was observed
- Thus, silver nitrate can be included as an ethylene inhibitor in the media used for the tissue culture studies in sugarcane

Keywords: Shoot morphogenesis, silver nitrate, ethylene, genotypic dependence

Sugarcane, one of the most efficient biofuel crops in the world, belongs to genus *Saccharum* and family *Gramineae*. India is the largest producer of cane sugar and second largest producer of cane next to Brazil. It is a widely cultivated crop in the subtropical belt across North India from Assam to Punjab. Sugarcane occupies a unique position in agro-industrial market by providing raw materials to produce sugar, jaggery and khandsari and producing co-products like alcohol, paper, cattle feed, electricity etc. Nowadays, tissue culture is widely used in sugarcane improvement programmes. A large

number of studies have been conducted in sugarcane dealing with shoot tip culture (Fitch and Moore 1993), callus induction (Rani *et al.* 2012) and direct plant regeneration (Mittal *et al.* 2013).

An efficient tissue culture system for the regeneration of plants from explants depends upon several aspects, of which shoot regeneration and rooting are the most important aspects (Purnhauser *et al.* 1987). In plant tissue culture and genetic transformation experiments, closed vessels are used for the purpose of avoiding contamination. As a result, ethylene (C_2H_4), a ubiquitous hormone, is produced which is associated with poor regeneration and recalcitrant behaviour of culture materialfurther affecting callus growth, shoot generation, rooting and somatic embryogenesis (Pua and Chi 1993). Most of the plant tissues have the tendency to produce ethylene. The effect of ethylene on *in vitro* morphogenesis, as with other phytohormones, depends on its concentration in and around the cultured tissues, as well as their sensitivity to it (Thorpe 1994). Silver nitrate is usually employed in *in vitro* studies for inhibiting ethylene action because of its water solubility and lack of phytotoxicity at effective concentrations (Beyer 1976).

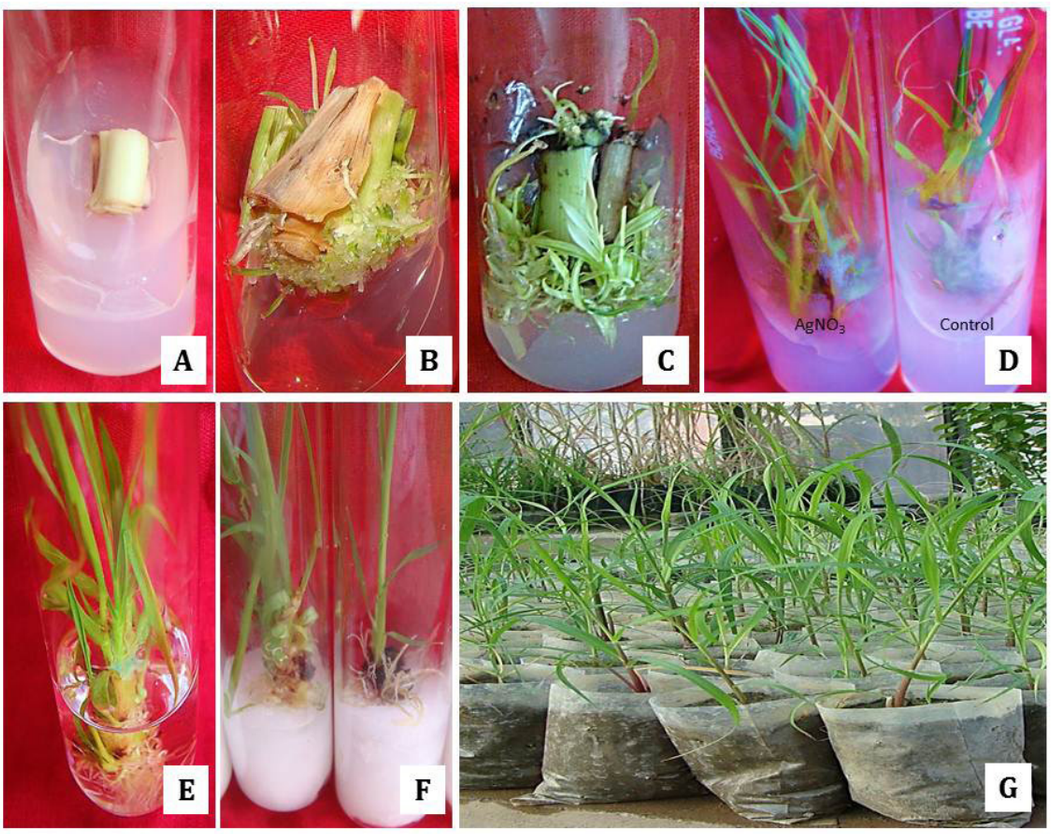

Figure 1 Establishment of shoot organogenesis and regeneration in sugarcane cv.CoJ 83

(A) Leaf roll segment placed onMS+ NAA (5 mgl-1) + Kin (0.5 mgl-1) +3.0% sucrose + 0.8% agar medium (B) Direct shoot regeneration from leaf roll segment after 14-15 days of culturing (C) Shoot elongation from leaf roll segment (D) Comparison of number of shoots formed on medium supplemented with 3 mgl-1 AgNO3 and control (E) Rooting of plantlets on liquid MS medium + NAA(3.0 mgl-1) + IBA (2.0 mgl-1) + sucrose (7%) medium (F) Hardening of rooted plantlet on pre-soaked cotton for 4 days under high light intensity (5000 lux) (G) Plantlets transferred to polybags containing soil and farmyard manure in 1:1 ratio

A number of studies have revealed that silver nitrate is known to enhance regeneration in many plants like *Vitexnegundo* (Steephen *et al.* 2010), *Decalepis hamiltonii* (Bais *et al.* 2000), *Cucumismelo* (Roustan *et al.* 1992). $AgNO_3$ has also been reported to enhance *in vitro* shoot formation in *Albizzia julibrissin* (Sankhla *et al.* 1995), reported microspore embryogenesis in *Brassica juncea* (Prem *et al.* 2005), higher frequency of multiple shoot formation in *Arachis hypogea* (Ozudogru *et al.* 2005) and plant regeneration in *Paspalum scrobiculatum* (Kothari-Chajer *et al.* 2008). In this investigation, we report the effects of an ethylene inhibitor, silver nitrate ($AgNO_3$) on *in vitro* regeneration in two different *cvs.* CoJ 83 and CoH 119 of sugarcane.

Materials and Methods

The investigation was carried out in Tissue Culture and Genetic Transformation laboratories at School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana during 2011-13. Shoot tops were collected from 6-8 month old field-grown, healthy sugarcane plants grown at University Seed Farm, Laddowal and from the field of School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana. After peeling of the outer mature leaves, shoot tops (spindles) were thoroughly washed with detergent 'Teepol' and then surface sterilized under a laminar air flow cabinet with 1% carbendazim (bavistin) to avoid fungal contamination followed by their sterilization with 0.1% mercuric chloride for 8-10 minutes to avoid bacterial contamination with gentle shaking and rinsed thrice with autoclaved distilled water. Afterwards, the spindles were treated with cefotaxime (500 mgl^{-1}) for half an hour. Further, preparation of explants was done from the sterilized spindles.

After removing the outer 2-3 leaf whorls and the nodal portions from the spindles, innermost leaf whorls were cut transversely into 1 cm long segments. Inoculations were performed under aseptic conditions. All the instruments (petridish, scalpel and forcep) were sterilized with spirit and heated over flame before use. For shoot organogenesis, leaf roll segments were placed on MS medium supplemented with α-Naphthalene Acetic Acid (NAA) [5.0 mgl^{-1} for CoJ 83 and 5.5 mgl^{-1} for CoH 119] supplemented with Kinetin (0.5 mgl^{-1}).

Table 1. Effect of different concentrations of silver nitrate on number of shoots, shoot length (cm), number of roots and root length (cm) in two cvs. of sugarcane (CoJ 83 and CoH 119)

	Number of shoots		Shoot length (cm)		Number of roots		Root length (cm)	
Variety	CoJ 83	CoH 119	CoJ 83	CoH 119	CoJ 83	CoH 119	CoJ 83	CoH 119
$AgNO_3$ (mgl^{-1})								
Control	6.23*	5.06	4.31	5.20	6.63	6.86	1.56	1.37
1	6.56	5.43	4.49	5.30	6.90	7.19	1.67	1.76
3	7.0	5.62	4.65	5.36	7.13	7.36	2.0	1.90
5	5.63	6.25	4.16	5.69	6.20	7.76	1.16	2.27
10	4.76	5.06	3.97	5.04	5.90	6.70	0.923	1.30
Mean	6.04	5.48	4.32	5.32	6.55	7.17	1.46	1.72
CD (5%)	0.346	0.547	0.104	0.164	0.228	0.360	0.239	0.377
CD (5%) [CoJ83 X CoH119]	0.774		0.232		0.509		0.534	

*All the values represent means of 15 cultures with 3 replications

These media were fortified with 3.0% sucrose and 0.8% agar. A single explant was inoculated per test tube (Figure 1A) and the cultures were incubated at 25 ± 2°C in an air-conditioned incubation room with 16:8 hours light: dark photoperiod regime. For rooting, 5-7 cm long shoots were cultured on MS medium + NAA (3.0 mgl^{-1}) + Indole-3-butyric acid (IBA; 2.0 mgl^{-1}) + sucrose (7%). The pH of the medium was set at 5.8 prior to autoclaving at 121°C for 20 min.

In order to investigate its effect, ethylene production was inhibited in the culture bottles using ethylene inhibitors *i.e.* $AgNO_3$. A stock solution of silver nitrate was prepared, autoclaved and poured into the autoclaved medium at their respective concentrations. Data was analysed using the CPCS-1 software (Cheema and Singh 1990). Fifteen leaf roll segments (three replicates for each concentration) were used for the present study.

Results and Discussion

The aim of this work was to study the influence of the ethylene inhibitor, $AgNO_3$ on shoot organogenesis and regeneration in sugarcane. For tissue culture studies of sugarcane, leaf roll segments served as a good source of explants (Figure 1A). After placing the leaf roll segments of sugarcane on shoot organogenesis medium, unwhorling occurred and the segments showed direct shoot regeneration [Figure 1 (B,C)] within 14-15 days of culturing. Both the cultivars exhibited extreme variation in response to ethylene inhibitor, silver nitrate ($AgNO_3$) with respect to the percentage of responding leaf roll segments, number of shoots formed per leaf roll segment, shoot length, number of roots and root length. The perusal of the data from Figure 2 showed that the percentage of responding explants increased to 82.3% (6.3% increase from control) in CoJ 83 variety on medium [MS+ NAA (5 mgl^{-1}) + Kin (0.5 mgl^{-1}) + 3.0% sucrose + 0.8% agar] supplemented with 3 mgl^{-1} of $AgNO_3$ and in case of CoH 119, increased to 79.16% (9.86% increase from control) on medium [MS+ NAA (5.5 mgl^{-1}) + Kin (0.5 mgl^{-1}) +3.0% sucrose + 0.8% agar] supplemented with 5 mgl^{-1} of $AgNO_3$.

This might be due to the fact that when ethylene is produced through cell division during establishment phase of leaf roll segments under *in vitro* conditions, Ag^+ ions are produced in the medium and an ethylene receptor, ETR1, contains one ethylene-binding site per homodimer which is further mediated by a single copper ion (Cu^+) present in the ethylene-binding site. The replacement of the copper co-factor by silver

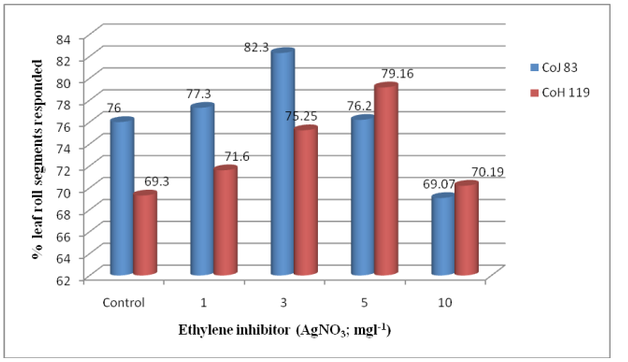

Figure 2. Per cent leaf roll segments responding with an increase in concentration of ethylene inhibitor (AgNO3; mgl-1) in the medium in two cultivars of sugarcane i.e. CoJ 83 and CoH 119

also serves to lock the receptor into a conformation such that it continuously represses ethylene responses (Zhao *et al.* 2002). Further, perusal of data from Table 1 shows that average number of shoots increased to 7 and 6.25 in case of CoJ 83 and CoH 119, respectively, which were significantly different from control as well as significant difference was observed between varieties. Similar observations were made for shoot length which was significantly higher in CoJ 83 in medium supplemented with 3 mgl^{-1} of $AgNO_3$ as compared to control and higher in COH 119 in medium supplemented with 5 mgl^{-1} of $AgNO_3$. Fei *et al.* (2000) observed that addition of the ethylene antagonist, silver nitrate ($AgNO_3$), into callus induction medium significantly enhanced embryogenic callus production (both induction frequency and callus growth) of field-collected male immature inflorescence cultures of buffalograss NE84-45-3 and 'Texoka'.

Earlier, silver nitrate has been known to influence *in vitro* shoot multiplication and root formation in

Vanilla planifolia (Giridhar *et al.* 2001). A similar study was conducted by Chae *et al.* (2012) in which ethylene inhibitors [aminoethoxyvinylglycine (AVG), cobalt chloride ($CoCl_2$) and silver thiosulphate (STS)] enhanced shoot organogenesis of *Gloxinia* (*Sinningiaspeciosa*). By using AVG and $CoCl_2$ in the culture medium at a concentration of 1 mgl^{-1}, an increase of 16% and 12% in the shoot number was observed, respectively.

Rooting of shoots was carried out on liquid MS medium + NAA (3.0 mgl^{-1}) + IBA (2.0 mgl^{-1}) + sucrose (7%) (Figure 1E). Rooting of shoots earlier placed on shooting medium supplemented with 3 $mgl^{-1}AgNO_3$ in CoJ 83 and 5 $mgl^{-1}AgNO_3$ in CoH 119 was also improved with respect to number of roots and root length. Significant variation was observed within the varieties as well as between the varieties. Average number of roots was increased to 7.13 from 6.63 in control with an average root length of 2.0 cm in CoJ 83 and increased to 7.76 from 6.86 (control) with an average root length of 2.27 cm in CoH 119 (Table 1). Plantlets with well-developed root and shoot system were hardened on pre-soaked cotton under high light intensity (5000 lux) for 4 days in test tubes under in vitro conditions (Figure 1F). The hardened plantlets were transferred to the soil with farmyard manure in the polybags (Figure 1G). The results reported in the present investigation showed that in two commercial cultivars of sugarcane, a significant enhancement in the per cent establishment of cultures, number of shoots formed per explant, length of shoots, number of roots and root length was observed. Therefore, it is concluded that, as an efficient regeneration system is essential for mass multiplication and genetic transformation studies, silver nitrate can be included as an ethylene inhibitor in the media used for tissue culture studies in sugarcane (Saccharum spp. hybrid).

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