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RESEARCH ARTICLE

Use of Sodium Hypochlorite as Media Sterilant in Sugarcane Micropropagation at Commercial Scale

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Abstract Sodium hypochlorite has been successfully utilized as media sterilant (at total active chlorine concentration 0.002% in the medium) in production of sugarcane plantlets by applying apical meristem culture technique. The process has been scaled up to produce 2.5 million sugarcane plantlets per year. Use of sodium hypochlorite in place of autoclaving has resulted in reducing considerable amount of electricity cost and ultimately reducing the cost of production of sugarcane plantlets.

Keywords Autoclaving · Cost reduction · Micropropagation · Sodium hypochlorite · Sugarcane · Shooting medium · Rooting medium

Introduction

Sugarcane, being a major source of sugar, is one of the most important commercial crops of several countries in tropical and subtropical regions. Micropropagation is a technique through which plants are multiplied rapidly by using apical meristem explants under controlled nutritional and environmental conditions. It helps in rapid seed multiplication of newly released sugarcane varieties which ultimately results in reducing the period required for covering large area with a newly released variety. Apical meristem culture was used by Coleman (1970) and Hendre et al. (1975) to obtain sugarcane mosaic virus free plants. Hendre et al. (1983) standardized apical meristem culture technique for rapid multiplication of leaf mosaic virus free plants of Co 740 variety. The technique when properly used, results in production of large number of disease free and true to type sugarcane plantlets within a shorter period of time. Vasantdada Sugar Institute has successfully used this technique for production of large number of plantlets of different sugarcane varieties. These plantlets are being supplied to sugar industry and sugarcane growers from Maharashtra and adjoining states for production of good quality planting material. The technology has been successfully incorporated in three tier system of seed multiplication in sugarcane.

Tissue culture technique is a labour intensive technique and also uses large quantity of electricity resulting in increased cost of production of sugarcane plantlets. Efforts were, therefore, made from time to time to reduce the cost of production of plantlets by periodically modifying the micropropagation protocol. Consequently the institute has been successful in reducing the production cost of sugarcane plantlet from Rs. 18 to 4 (1US$ = 45 INR) per plantlet.

For maintaining sterility of the culture media, autoclaving is generally performed which consumes large quantity of electricity. For minimizing the electricity cost it was thought worthwhile to adopt chemical sterilization by incorporating sodium hypochlorite in the media.

Sodium hypochlorite, a chemical compound with the formula NaOCl, is frequently used as a disinfectant or a bleaching agent. It is effective against many bacteria and some viruses. In plant tissue culture it is used for surface sterilization of explants. In the present study effect of different concentrations of sodium hypochlorite incorporated in growth media was examined on the in vitro shooting and rooting in sugarcane varieties CoC 671 and Co 86032 and compared with that of autoclaved sterilized media.

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Plate 1 Effect of varying concentrations (C%) of sodium hypochlorite incorporated in shooting medium (SH) on the growth of CoC 671 explants

Materials and Methods

The apical meristems of the two sugarcane varieties (CoC 671 and Co 86032) were obtained from the 4 month old tillers produced by field growing crop. These apical meristems were surface sterilized with 0.1% mercury chloride ($HgCl_2$) solution and were given three changes of sterile distilled water to get rid of traces of mercury chloride. The apical meristems thus obtained were inoculated on modified liquid MS medium (Murashige and Skoog 1962) containing cytokinins (Kinetin and BAP). They were allowed to grow in the medium for 25–30 days after which they were transferred to bottles containing same growth medium, and incubated on illuminated shakers for a further period of 25 days. The growth thus obtained was further used as explants in the following experiments.

Effect of Different Concentrations of Sodium Hypochlorite Incorporated in Shooting and Rooting Media on the Growth of Explants

With a view to study the growth of explants of two varieties in shooting and subsequent rooting medium the following types of media were used for inoculation.

(i) Shooting medium (liquid)—autoclaved (control).
(ii) Shooting medium (liquid) with sodium hypochlorite (4% w/v available chlorine) at the concentrations 0.1,

Plate 2 Effect of varying concentrations (C%) of sodium hypochlorite incorporated in shooting medium (SH) on the growth of CoC 86032 explants

0.2, 0.3, 0.4 or 0.5% corresponding to 0.004, 0.008, 0.012, 0.016 or 0.020% active chlorine. This was then autoclaved.

(iii) Shooting medium (liquid) as described in (ii) above was prepared but it was not autoclaved.

Eight explants of each of the two varieties were used for inoculating each of the above mentioned media in culture bottles.

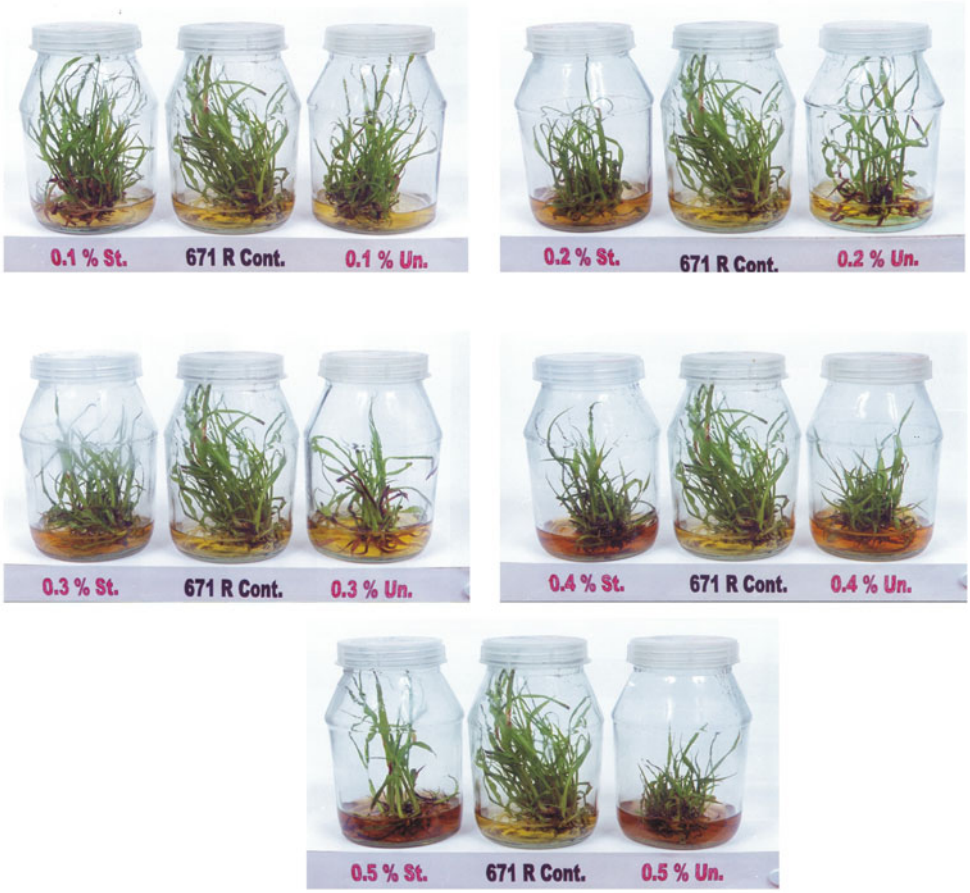
The growth obtained in the bottles containing above media was used as explants for inoculating bottles containing rooting medium. The rooting medium (MS medium with NAA) was prepared and eight bottles of each type of medium were inoculated by using explants obtained from the shooting experiment as described above. The following three types of rooting media were prepared.

(i) Rooting medium (liquid)—autoclaved (Control).

(ii) Incorporation of various concentrations of sodium hypochlorite (as indicated above) to rooting medium followed by autoclaving.

(iii) Rooting medium (liquid) as described in (ii) above was prepared but it was not autoclaved.

Bottles inoculated with the explants from shooting experiment were first incubated on illuminated shakers and



C% St = C% sodium hypochlorite incorporated in rooting medium (R) and then autoclaved.
SH Cont = Autoclaved rooting medium (R) kept as control.
C% Un = C% sodium hypochlorite incorporated in rooting medium (R) but not autoclaved

Plate 3 Effect of varying concentrations (C%) of sodium hypochlorite incorporated in rooting medium (R) on the growth of CoC 671 explants

then transferred to illuminated racks. The observations on rooting were recorded after 25–30 days of inoculation.

Effect of Sodium Hypochlorite as Media Sterilant Under Unsterile and Sterile Conditions

Since sodium hypochlorite was found to be good sterilant, it was thought worthwhile to study the effect of elimination of procedure of autoclaving empty bottles, which were subsequently used for filling up different types of media.

Experiments were conducted with both shooting and rooting media in which sodium hypochlorite was incorporated at 0.1% concentration (Corresponding to 0.004% of active chlorine). The explants (as described above) used in this experiment either treated with mercury chloride (+Hg) or without mercury chloride (−Hg) were inoculated on these media either in laminar flow (+LF) or outside laminar flow (O). Effect of these media and inoculation conditions was studied in explants of both the varieties.

Optimum Dose of Sodium Hypochlorite as Media Sterilant

Sodium hypochlorite at a concentration of 0.1% (corresponding to 0.004% active chlorine) emerged as an effective media sterilant and therefore an experiment was conducted with various concentrations of sodium hypochlorite ranging from 0.01 to 0.1% (v/v) to optimise the dose of sodium hypochlorite.

Results and Discussion

Effect of Different Concentrations of Sodium Hypochlorite Incorporated in Shooting and Rooting Media on the Growth of Explants

It could be seen from Plate 1 that when sodium hypochlorite was incorporated in shooting medium at different concentrations ranging from 0.1 to 0.5% there was good

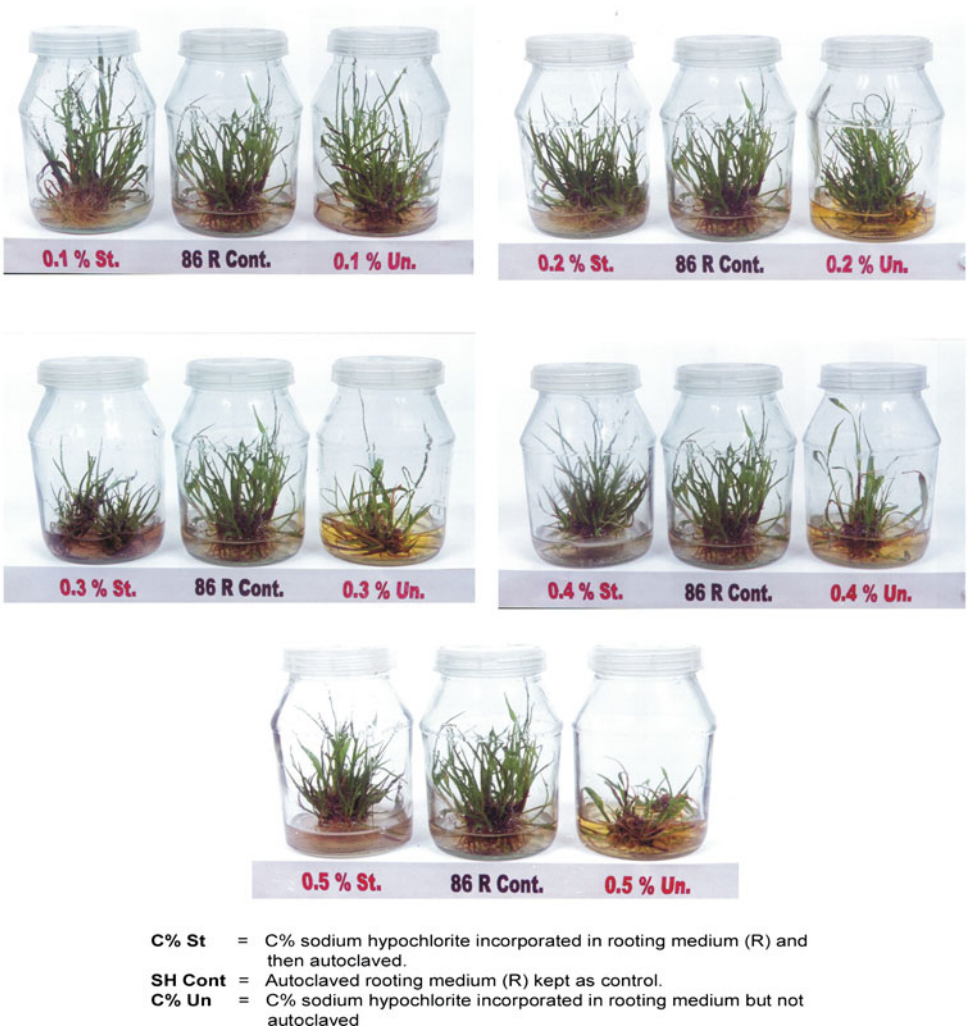


Plate 4 Effect of varying concentrations (C%) of sodium hypochlorite incorporated in rooting medium (R) on the growth of CoC 86032 explants

comparable growth on unautoclaved medium added with definite concentration of sodium hypochlorite. Results were comparable with those obtained in media, which were either autoclaved after addition of sodium hypochlorite (St) or autoclaved without addition of sodium hypochlorite (Cont). Similar results were obtained for both the varieties. Here it is necessary to mention that growth of CoC 671 explants in shooting medium was more pronounced with production of few tillers as compared to that in Co 86032 in which many tillers were produced giving it a bunchy appearance (Plate 2). This was due to varietal characteristics.

It is evident from the Plate 3 that when different concentrations (0.1–0.5%) of sodium hypochlorite were incorporated in rooting medium there was good growth of the explants of CoC 671 which was comparable to that with sterilized and control medium. The roots were very pronounced in Co 86032 (Plate 4) as compared to those produced by CoC 671 explants (Plate 3). Incorporation of 0.1% sodium hypochlorite in the rooting medium was found to be little bit detrimental for root production as compared to sterilized or autoclaved control medium. Root production in medium containing 0.1% sodium hypochlorite was scanty (Plate 6).

Effect of Sodium Hypochlorite as Media Sterilant Under Unsterile and Sterile Conditions

In this set of experiments, the shooting medium was incorporated with 0.1% sodium hypochlorite and the bottles were not autoclaved (Un). The growth of explants was compared with that in sterile shooting medium (SH St) as well as with that in which sodium hypochlorite was not added and it was also not autoclaved (Cont.). The inoculations were either performed under laminar flow hood (LF) or outside laminar flow (O). Before incorporating explants in the culture media bottles, they were either surface sterilized with mercury chloride (+Hg) or not surface sterilized with mercury chloride (−Hg). The results showed that in 100% bottles containing unsterile medium, without incorporation of sodium hypochlorite and which

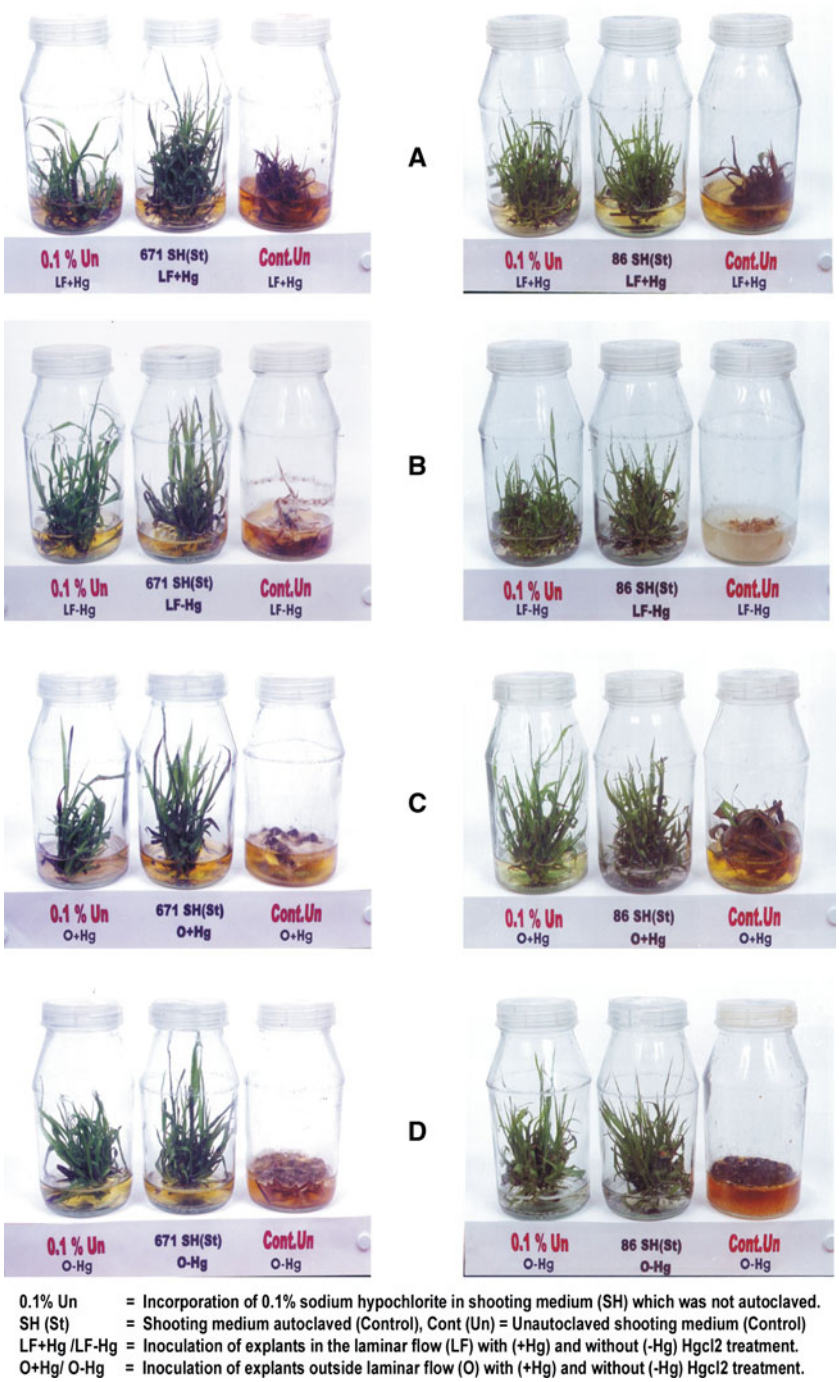
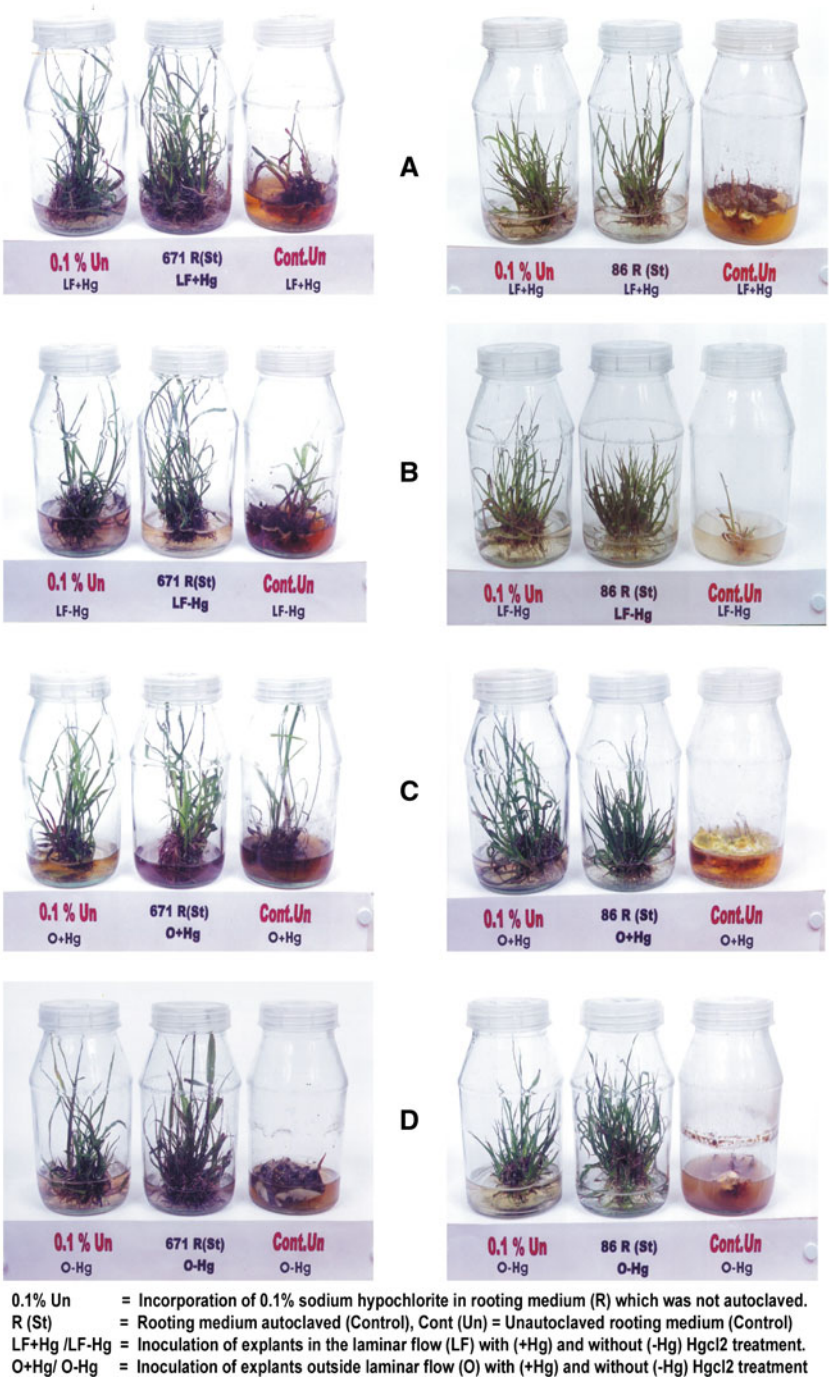


Plate 5 Effect of incorporation 0.1% sodium hypochlorite in unsterilised shooting medium (SH) on the growth of CoC 671 and Co 86032 explants

were not autoclaved, there was scanty growth of explants or they were totally contaminated (Plate 5 right hand side bottles at A–D). The explants when incorporated in unsterile shooting medium containing 0.1% sodium hypochlorite gave good growth (Plate 5 left hand side bottles at A–D), which was comparable with the growth of explants in the sterile shooting medium (Plate 5 middle bottles at A–D). When similar concentration (0.1%) of sodium hypochlorite was incorporated in unsterile rooting medium there was comparable growth of explants (Plate 6 left hand side bottles at A–D), with that growing in the autoclaved sterilized rooting medium (Plate 6 middle bottles at A–D). In this case also unsterilized rooting medium without incorporation of sodium hypochlorite and which were not autoclaved, gave very scanty growth or total contamination (Plate 6 right hand side bottles at A–D).

It is apparent from Table 1 that when empty bottles were not autoclaved and were used for shooting medium the growth of the explants under different treatments (LF + Hg, LF − Hg, O + Hg, O − Hg) was comparable



0.1% Un = Incorporation of 0.1% sodium hypochlorite in rooting medium (R) which was not autoclaved.
R (St) = Rooting medium autoclaved (Control), Cont (Un) = Unautoclaved rooting medium (Control)
LF+Hg /LF-Hg = Inoculation of explants in the laminar flow (LF) with (+Hg) and without (-Hg) Hgcl2 treatment.
O+Hg/ O-Hg = Inoculation of explants outside laminar flow (O) with (+Hg) and without (-Hg) Hgcl2 treatment

Plate 6 Effect of incorporation 0.1% sodium hypochlorite in unsterilised rooting medium (R) on the growth of CoC 671 and Co 86032 explants

to that when empty bottles were autoclaved and were used for shooting medium. Similar results were obtained when such autoclaved and unautoclaved bottles were used for rooting medium (Table 2).

Optimum Dose of Sodium Hypochlorite as Media Sterilant

It could be seen from the Table 3 that when sodium hypochlorite was incorporated at 0.01 and 0.05% concentrations in shooting and rooting media there was good growth of explants which was comparable to that obtained in the media containing 0.1% sodium hypochlorite or in the control autoclaved media. Though there was good growth in all the bottles (five of each type) containing media supplemented with 0.01% sodium hypochlorite after prolonged incubation some of the bottles (10–15%) got contaminated. No such contamination was observed in the media in which sodium hypochlorite was incorporated at 0.05% concentration even after prolonged incubation.

Table 1 Contamination (%) under different treatments in shooting medium

Treatments	Unautoclaved empty bottles + 0.1% NaOCl		Autoclaved empty bottles + 0.1% NaOCl		Control (autoclaved media)		Control (unautoclaved media)	
	CoC 671	Co 86032	CoC 671	Co 86032	CoC 671	Co 86032	CoC 671	Co 86032
LF + Hg	0	0	0	0	0	0	100	63
LF − Hg	0	0	0	0	0	0	100	100
O + Hg	0	0	0	0	0	0	100	100
O − Hg	0	0	0	0	0	0	100	100

LF + Hg = Inoculation in laminar flow with $HgCl_2$ treatment; LF − Hg = Inoculation in laminar flow without $HgCl_2$ treatment; O + Hg = Inoculation outside laminar flow with $HgCl_2$ treatment; O − Hg = Inoculation outside laminar flow without $HgCl_2$ treatment

Table 2 Contamination (%) under different treatments in rooting medium

Treatments	Unautoclaved empty bottles + 0.1% NaOCl		Autoclaved empty bottles + 0.1% NaOCl		Control (Autolaved media)		Control (Unautoclaved media)	
	CoC 671	Co 86032	CoC 671	Co 86032	CoC 671	Co86032	CoC 671	Co 86032
LF + Hg	0	0	0	0	0	0	100	100
LF − Hg	0	0	0	0	0	0	100	100
O + Hg	0	0	0	0	0	0	100	100
O − Hg	0	0	0	0	0	0	100	100

LF + Hg = Inoculation in laminar flow with $HgCl_2$ treatment; LF − Hg = Inoculation in laminar flow without $HgCl_2$ treatment; O + Hg = Inoculation outside laminar flow with $HgCl_2$ treatment; O − Hg = Inoculation outside laminar flow without $HgCl_2$ treatment

Table 3 Growth of explants of CoC 671 and Co 86032 on shooting and rooting media supplemented with different concentrations of sodium hypochlorite

4% (w/v) of sodium hypochlorite added to the culture medium (%)	Active chlorine added to the culture medium (%)	Shooting medium CoC 671	Co86032	Rooting medium CoC 671	Co86032
0.01(untoclaved)	0.0004	G	G	G	G
0.05(untoclaved)	0.002	G	G	G	G
0.1 (untoclaved)	0.004	G	G	G	G
Control (autoclaved)	–	G	G	G	G
Control (untoclaved)	–	C	C	C	C

G Good growth, *C* contamination

Therefore, it was decided to use 0.05% sodium hypochlorite corresponding to 0.002% active chlorine for chemically sterilizing different media. It is noteworthy that the empty bottles used in this experiment also were never autoclaved they were thoroughly washed, dried and used directly for filling the different types of media.

Teixeira et al. (2006) reported that sodium hypochlorite could be used for sterilizing nutrient medium and were successful in growing pineapple, banana, eucalyptus and orchid explants in such medium. Teixeira and Torres (1998) tried microwave oven for sterilizing the medium, with no success. The present results that addition of 0.002% active chlorine (corresponding to 0.05% of 4% w/v sodium hypochlorite) was found effective in sterilizing both shooting and rooting media, is in conformity with the finding of Teixeira et al. (2006) who found that 0.0003% active chlorine when added to culture medium resulted in getting cultures without any contamination.

Yanagawa (2006) reported the effect of sodium hypochlorite in maintaining sterility in unautoclaved culture vessels. In the present investigation also the effect of eliminating empty bottles autoclaving was studied and it was found that the medium in which the sodium hypochlorite was incorporated at 0.05% concentration (corresponding to 0.002% active chlorine) resulted in getting rid of contamination.

The economics of the two methods viz (i) autoclaving of media and (ii) using sodium hypochlorite as a media sterilant was worked out. The production capacity of Tissue Culture laboratory at Vasantdada Sugar Institute is

2.5 million plantlets per year and on this basis the economics has been worked out. It was found that in a year for production of 2.5 million plantlets an electricity consumption worth Rs. 0.75 million (1US$ = INR 45.00) is required for autoclaving @ Rs. 0.3 per plantlet. As against this if sodium hypochlorite was used for sterilizing media the cost of sodium hypochlorite comes to Rs. 1200 per year for production of 2.5 million plantlets. Thus about Rs. 0.75 million can be saved on electricity bills in a year.

Conclusion

Sodium hypochlorite has been found to have medium sterilization property. Active chlorine concentration of 0.002% in the medium was found to be effective in complete sterilization of the medium. It has been successfully utilized for production of sugarcane plantlets on large scale. This has helped in reducing the cost of electricity to a considerable extent.

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