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Effects of culture media pH on *In Vitro* shoot multiplication in sugarcane

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Abstract
The present study deals with the effects of culture media pH on *in vitro* shoot multiplication in two commercially cultivated sugarcane genotypes *viz.,* Co 86032 and Co 94008. The results observed in this study clearly indicate that the culture media pH contributes significant effects on *in vitro* shoot multiplication in sugarcane. The highest shoot multiplication in both genotypes was observed on the culture media having pH 6.0. This study suggests that it is necessary to standardize the culture media pH separately for individual sugarcane genotypes prior to develop efficient *in vitro* regeneration protocols for commercial purposes.

Keywords: Culture media pH, genotype, micropropagation, sugarcane

Introduction
Sugarcane (*Saccharum officinarum* L.), a member of family Poaceae is commercially cultivated in many tropical and sub-tropical countries especially for chewing, sugar production and biofuel production. Sugarcane alone produces about 70% of the total sugar produced worldwide (LMC International, 2008) [1]. Brazil has the largest area and production of this cash crop whereas, India ranked second in terms of area and production. Being a polyploid crop, its improvement is tedious than other field crops but the efforts are on the way. Sugarcane is generally propagated through setts but their slow multiplication rates and rapid spreading of diseases are major constraints to the crop production. Thus, the production of sufficient quality planting material through conventional methods takes several years. The use of infected planting material for sowing purposes wastes farmer's money and efforts at a large scale every year. Some major sugarcane diseases *i.e.* red rot, smut, grassy shoots are generally inherited generation to generation through infected planting materials and reduces crop yield at commercial scales. So, the maintenance of disease-free and quality planting material in this crop is highly required but challengeable. *In vitro* clonal propagation or micropropagation may be a best option for obtaining quality planting material, because in this technique the plants are mainly grown under aseptic conditions and their multiplication rate is very high as compared to vegetative methods of propagation. Micropropagation technique has been used for rapid and large scale production of disease free planting materials in a number of crops including banana and sugarcane. In our country, several research institutions and agro-industries are engaged in obtaining disease-free planting material of newly released, disease tolerant and commercially important genotypes of sugarcane through micropropagation techniques (Yadav *et al.,* 2004) [2]. High frequency *in vitro* plant regeneration in sugarcane through shoot tip culture has been reported earlier (Ramanand and Lal, 2004; Khan *et al.,* 2008; Mishra, 2011; Sengar *et al.,* 2011; Sharma *et al.,* 2015) [3, 4, 5, 6, 7]. *In vitro* micropropagation mainly depends upon various environmental and culture conditions. The media pH is also a critical factor among them that is directly correlated with plant growth under *in vitro* conditions. In this regard, a little but appreciable work has been done by various researchers (Ramanand and Lal, 2004; Singh, 2005; Mishra, 2011; Sengar *et al.,* 2011; Sharma *et al.,* 2015) [3, 5, 6, 7, 8]. Therefore, the present investigation was proposed to study the effects of culture media pH on *in vitro* shoot multiplication responses from shoot tip explants of two commercially cultivated sugarcane genotypes *viz.* Co 86032 and Co 94008.

Materials and Methods
In Vitro establishment of cultures
Very young and healthy tops (ca. 10-12 cm) of sugarcane genotypes *i.e.* Co 86032and Co

94008 excised from 4-6 months old field grown plants, maintained under the natural conditions in rain out shelter at Agricultural Biotechnology Research Station, S.V.P. University of Agriculture & Technology, Meerut, Uttar Pradesh, were used as explants to initiate the cultures. All the open green leaves were removed and about 5-6 cm long spindle segments were dissected and washed thoroughly under running tap water for 30 min containing 2-3 drops of Tween-20 (Hi-Media, India). Thereafter, the explants were surface sterilized with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 3 min followed by the treatments for 3 minute with fungicide [Carbendazim, 0.1% (w/v)] and bactericide [Streptomycin, 0.1% (w/v)] and then with 70% ethanol for 45 seconds. Finally, the explants were rinsed 4-6 times with sterilized distilled water under the aseptic conditions to remove the traces of the above mentioned surface sterilizing agents. About 1.5-2.0 cm long shoot tip explants containing apical meristematic dome along with 1-2 leaf primordia were carefully excised from the sterilized segments and immediately inoculated on to the full strength Murashige and Skoog's medium (Murashige and Skoog, 1962) [9] fortified with 100 mg/L myo-inositol, 30 g/L sucrose, N^6-benzylaminopurine (BAP) and N^6-furfuryladenine (Kinetin) [0.5 mg/L each] and solidified by 0.8% (w/v) bacteriological grade agar (Hi-media, India). The pH of the culture media was adjusted to 5.8 prior to autoclaving at 121ºC and 1.06 kg cm^{-2} for 20 min. All the cultures were incubated at 25±2ºC and 40 μmol^{-2} s^{-1} for 16h/8h light/dark photoperiod, provided by cool white fluorescent tubes (Philips, India). After six weeks of inoculation, established shoot cultures were transferred onto fresh MS media supplemented with higher concentrations of both 2.0 mg/L BAP + 2.0 mg/L Kinetin [MS-2 Media] for further multiplication. Thereafter, Four weeks old multiplicated shoots (1.0-2.0cm) were isolated and individually subcultured onto same MS media to obtain enough mother stock cultures for various experiments. All the above mentioned *in vitro* practices were carried out in Sugarcane Tissue Culture Laboratory at the same University.

Effects of media pH on shoot multiplication

To investigate the effects of pH on growth and multiplication of shoot cultures, the established plantlets were subcultured separately on media having pH values 5.2, 5.4, 5.6, 5.8, 6.0, 6.2 and 6.4. The pH was adjusted with 0.1 *N* NaOH or 0.1 *N* HCl before autoclaving. Other physical and chemical parameters were kept constant.

Statistical Analysis

Data on shoot multiplication was recorded after 6-weeks of treatment for all the cultured plants. All experiments were conducted in three replications with 12 test tubes for each treatment (n=12). The effect of different treatments on shoot multiplication was quantified and the data was statistically analyzed by using software OPSTAT 1.0.

Results & Discussion

The present investigation suggests that the manipulations in culture media pH significantly affect *in vitro* shoot multiplication in sugarcane. The optimization of culture media pH is a necessary but critical step to achieve rapid and efficient growth of plantlets by using *in vitro* practices. In the present study the growth responses observed in both the sugarcane genotypes in terms of culture media pH are illustrated here in detail. Data presented in Table-1 clearly demonstrates the effects of culture media pH (5.2-6.4) on *in vitro* shoot multiplication in two sugarcane genotypes *viz.* Co 86032and Co 94008. According to the results obtained in the present study, it is concluded that the rate of shoot multiplication and shoot growth was highly influenced by the pH of culture media. Best shoot regeneration responses were obtained at media pH 6.0 in both the genotypes studied followed by media pH 5.8. In the present study, genotype Co 86032showed highest shoot regeneration with 16.4±1.13 shoots/culture whereas; Co 94008 showed 13.8±1.43 shoots/culture at the same pH. At lower (5.2) and higher (6.4) pH both genotypes showed reduced shoot regeneration [Fig.-1]. Shoot growth was also poor on this pH. At pH 5.8-6.2, both genotypes showed vigorous and green shoots with a good growth. The results obtained in the current study showed that increasing in pH significantly increased shoot regeneration in both genotypes whereas the higher pH reduced shoot growth.

Table 1: Effects of culture media pH on *in vitro* shoot multiplication in two sugarcane genotypes after 6-weeks of explant inoculation on MS-2 media (Mean±SD)

pH of culture media	Co 86032		Co 94008	
	Average number of shoots/culture	Shoot growth	Average number of shoots/culture	Shoot growth
5.2	4.7±0.52	+	3.1±0.46	+
5.4	6.3±0.86	+	5.7±0.64	+
5.6	8.4±1.05	++	7.9±1.14	++
5.8	11.4±1.19	+++	10.7±1.26	+++
6.0	16.4±1.13	+++	13.8±1.43	+++
6.2	10.5±0.78	+++	9.4±0.63	+++
6.4	7.3±0.94	++	5.2±0.57	++

Here, + (Poor), ++ (Moderate), +++ (Good) shoot growth

Present study suggested that the responses regarding multiplication of shoot cultures were also influenced due to pH of the culture medium. Our results were in conformity with the reports of some earlier studies (Ramanand and Lal, 2004; Singh, 2005; Mishra, 2011; Sengar *et al.*, 2011; Sharma *et al.*, 2015) [3, 5, 6, 7, 8]. In their studies, Ramanand and Lal (2004) [3] and Singh (2005) [8] reported that the shoot multiplication rate was higher at pH 6.0 whereas, culture media pH 5.2 and 6.4 gave poor shoot regeneration responses. Therefore, the present study suggests that it is necessary to investigate the effects of culture media pH on plant growth and development before establishing efficient *in vitro* plant regeneration protocols for commercial purposes in this bio-energy crop due to its genotype dependent nature.

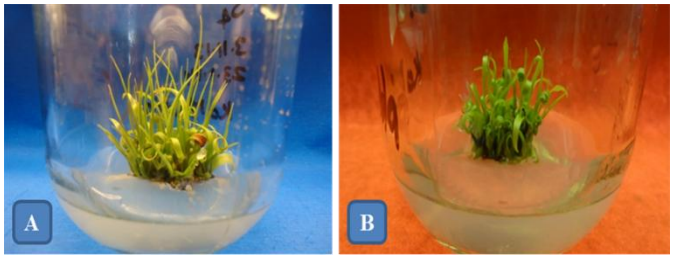

Fig 1: *In vitro* shoot multiplication in sugarcane varieties after 4 weeks at 25ºC, pH 5.8 and 16h photoperiod 4000 lux. Here, (A) Co 86032 (B) Co 94008. Full strength MS media was supplemented with BAP + Kinetin (2.0 mg/L each) + 30 g/L Sucrose + 100mg/L Myo-inositol + 8.0 g/L Agar.

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