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Screening and Determination of Sterilization Protocol and Optimum Growth Regulators for Micropropagation of Sugar Cane (*Saccharum officinarum* L.) in Ghana

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Abstract:
Micropropagation is a more rapid means to generate adequate healthy sugar cane planting materials towards increased sustainable production of the crop for industrial processing to produce sugar. However, stable axenic cultures and optimum growth regulators are critical to in vitro propagation of sugar cane. The study was to determine the optimum concentration of sodium hypochlorite, and some organic supplements to establish and multiply sugar cane in vitro. Murashige and Skoog (MS) media were supplemented with different concentrations and combinations of 6-Benzylaminopurine (BAP), 1-Naphthaleneacetic acid (NAA), Gibberellic acid (GA), Indole-3-butyric acid (IBA), kinetin and sucrose with blank medium as control. The media were inoculated with sterile shoots derived from initiated cultures of a local variety of sugar cane (B41227) and incubated at 26 ± 2^{o}C, 2500 Lux and 16 hours photoperiod for 6 weeks. The experiment was repeated with 15 cultures per treatment in three replications. 2.0% sodium hypochlorite was most effective to sterilize shoot explants with 68 % surviving cultures. A significantly ($P \leq 0.05$) highest mean number of shoots and fresh weight of 25.2 and 2.02g were induced by 2.0 mg/l BAP plus 2.0 mg/l GA with the least number of shoots (4.0) and fresh weight (0.32g) for control cultures. Rooting was significantly ($P \leq 0.05$) highest (10.3 per culture) for 45 % sucrose followed by 3.5 mg/l IBA (6.5 per culture). Indeed, 2.0 mg/l BAP plus 2.0 mg/l GA, and 45 g/l sucrose or 2.0 mg/l IBA were optimal for shoot proliferation and rooting respectively.

Keywords: *Micropropagation, propagation, axenic cultures, explants, proliferation*

1. Introduction

Sugar cane is an herbaceous perennial crop widely cultivated in the tropical and subtropical regions of the world (DSD, 2013; FAO, 2009; Guimarces & Sobral, 1998; Jahangir & Nasir, 2010). Nutritionally, it is a rich source of energy, carbohydrate, protein, calcium, iron, potassium and sodium. Sugar cane is the major source of sugar and contributes to 80% of the world sugar production with the remaining 20% from sugar beet (FAO, 2009). Africa, South Africa and Egypt are the major sugar producing countries with more than 15 million metric tonnes and 12 million metric tonnes respectively (Tarimo & Takamura, 1998). The use of sugar cane in the production of paper, antibiotics, dextran, waxes and fats, epoxy polymers and biofertilizer has also been reported (DSD, 2013; FAO 2014; Girma & Awulachew, 2007; Jalaja et al., 2008; Netafim, 2013). Sugar cane is propagated vegetatively by stem cutting, with 30-40 cm long fresh setts (Behera & Sahoo, 2009; Verheye, 2010). However, the rate of generating planting material for large scale production is low and may take a long time. Besides, there is also problem of contamination by systematic pathogens which reduce sugarcane yield and quality of the processed sugar when elite genotypes are multiplied in the open field using the conventional method (Behera & Sahoo, 2009; Schenk & Lehrer, 2000; Tarimo & Takamura, 1998). It takes a multi-stage selection scheme over a period of 6-10 years to develop suitable varieties of sugar cane for commercial use and industrial purpose (Khan *et al*., 2006; Singh *et al*., 2001). In Ghana, the sugar cane industry is being revamped with the re-establishment of the Komenda sugar factory but there is lack

of sugar cane raw material to feed this newly built sugar factory. The conventional method of generating planting material is woefully inadequate to establish large scale sugar cane plantations.

Clonal propagation through tissue culture is one of the most widely accepted methods used for commercial production of genetically improved plants (George, 1993; Tiwari *et al*., 2012). The development of protocols for sugar cane tissue culture has been reported (Anita *et al*., 2000; Barba *et al*., 1977; Jalaja *et al*., 2008; Naz, 2003) and remains the only realistic means to produce large quantities of disease free planting material of elite sugar cane clones. Axillary bud culture was applied by Sauvaire & Galzy (1978) to produce true to type clones in many sugar cane varieties. An apical meristem culture technique for rapid multiplication of mosaic virus free plants of sugarcane has been standardized (1983; Coleman, 1970; and Hendre *et al.* 1975). Sreenivasan & Jalaja (1981) also standardized micropropagation technique based on the use of apical meristem with two or three leaf primodia as the explant. Attempts made to decontaminate both epiphytic and endophytic contaminants from sugarcane bud explants with the use of heat treatment coupled with surface sterilization prior to the culture of sugarcane bud explants failed (Waterworth & Kahn, 1978). Similarly, Ahloowalia & Meretzeki (1983) also failed in the attempt to use a combination of benylate and sodium hypochlorite solution followed by surface sterilisation with 95% ethanol. Though micropropagation is a more reliable method for rapid multiplication of sugar cane planting material, it is challenged by microbial contamination of cultures and requirement for optimum growth regulators to enhance regeneration of cultures and planting materials towards commercial production of the crop to meet industrial needs. The current study sought to employ *in vitro* propagation protocol with optimized sterilization reagents and growth regulators for rapid multiplication of sugar cane for farmers to cultivate to meet industrial demand in Ghana.

2. Materials and Methods

The study was conducted in the laboratory of Department of Molecular Biology and Biotechnology, University of Cape Coast, Ghana. Samples of stem cuttings of sugar cane genotype B41227 were obtained from a plantation of Komenda sugar factory. The stock plant materials were established in plastic pots in the Botanical garden. The young axillary and terminal shoot tips were excised with a pair of knife and transferred to the laboratory. The outer leaves were removed and the spindle was washed with a solution of two drops of Tween 80 under running tap water. The explants were then placed in a beaker and treated with different concentrations of sodium hypochlorite (NaOCl) solution (1.0%, 1.5%, 2.0%, 2.5% and 3.0%) for 10 min and further dipped in 70% ethanol for 1 min and then rinsed with four changes of sterile distilled water to remove all traces of chemicals. The explants were then inoculated onto 15 ml Murashige and Skoog (MS) basal medium supplemented with 1g/l BAP and 30g/l sugar in 25 x 250 mm test-tubes. The medium was adjusted to pH of 5.8, solidified with 7g/l agar and then sterilized at 121 ^{o}C for 15 minutes. The cultures were incubated at 26 ± 2^{o}C, 2500 Lux and 16-hour photoperiod for 6 weeks. The established sterile developing buds were transferred onto MS basal medium supplied with 1.0 mg/l BAP (6-Benzylaminopurine), 2.0 mg/l BAP, 2.0 mg/l BAP + 0.5 mg/l NAA (1-Naphthaleneacetic acid), 2.0 mg/l BAP + 0.5 mg/l IBA (Indole-3-butyric acid), 2.0 mg/l BAP + 0.5 mg/l Kin (Kinetin), 2.0 mg/l BAP + 2.0 mg/l GA (Gibberellic acid), and O.5 mg/l BAP + O.5 mg/l Kin + 0.5 mg/l GA for shoot multiplication and fresh weight (Behera & Sahoo, 2009; Biradar *et al*., 2009; Mekonnen, 2014; Singh *et al*., 2001): For root inducement, the developing buds were transferred onto half strength MS basal medium supplemented with different concentrations of IBA (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 mg/l), and NAA (0.5, 3.0, 4.0, 5.0 mg/l) as well as different concentrations of sucrose (30.0, 45.0, 40.0, 45.0, 50.0, 55.0, 60.0, 65.0 g/l). One explant was cultured in each of 25 x 250 mm tubes which represented an experimental unit and this was replicated 15 times per treatment in a randomized complete block design outlay.

3. Results and Discussions

The survival rate and decontamination of the initiated cultures differed significantly ($p \leq 0.05$) in response to different concentrations of NaOCl used for the surface sterilization. There was 100% decontamination of sugar cane explants but low survival of explants (47 %) in response to 3.0 % NaOCl whilst 68 % and 67 % surviving and developing cultures were obtained for 2.5% and 2.0% NaOCl respectively. The least decontamination and survival rates were observed in 1.0 % NaOCl followed by 1.5% NaOCl (Fig. 1). No significant ($P > 0.05$) difference exists between the effects of 2.0% NaOCl and 2.5 % NaOCl. Wang and Juang (1971) used 3.0 % NaOCl for sterilization of sugar cane explant and reported callus tissues formation which grew weakly and eventually turned brown and deteriorated after transfer unto fresh media. In contrast, Danso *et al*. (2011) obtained 30 % sugar cane explant survival through a double sterilization treatment using NaOCl at 3.5 % for 7 min and 1.75 % for 3 min. According to Jalajaj et al. (2008), a 10 % NaOCl solution and 20 min treatment is most effective to surface sterilize sugar cane explant.

A significantly ($P \leq 0.05$) highest shoot multiplication (25.2 per culture) was observed for 2.0 mg/l BAP + 2.0 mg/l GA followed by 1.0 mg/l BAP (19). Shoot fresh weight were similar on 2.0 mg/l BAP + 2.0 mg/l GA (2.02 g) and 1.0 mg/l BAP (1.62 g) (Fig. 2 and 3). The 2.0 mg/l BAP induced similar average number of shoot proliferation (17.4) as 2.0 mg/l BAP + 0.5 mg/l Kin (17.2) but a lower shoot fresh weight (1.54 g) than 2.0 mg/l BAP + 0.5 mg/l Kin (1.62 g). The concentration of BAP required for shoot initiation and establishment has been reported to be dependent on the crop genotype (Biradar *et al*., 2009). The combination of BAP and Kin in a medium is important for shoot multiplication than only BAP (Mekonnen *et al*., 2014), however, this was contrary to the observation made in the current study (Fig. 2). BAP at a concentration of 1.0 mg/l showed a maximum frequency of shoot and fresh weight establishment than 2.0 mg/l but according to Biradar *et al*. (2009), BAP promoted higher shoot multiplication at 2.0 mg/l concentration. The control recorded the lowest mean for both the number of shoots (4.0) and fresh weight (0.32). However, this emphasize the need to supplement MS medium with the requisite growth regulators, which conforms to similar reports by Mekonnen *et al*. (2014) and Biradar *et al*. (2009). Besides, Danso *et al*. (2011) established 5mg/l BAP, 2mg/l IAA, 2mg/l GA3 and 3g/l activated charcoal as well as antibiotics as nutrient supplemented to the MS medium to obtain single or multiple shoot axenic cultures.

Maximum rooting in response to IBA, NAA, and Sucrose effects were recorded at 3.5 mg/l, 5.0 mg/l, and 45.0 mg/l with a mean of 6.5, 5.0, and 10.33 respectively (Fig. 4, 5 and 6). There were no roots development in response to 5.5 mg/l and 6.0 mg/l (IBA), 30.0 g/l and the control (Sucrose free medium). It is obvious that sucrose alone could induce higher rooting than the auxins used in the current study. This implies that optimum sucrose concentration may influence endogenous auxin synthesis as well as enhance biosynthesis to promote development of sugar cane plantlets. Indeed, rooting of cultures is necessary to establish micropropagated plants *ex-vitro*. The use of 45 mg/l sucrose supplement in MS medium may be optimum for rooting of sugar cane cultures which could be a low-cost option compared to exogeneous auxin supplemented MS medium.

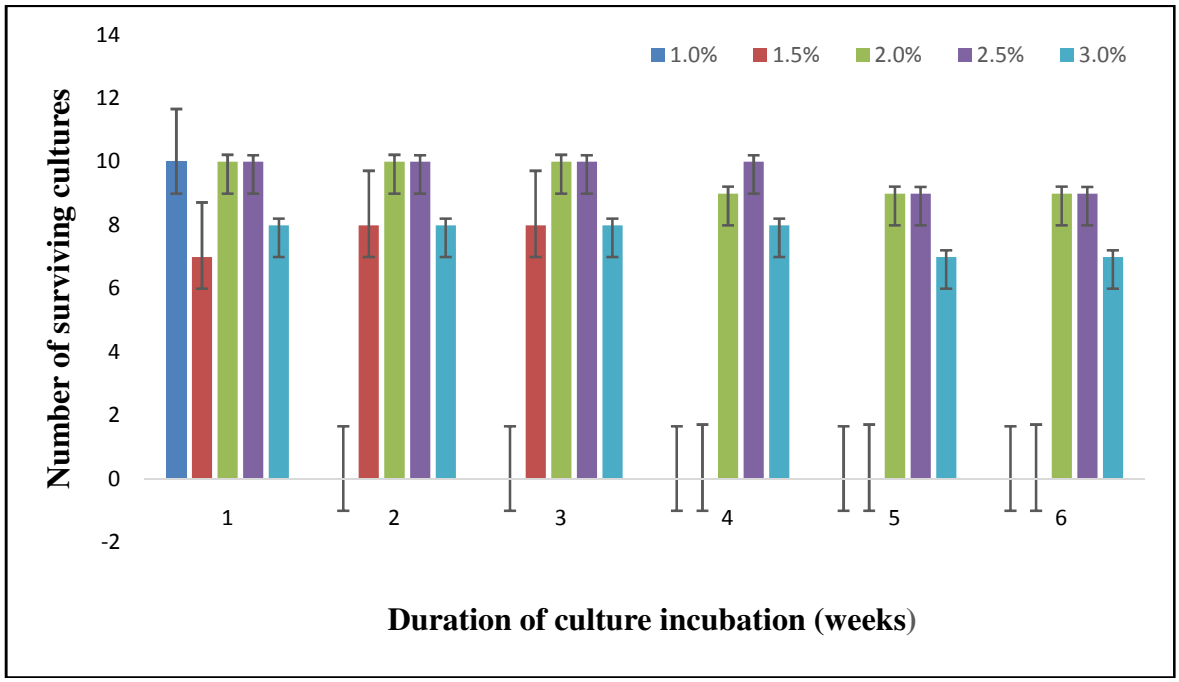

Figure 1: Effect of different concentrations of sodium hypochlorite on the survival rate of sugar cane shoots.

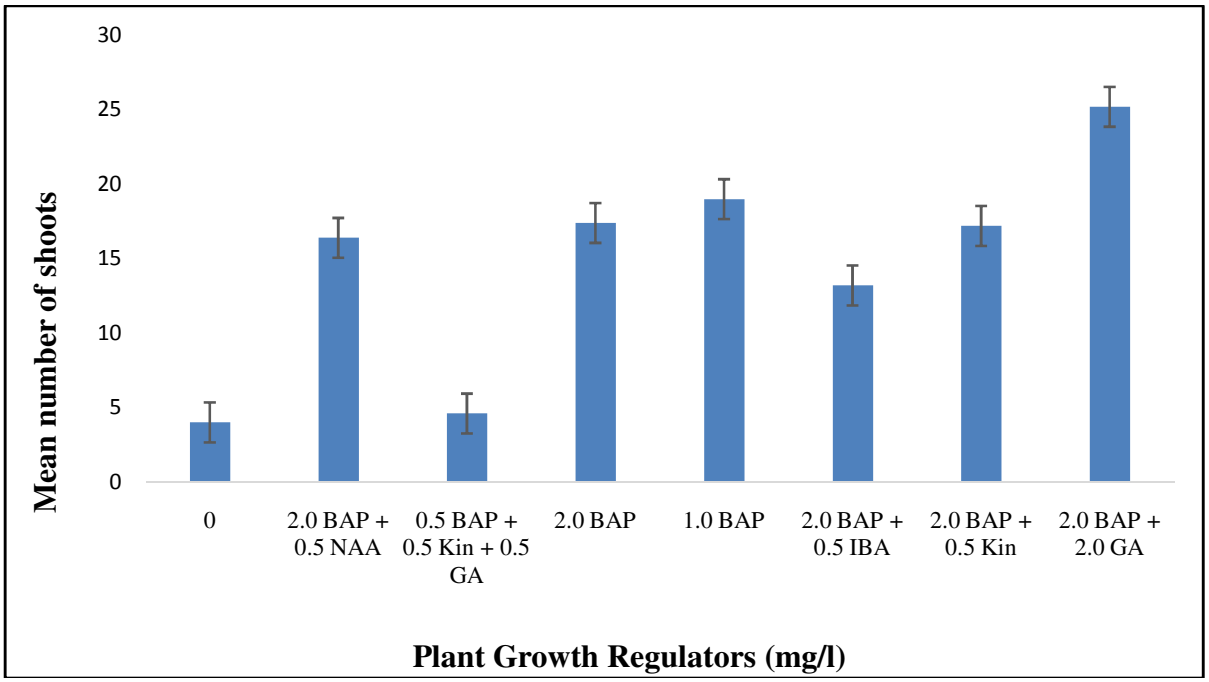

Figure 2: Effect of growth regulators on shoot multiplication. Zero indicates the absence of hormone (control).

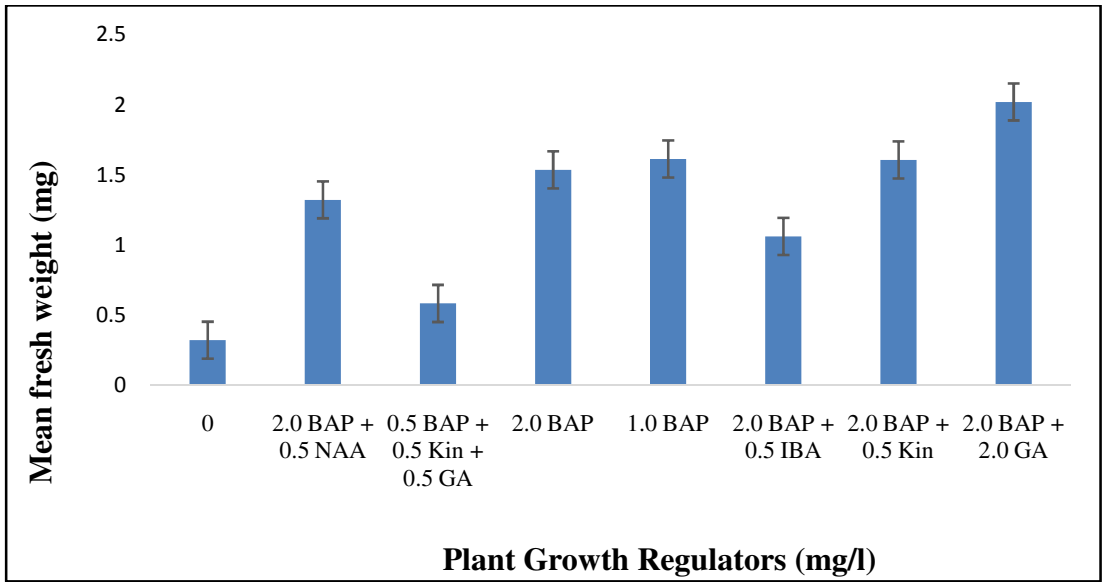

Figure 3: Effect of growth regulators on fresh weight. Zero indicates the absence of hormone (control).

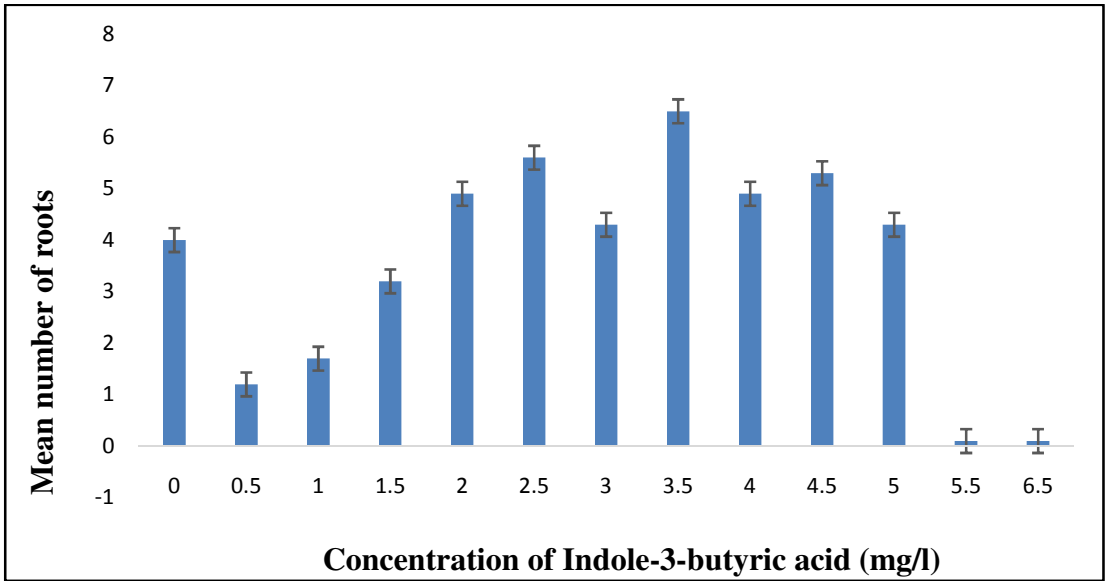

Figure 4: Effect of Indole-3-butyric acid (IBA) on root development. Zero indicates the absence of growth regulator (control)

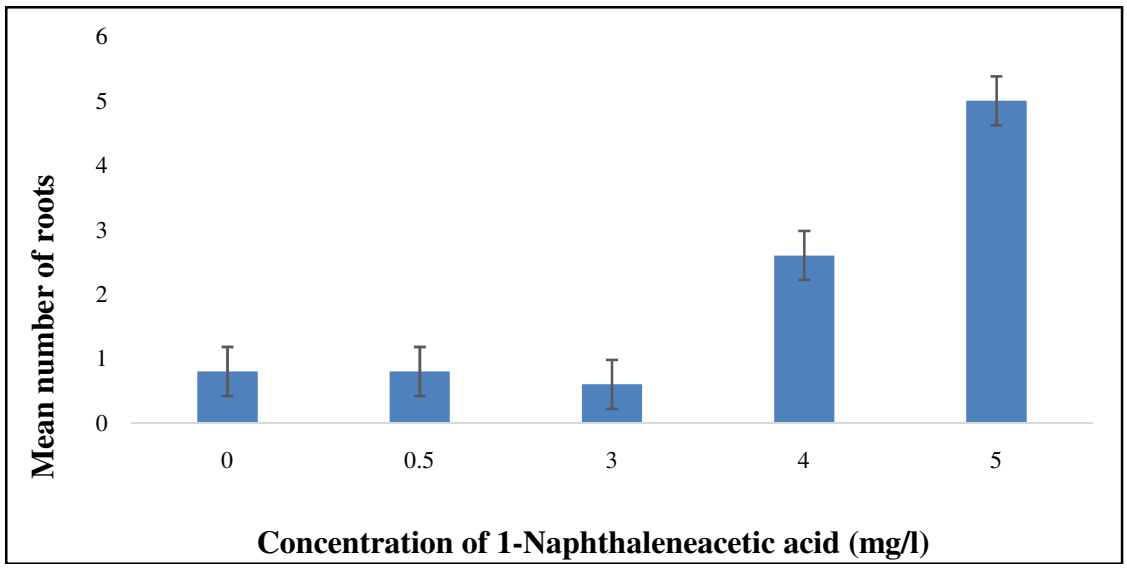

Figure 5: Effect of 1-Napthaleneacetic acid (NAA) on rooting of sugar cane cultures. Zero indicates the absence of hormone (control)

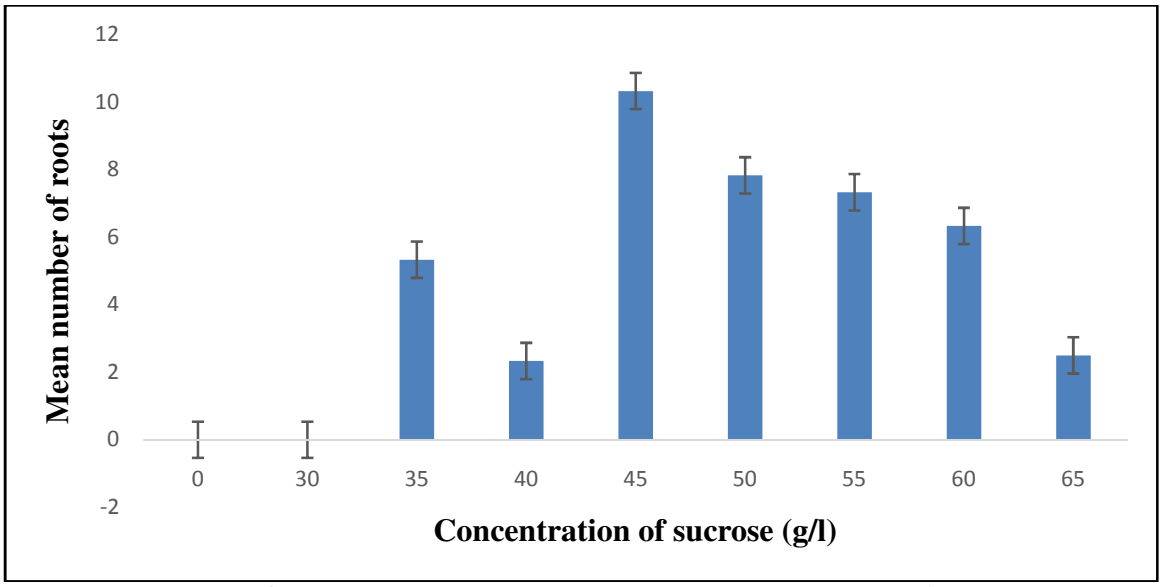

Figure 6: Effect of sucrose concentration on rooting of sugar cane cultures

4. Conclusion

On the whole, 2.0% sodium hypochlorite (NaOCl) solution was most effective for sterilization of sugar cane genotype B41227 shoot tip explants to establish sterile cultures. The concentration and type of plant growth regulator significantly ($P \leq 0.05$) influenced shoot and root multiplication. The best performance for shoot multiplication and fresh weight was in response to media supplemented with 2.0 mg/l BAP and 2.0 mg/l GA. Low amounts of NAA and high amounts of IBA and sucrose reduced root development. In all, 2.0 mg/l IBA, 5.0 mg/l NAA and 45.0 g/l sucrose were optimal to induce significantly ($P \leq 0.05$) high rooting response of sugar cane cultures. The findings obtained will facilitate rapid propagation of high quality sugar cane planting materials to scale up commercial production of the crop in Ghana. It will also enhance storage of sugar cane germplasm under aseptic conditions.

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