Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.11, No.3, 2021

Development of In Vitro Callus Induction and Regeneration Protocol for Newly Released Sugarcane (Saccharum officinarum L.) Genotypes from Inner Leaf Sheath

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Abstract
Improvement of sugarcane through conventional breeding takes longer time (8-10 years) to release new improved genotypes. Callus induction and regeneration protocol is a prerequisite for genetic improvement through genetic engineering. Hence, this study was initiated to develop a protocol for in vitro callus induction and regeneration of three sugarcane genotypes (C86-56, C132-81 and SP70-1284) using immature young leaf as a source of explants. Seed cane of 30 cm size having 2 nodes each were obtained from Metehara Research and Development, operating under Ethiopian Sugar Corporation. They were then brought to the National Agricultural Biotechnology Research center and planted in the greenhouse to serve as sources of explants. The treatments were arranged in a factorial experiment laid out in a completely randomized design. For callus induction MS medium supplemented with five levels of 2,4-D (2.0, 2.5, 3.0, 3.5 and 4.0 mg/l) was used. Immature leaves were collected from the green house grown plants 3 months after planting and were cultured on callus induction medium after sterilizing with 5% berekina for 15 minutes and then followed by 70% alcohol for 30 seconds under the biological safety cabinet. For plant regeneration four levels of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) were used in combination with four levels of IBA (0.0, 0.25, 0.5 and 0.75 mg/l). Callus induction experiment was replicated four times and each replicate was represented by four explants per a culture jar and shoot regeneration experiment was replicated three times, 2 embryogenic calli per jar each for regeneration. Data were recorded for all parameters and analyzed using Statistical Analysis System (SAS) Software version 9.3. Means were separated using least significant differences (LSD) at 0.001 probability level. Analyses of variance indicated that days to callus initiation, frequency of callus induction and callus weight were affected significantly ($p \leq 0.001$) by genotype, 2, 4-D levels and their interaction. Genotype C132-81 was identified as the best for early callus initiation, high frequency of callus and callus weight. The highest percentage (92.31%) of callus initiation was recorded on medium supplemented with 3.0 mg/l of 2,4-D with higher callus weight (454.5 mg). Regeneration percentage, number of shoots, shoot length and number of leaves per shoot were also significantly ($p \leq 0.001$) affected by genotype, BAP, IBA and their interaction effect. Genotype C132-81 also recorded significantly higher number of shoots (43.44±0.5) on MS medium supplemented with 1 mg/l BAP in combination with 0.5 mg/l IBA. Genotype C86-56 gave higher shoot length (6.38±0.08) cm while C132-81 gave the higher number of leaves (8.24±0.06) at 2.0 mg/l BAP+0.25 mg/l IBA. In conclusion, 3.0 mg/l and 3.5 mg/l 2,4-D concentrations were found optimum for callus induction and development, while 1.5 mg/l BAP+0.5 mg/l IBA was the best for plant regeneration.
Keywords: genotype, explant, callus induction, regeneration
DOI: 10.7176/JBAH/11-3-05
Publication date: February 28th 2021

INTRODUCTION

Sugarcane (*Saccharum officinarum L.*) is a major industrial cash crop widely cultivated in tropical and subtropical countries of the world for sugar and bioethanol production. It is a perennial tropical crop that tillers at the base to produce unbranched stems of 3-4 m in length or more with a thickness of around 5 cm in diameter (Dereje *et al*., 2015).

Currently, in the world more than 130 countries produce either sugarcane or sugar beet, and from that on average, sugarcane produces about 80% of global sugar production (ISO, 2017**)**. Globally, over an area of 26.5 million hectares of land is used for sugarcane cultivation with an annual production of 1.9 billion tons cane and productivity of 71.7 tons/ha. In Africa, above 1.58 million hectares of land area is covered by sugarcane with 97.17 million tons of cane production, and an average yield of 56.81 tones/ha (FAOSTAT, 2014**).** In Ethiopia, Sugarcane is planted in different parts of the country and currently covers an area of 102,741 hectares and production of 400,000 tons of sugar and 25,388 m^3 of ethanol per annum (Esayas *et al*., 2018).

In commercial scale cultivation, stem cutting is the main propagation method. However, the amount of planting material provided by this method is limited as it has a 1:10 hectare ratio (Belete *et al*., 2017). It is very

low in quick multiplication of new genotypes and takes 10-12 months to use as a seed cane to get best sprouting. In addition, there is a high risk of disease transmission during seed cane preparation, distributions within and among sugar estates leading to yield loss. Nowadays, unlike the conventional propagation method, micro propagation is the only realistic means of achieving rapid and large scale production of disease free quality planting materials in sugarcane and alternative approach for fast multiplication of a variety maintaining its genetic identity (Belay *et al.*, 2014).

In vitro propagation and other *in vitro* techniques are commonly used in agriculture and horticulture for resolving several problems related to plant and mass propagation of crop plants. *In vitro* propagation of sugarcane is a model system to overcome weed control via herbicide tolerance, sugar uptake, disease control, sugar metabolism and improvement of yield components (Jamil *et al*., 2017). Use of plant tissue culture is a preferable technique to solve the agricultural problems over conventional methods, as plants are grown in controlled environments.

Plant regeneration from tissue culture of sugarcane has been successfully applied to breeding programs for rapid screening of clones for disease resistance, salt tolerance, drought tolerance, herbicide resistance and early maturity and high sugar (Sharma *et al*., 2002). Callus induction is a very important phenomenon in tissue culture. Since in tissue culture different genotypes of the same species respond differently to media (Roy, 2000), it is recommended that an efficient protocol is needed for every new genotype or clone of sugarcane to get rapid callus induction and development (Behera and Sahoo, 2009).

Thus, it is of paramount importance to develop specific *in vitro* callus induction protocols for the major commercial sugarcane genotypes. Several studies have been done on callus induction of sugarcane using different source of explant (Ali *et al.,* 2007; Gandonou *et al*., 2015; Patel *et al*., 2015; Rao and Arjun 2015; Jamil *et al*., 2017 and MM *et al*., 2018). But in Ethiopia, very few studies were carried out on micro propagation of sugarcane through direct organogenesis and no study was done on callus induction from inner leaf sheath. The present study was initiated to optimize conditions for callus induction and regeneration in sugarcane. This optimized protocol will help still in maximizing multiplication factor to regenerate plantlets within a shorter time, large scale production of disease free planting and create novel genetic variation for improvement of genotypes from callus culture through genetic engineering. So, the next researchers can be able to improve genotypes using this established and efficient protocol in screening for various stresses such as resistance to diseases, insect pests and for achieving high yielding genotypes. This study was carried out to test the ability of three genotypes of sugarcane (C132-81, SP70-1284 and C86-56) for callus induction, embryogenic callus production and plant regeneration using inner leaf sheath explants and to identify the genotypes with high regeneration capacities for *in vitro* selection programs. Therefore, the current study was initiated to develop a protocol for *in vitro* callus induction and regeneration of three sugarcane genotypes using immature young leaf as a source of explants.

MATERIALS AND METHODS

Plant materials and explants

Three sugarcane genotypes, viz., C86-56, C132-81 and Sp70-1284 were used in the study. The genotypes were imported from Cuba in 2006 and passed through agronomic performance evaluation and were then planted at Metehara Research and Development for Commercialize sugar production (Feyissa *et al*., 2014). They were selected for the study based on their high yield performance and sugar quality. The study was conducted at the National Agricultural Biotechnology Research Center (NABRC) Plant Tissue Culture laboratory located at Holeta. Seed cane of 30 cm size having 2 nodes each were obtained from Metehara Research and Development, operating under Ethiopian Sugar Corporation and were then planted in the greenhouse at NABRC to serve as sources of explants Before planting, the seed canes were treated with hot water at 50^{o}C for 2 hours.

For growing the seed canes in the greenhouse soil was prepared by mixing sand, forest soil and compost at the ratio of 1: 1: 1 and filled into pots. Then the soil was watered by applying water until they were wet enough for planting. In each of the pots two bud sets were planted and then the pots were labeled with the name of the genotypes and the dates on which they were planted. Then watering and other agronomic activities were carried out regularly as required. Immature leaves from inner part of the leaf sheath were collected 3-4 months after planting to serve as explants in *in vitro* culture.

Explant Preparation and Surface Sterilization

Explants were prepared following the method by Tilahun *et al*. (2013) by removing the surrounding leaf sheaths carefully one by one until the inner white sheaths were exposed. Then, 10 cm long tops were collected by cutting off at the two ends, locating the growing point somewhere in the middle of the top. The inner leaf sheaths were washed under running tap water for 30 min with soap solution and treated with 0.3% kocide (fungicide solution) for one and a half hours under laminar air flow cabinet. After decanting kocide solution, inner leaf sheaths were washed three times with sterile distilled water and further immersed in 70% ethanol for 30 s and rinsed three times with sterile distilled water to remove ethanol.

To increase efficacy, two drops of Tween-20 solution were added into Berekina solutions. Tween-20 solution is a wetting agent added to the disinfectants to reduce surface tension and allow better surface contact. Decanting the sterilizing solutions under safe condition, the explants were washed three times each for 5 min with sterile distilled water and left for 10 min to make the surface dry. Thereafter, leaf sheaths damaged during sterilization were removed using sterilized forceps. Finally, 2 cm long leaf whorl were excised with sterilized surgical blade. The outer two to three whorls of leaves were removed and the innermost whorls, the immature leaf rolls were cut into transverse sections/segments (Ali *et al*., 2012). The discs obtained from immature leaf rolls were aseptically transferred/cultured on Murashige and Skoog (1962) medium solidified with agar (40 ml in 150 ml jars).

Culture Media Preparation

The stock solutions were first prepared by dissolving the required quantity of chemicals in double distilled water and stored under refrigerated conditions. They were done by adding the required amount of MS stock solution and sucrose (30 g/l) in a conical flask.

Types of stock	Compound	Mg/l	100x	Amount required	1000x	Amount required
Stock I /Macro Stock	$KN0_3$	1900	190	10mg/l	1900	1mg/l
	NH_4N0_3	1650	165		1650	
	KH_2P0	170	17		170	
	$MgS0_4{\cdot}7H_2O$	370	37		370	
Stock II	$CaCl_2{\cdot}2H_20$*	440	44	10mg/l	440	1mg/l
Stock III /Micro stock	$MnS0_4{\cdot}4H_20$	22.3	2.23		22.3	1mg/l
	KI	0.83	0.083	10mg/l	0.83	
	H_3B0_3	6.3	0.63		6.3	
	$ZnS0_4{\cdot}7H_20$	8.6	0.86		8.6	
	$CuS0_4{\cdot}5H_20$	0.025	0.0025		0.025	
	$Na_2Mo0_4{\cdot}2H_20$	0.025	0.0025		0.025	
	$CoCl_2{\cdot}6H_2O$	0.025	0.0025		0.025	
Stock V /Iron stock	$FeS0_4{\cdot}7H_2O$	27.8	2.78	10mg/l	27.8	1mg/l
	Na_2EDTA	33.6	33.6		33.6	
Stock VI / Vitamin Stock	Nicotinic acid	0.05	0.005	10mg/l	0.05	1mg/l
	Pyridoxine-HCI	0.05	0.005		0.05	
	Thiamine-HCI	0.05	0.005		0.05	
	Inositol*	100	10		100	
	Glycine	0.2	0.02		0.2	

The medium (40 ml) was dispensed into glass culture jar and stored under aseptic condition at + 4^0C until use. The pH of the medium were adjusted at 5.8 by using 0.1N NaOH or 0.1N HCl. To solidify the media 2.3 g/l phytagel were added and the medium boiled by oven. Finally, the medium was dispensed in to bottles or test tubes and sterilized using autoclave at a temperature of 121°C with a pressure of 105 KPa for 20 minutes and the cultures were incubated in dark which was adjusted at 25 ± 2°C and relative humidity of 70-80%.

Treatments and Experimental Design

Callus induction

The prepared leaf whorl was cultured on the MS basal medium supplemented with different levels of 2, 4-D (2, 2.5, 3, 3.5 and 4 mg/l). The treatments were arranged in a 3 X 5 factorial experiment laid out in a completely randomized design with four replications. For callus induction the cultures were maintained in dark for four weeks. Distinction between embryogenic and non-embryogenic callus were performed on the basis of callus external aspect. Embryogenic calli are of glossed aspect, compact, characterized by their white to cream colour and their nodular structure, while non-embryogenic callus are of wet aspect, translucent or of brownish colour. After 4 weeks of culture, data were recorded on initiation of callus, percentage of explants that induced callus, number of days taken for callus initiation and weight of callus. The callus masses obtained in the various concentrations of 2, 4-D were multiplied and maintained by sub culturing. Every two weeks the healthy looking and fast growing tissue fragments from the callus mass was sub cultured on MS medium supplemented with 2,4-D (2, 2.5, 3, 3.5 and 4 mg/l). Callus induction efficiencies were calculated by applying the following formula as given below.

$$\text{Callus induction efficiency} = \frac{\text{Number } of \text{ explants induced callus}}{\textit{Total} \text{ number } of \text{ cultured } \textit{explants}} x100$$

Shoot regeneration

For plant regeneration embryogenic calli were transferred to full strength MS basal medium. Supplemented with four levels of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) and four levels of IBA (0.0, 0.25, 0.5 and 0.75 mg/l) after 4 weeks. The treatments were arranged in a 3 X 4 X 4 factorial experiment with three replication each replication having plants per culture jar. The cultures were incubated in growth cabinet at 25±2°C under 16/8 h light/dark photoperiod. Calli that regenerated plantlets were recorded after 5 weeks and the data were expressed as a percentage of regenerated calli per total number of calli transferred for regeneration. Considering the very high number of plant obtained per callus, plant density per callus was estimated visually. Capacity of plant regeneration was assessed by calculating plant regeneration efficiency as given below:

$$\text{Plant regeneration efficiency} = \frac{\text{Number of explants regenerated shoots}}{\textit{Total} \text{ number } \textit{of} \text{ cultured callus induced explants}} \; x100$$

Data analysis

Analysis of variance was done using Statistical Analysis System (SAS) Software version 9.3. Comparisons of the treatment means were done using the least significant difference (LSD) test at 1% level of significance.

RESULT AND DISCUTION

Callus induction

Swelling of the explants was observed one week after inoculation. However, callus initiation was observed in the second week after inoculation. Three weeks later, callus initiated at the cut edge of the explants and developed into a full grown callus. The callus was morphologically found to be yellowish-white, compact, dry and nodular. Number of days taken for callus induction was significantly ($P<0.001$) affected by genotype, levels of 2,4-D and their interaction. It ranged from 10.5 to 19.25 days where the highest number of days (19.25) was taken by treatment T_1 (MS medium supplemented with 2.0 mg/l of 2, 4-D) for the genotype C86-56. The earliest callus induction (10.5 days) was noticed in the fifth treatment (T_5) in which genotype C132-81 was cultured on MS medium supplemented with 4.0 mg/l of 2, 4-D. The variation might be due to the genotypic difference in response to growth regulators and the difference in performance of different growth regulators towards the explant *in vitro.*

Increasing 2, 4-D from 2 to 4 mg/l showed a significant decrease from 19.25 to 11.25, 17.25 to 10.5 and 18.25 to 11.75 in number of days taken for callus initiation in C86-56, C132-81 and Sp70-1284 genotypes respectively (Table 1). Different studies (Kureel *et al.* (2006), Behera and Sahoo (2009), Tahir *et al.* (2011), Parmar Rina *et al*. (2017), Rahman *et al*. (2018) and Kona *et al*. (2019)) reported that number of days taken to callus induction increased with increasing concentration of 2,4-D from the 4 mg/l to 6 mg/l. As shown in Table 1although in all concentrations of 2, 4-D, callus induction was triggered significantly observed at 2, 4-D concentration between 2mg/l and 4mg/l. But embryogenic callus formation was higher with 2, 4-D levels of 3 mg/l and 3.5 mg/l.

In general significantly higher percentage of the callus was initiated in between 2.5mg/l and 3.5mg/l 2, 4-D levels which also resulted in better embryogenic callus formation and successful shoots regeneration. The cumulative frequency of callus initiation varied from 44.93 to 92.31 % among the genotypes (Table 1; Figure 1). This different response might be due to differences in the capability of the genotypes to induce callus, culture media and their interaction with different concentration levels of 2, 4-D hormone. The highest percentage (92.31%) of callus initiation was observed in 3.0 mg/l of 2,4-D in the genotype C132-81 followed by 85.12% in 3.5 mg/l in the genotype Sp70-1284 and 80.43% in 2.5 mg/l of 2, 4-D in the genotype C132-81, while C86-56 gave the lowest callus induction (44.93%) in 2mg/l of 2,4-D. This could be due to variation in the endogenous level of auxin hormone in the three genotypes which then required varied levels of supplemental auxin hormone through the medium for callus initiation.

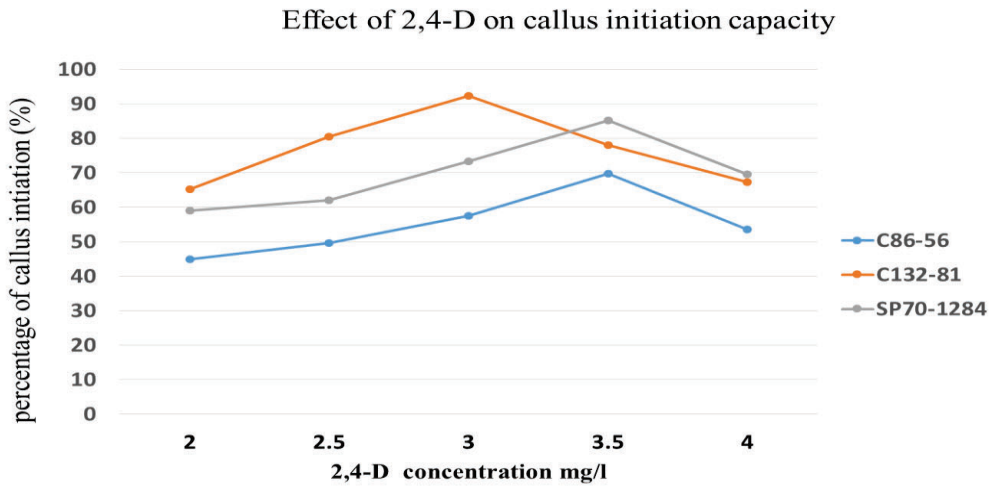


Figure 1. The value of Frequency of callus induction in three sugarcane genotype increased with increasing of 2, 4-D from 2 to 3 mg/l in the three genotypes, also increased with increasing 2, 4-D from 2 to 3.5 mg/l in both genotypes Sp70-1284 and C86-56.

Embryogenic characteristic of callus is a very important parameter. It shows the ability of the callus to regenerate plant from one cell or few numbers of cells. Differences between embryogenic and non-embryogenic callus was observed on the basis of external appearance of the callus. From the result two types of calli were observed based on morphological appearance. (I) Type A-yellowish white, friable, dry and nodular (Figure 2) and (II) type B- whitish globular, compact and wet (Figure 3). These types of calli have also been reported by Khan *et al.* (2008) and Khatri *et al.* (2002).

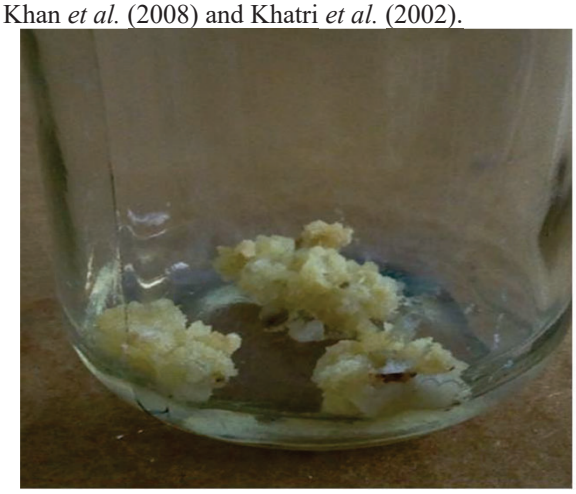


Figure 1. Type A callus

Figure 2. Type B callus

After four weeks of culture, a considerable mass of white, compact and yellow, fast-growing, friable callus had accumulated on all the explants cultured.

Callus weight

Callus weight was also significantly ($P<0.001$) affected by genetype, levels of 2,4-D and their interaction. Callus weight and proliferation are the main parameters used for callus induction, as they have direct effect on final callus. (Table 1). The genotypes responded to the levels of 2,4-D in terms of callus weight the same way they responded for callus induction.

Higher callus weight (454.5mg) was obtained on MS media supplemented with 3 mg/l 2, 4-D and followed by (430.75 mg) obtained on MS media supplemented with 3.5 mg/l 2, 4-D in genotype in C132-81 and the lower callus weight was obtained in genotype C86-56 (114.25mg) on MS media supplemented with 2 mg/l 2, 4-D. Genotype Sp70-1284 had intermediate callus weight relative to the two genotypes with higher callus weight (378.75mg) on MS media supplemented with 3.5 mg/l 2, 4-D and less callus weight (189.75mg) was recorded on 2 mg/l 2, 4-D. For C86-56 genotype, maximum weight of callus (349.25mg) was recorded on MS media supplemented with 3.5 mg/l 2, 4-D and the least callus weight (114.25mg) was obtained for the same genotype

on 2 mg/l 2, 4-D (Table 1).

Comparative results were accounted for by Gemechu *et al.* (2014) produced maximum callus weight (600mg) on MS media supplemented with 3 mg/l auxin/2, 4-D and less callus weight (200mg) was recorded on 2 mg/l 2, 4-D. Contrary to this result, Khan *et al.* (2008) observed maximum callus weight on 4 mg/l 2, 4-D for the varieties they tested. Rashid *et al.* (2009) obtained maximum callus size on MS media supplemented with 2, 2.5, and 3 mg/l 2, 4-D. The present work is quite different from that of Gopitha *et al.* (2010) found best result of callus induction at lower concentration of 2, 4-D.

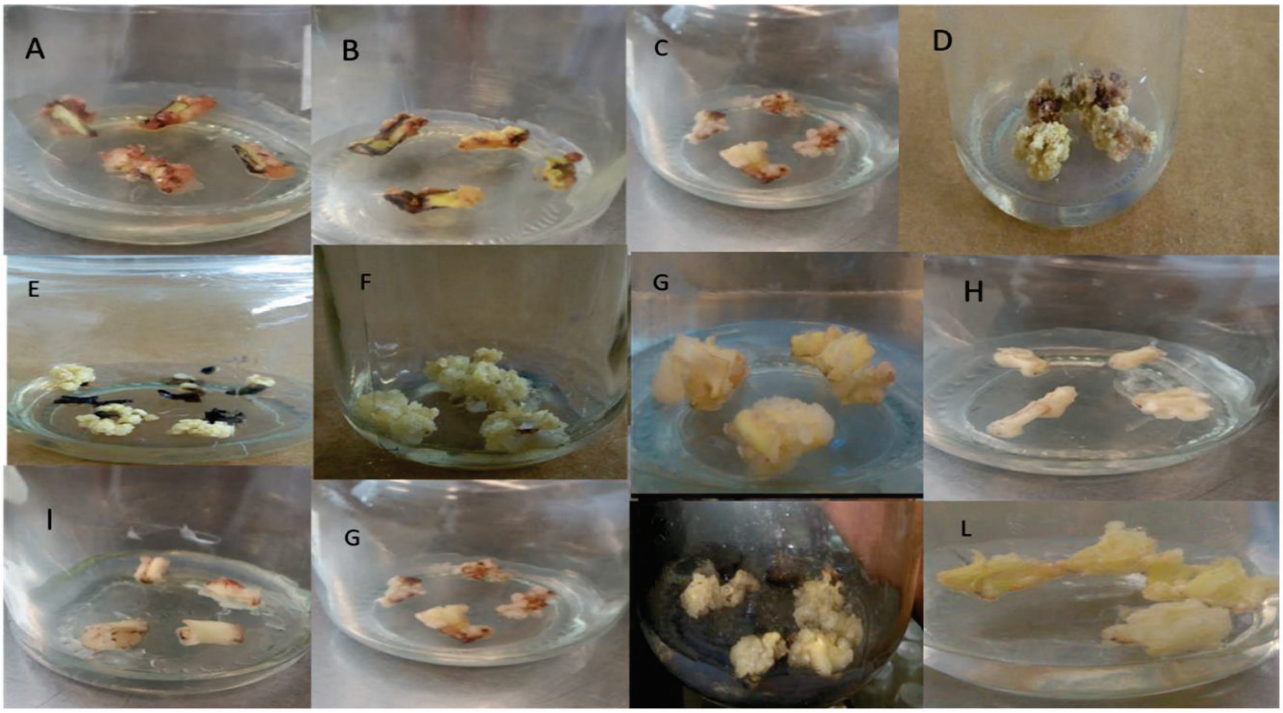
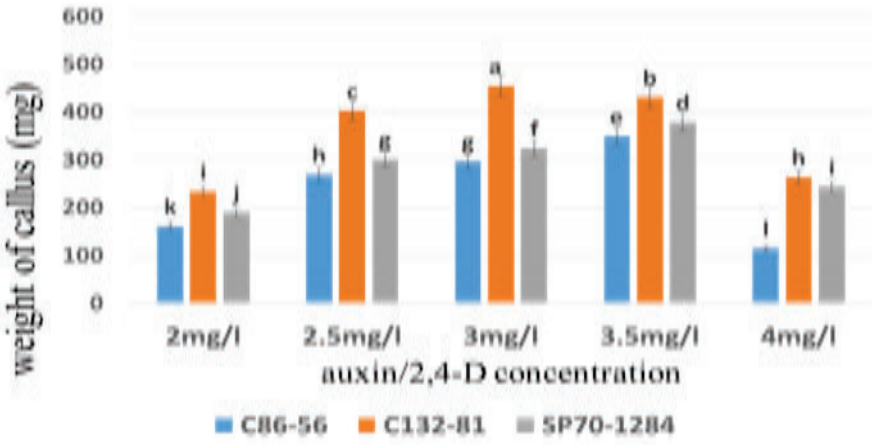


Figure 4. Callus induction and development on MS medium supplemented with different concentration of 2, 4-D. **(A)** MS medium supplemented with 2 mg/l in C86-56; **(B)** MS medium supplemented with 2.5 mg/l in C86-56; **(C)** MS medium supplemented with 3mg/l in C86-56; **(D)** MS medium supplemented with 3.5 mg/l in C86-56. **(E)** MS medium supplemented with 2.5 mg/l in C132-81 **(F)** MS medium supplemented with 3 mg/l in C132-81; **(G)** MS medium supplemented with 3.5 mg/l in C132-81; **(H)** MS medium supplemented with 4mg/l in C132-81; **(I)** MS medium supplemented with 2 mg/l in Sp70-1284; **(G)** MS medium supplemented with 2.5 mg/l in Sp70-1284; **(K)** MS medium supplemented with 3 mg/l in Sp70-1284; **(L)** MS medium supplemented with 3.5mg/l in Sp70-1284.



Note; the values assigned by the same letter are not significantly different ($p \leq 0.001$)

Figure 5. Effect of 2, 4-D concentration on weight of callus/explant

Table 1. Effect of different concentration of 2, 4-D on callus initiation

Treatments	Number of days taken for callus initiation			Frequency of callus induction			Weight of callus/explant		
2,4-D mg/l	Genotypes			Genotypes			Genotypes		
	C86-56	C132-81	Sp70-1284	C86-56	C132-81	Sp70-1284	C86-56	C132-81	Sp70-1284
2	19.25^{a}	17.25^{c}	18.25^{b}	44.93^{l}	65.31^{fg}	59.06^{hi}	161.00^{k}	324.00^{i}	189.75^{j}
2.5	16.25^{d}	14.50^{e}	15.50^{d}	49.68^{k}	80.43^{c}	62.12^{gh}	269.5^{h}	402.00^{c}	301.25^{g}
3	14.25^{e}	12.25^{g}	13.25^{f}	57.56^{i}	92.31^{a}	73.31^{d}	296.75^{g}	454.50^{a}	324.00^{f}
3.5	12.25^{g}	11.50^{gh}	12.25^{g}	69.81^{de}	78.06^{c}	85.12^{b}	349.25^{e}	430.75^{b}	378.75^{d}
4	11.25^{gh}	10.50^{hi}	11.75^{i}	53.50^{j}	67.37^{ef}	69.62^{e}	114.25^{l}	263.25^{h}	243.00^{i}
LSD	**0.9**	**0.9**	**0.9**	**1.59**	**1.59**	**1.59**	**3.51**	**3.51**	**3.51**
CV (%)	**4.54**	**4.54**	**4.54**	**3.72**	**3.72**	**3.72**	**3.08**	**3.08**	**3.08**

Note; the values assigned by the same letter are not significantly different ($p \leq 0.001$

Table 2. ANNOVA Summery for callus induction and development.

Source	DF	Days to initiation callus	Percentage of callus induction	Callus weight
		MS	MS	MS
Genotype	2	11.017***	2436.81***	71198.02***
2,4-D	4	97.97***	901.69***	92970.24***
Genotype*2,4-D	8	0.60***	176.79***	3682.24***
CV (%)		4.57	3.72	3.08

Note ***=very highly significant at $P \leq 0.0001$, ** =highly significant at $p \leq 0.01$, *= significant at $p \leq 0.05$ and ns = non-significant where, P= Probabilities Value at $P \leq 0.05$, MS = Mean square, DF = Degree of freedom, CV = Coefficient of Variation.

Shoot regeneration

Embryogenic calli obtained from 3 mg/l and 3.5 mg/l levels of 2,4-D for all genotypes were transferred to full strength MS basal medium. Regeneration started with the appearance of green dots on callus within a week on regeneration medium. Shoot regeneration capacity and number of shoots/calli were significantly ($P<0.001$) affected by genotype, levels of BAP, levels of IBA and their interaction. The maximum number of shoots per callus of 43.44±0.5 with shoot regeneration capacity of (94.08%) were obtained when calli were cultured on the MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA for the genotype Sp70-1284. Whereas, genotype C132-81 gave the highest shoot regeneration capacity of (100%) with 39.71±0.62 shoots per callus were cultured on the MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA. The minimum regenerated plantlets per callus (8.64±0.37) with shoot regeneration capacity (38%) was noticed by C86-56 where cultured on the MS medium supplemented with 0.5 mg/l BAP and 0.0 mg/l IBA.

Increasing BAP alone from 0.5 to 2 mg/l showed a significant increased both shoot regeneration capacity (%) and number of regenerated shoots per callus. The increasing BAP alone from 0.5 to 2 mg/l was significantly increased from 38.00 to 76.67%, 36.63 to 89.78% and 35.94 to 84.42% shoot regeneration capacity in C86-56, C132-81 and Sp70-1284 genotypes respectively. Similarly, the increasing BAP alone from 0.5 to 2 mg/l was significantly increased from 8.64±0.37 to 24.36±0.81, 9.76±0.39 to 26.68±0.47 and 12.79±0.74 to 31.45±0.27 number of regenerated shoots per calli in C86-56, C132-81 and Sp70-1284 genotypes respectively.

From the reports and present result it was observed that for shoot induction from callus cultures of sugarcane, either BAP alone or combination of BAP with IBA were essential. But the combination of BAP with IBA gave higher result to both regeneration capacity and number of shoots per callus. Thus, the ratio of cytokinin and auxin balance is proved to be more important with respect to morphogenesis in sugarcane. Although cytokinins are known in stimulating cell division, they do not induce DNA synthesis. Nevertheless, addition of auxin at low concentration is very important to promote cell division and elongation, and has an ability to induce DNA synthesis (Belete *et al*., 2017). Hence, the presence of auxin with cytokinin stimulates cell division and control morphogenesis thereby influences regenerate shoot production. Increase in BAP concentrations led to the increase in number of shoots, a phenomenon that also reported by other researchers on

tissue culture of other plants.

The current study was similar with khamrit *et al*. (2012) reported best calli exhibited shoot formation and multiplication where cultured on 1mg/l BAP and 0.5 mg/l IBA. Gopitha *et al.* (2010) another study supported that shoot regeneration of sugarcane cultivar CO 671 from calli could be achieved by the application of 1.0 mg/l BAP combined with 0.5 mg/l IBA. Islam *et al*. (1982) also reported that shoot initiation and multiplication of sugarcane genotypes Isd-16 and Isd-28 were successful using 1.0 mg/l BAP combined with 0.5 mg/l IBA and Karim *et al.* (2002) reported that the combination of BAP and IBA promoted shoot formation in sugarcane. Result of the present study disagrees with that of Behera and Sahoo, (2009) who reported the best for shoot formation in sugarcane cultivar Nayana with MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA.

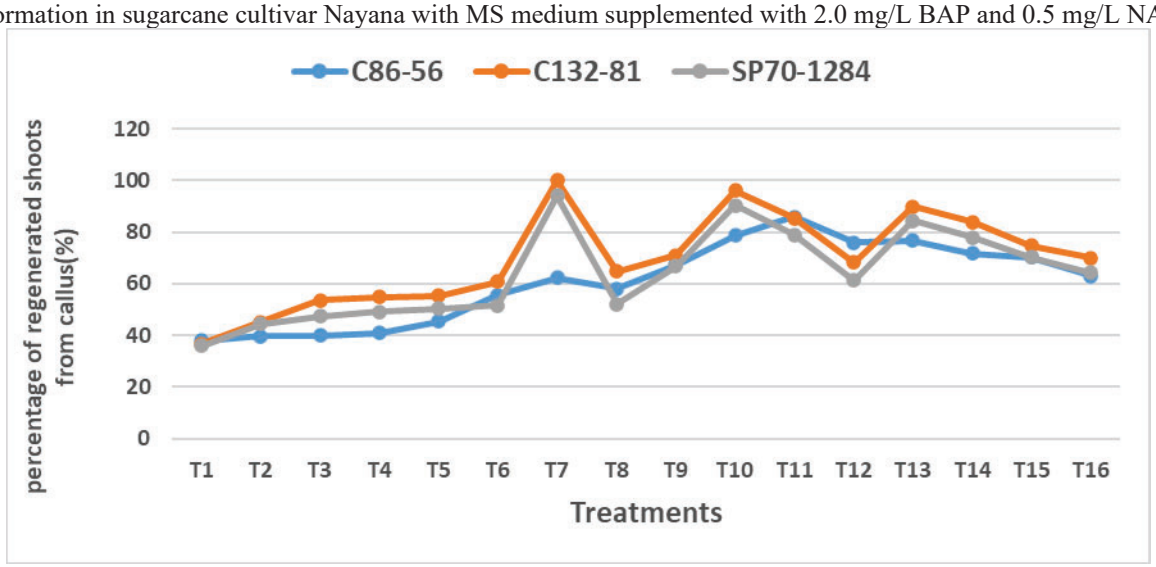


Figure 3. Effect of BAP and IBA concentration on percentage of regenerated shoots from callus.

The value of frequency of regenerated shoots increased with increasing BAP concentration alone from 0.5 to 2 mg/l (T1=0.5mg/l, T5=1mg/l,T9=1.5mg/l and T13=2mg/l). Frequency of regeneration shoots also increased with increasing combination of BAP from 0.5 to 1 mg/l and IBA from 0.0 to 0.75 mg/l for the three genotypes. Whereas, the value of frequency of regeneration shoots decreased with increasing combination of BAP from 1.5 to 2 mg/l and IBA from 0.25 to 0.75 mg/l (from T13=2 BAP with 0 mg/l IBA to T16=.2 BAP with 0.75mg/l).

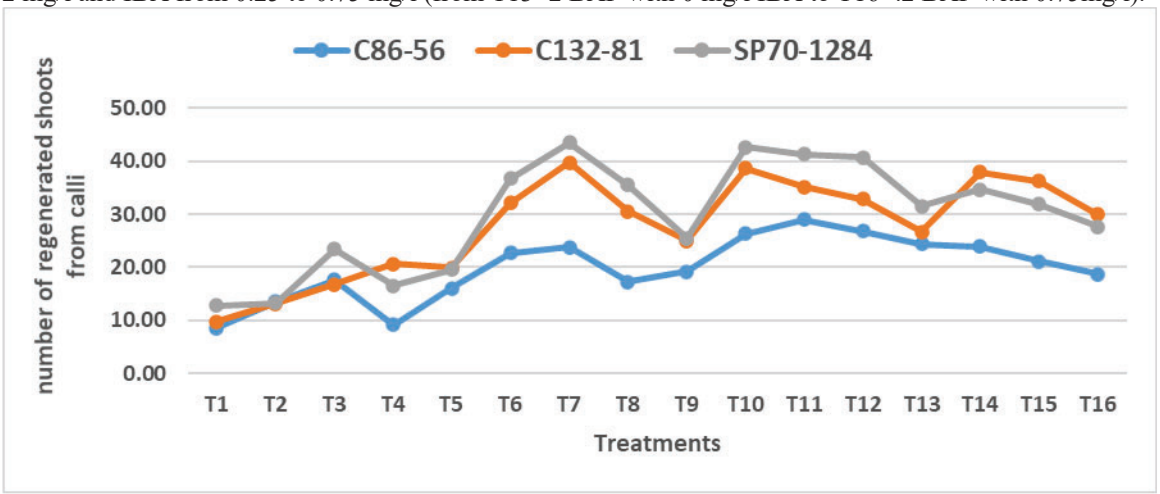


Figure 4. Effect of BAP and IBA on number of regenerated shoots.

Sp70-1284 gave the highest number of shoots (43.44±1.00) on MS media supplemented with 1 mg/BAP and 0.5 mg/l IBA followed by (42.64±0.19) with supplemented on MS media 1 mg/l and 0.25 mg/l IBA. C132-81 was the intermediator to response number of shoots from calli. Whereas, C86-56 gave the lowest value of number of regenerated shoots from calli.

Table 3. Effect of BAP and IBA on shoot regeneration

Hormone combination		Shoot regeneration capacity (%) Genotypes			Number of shoots/calli mean±SD Genotypes		
BAP mg/l	IBA mg/l	C86-56	C132-81	Sp70-1284	C86-56	C132-81	Sp70-1284
0.5	0	38.00^{xy}	36.63^{xy}	35.94^{y}	8.64±0.37^{z}	9.76±0.39^{z}	12.79±0.74^{y}
0.5	0.25	39.67^{wxy}	45.15^{uv}	44.52^{uvw}	13.47±0.47^{y}	13.16±0.36^{y}	13.25±042^{y}
0.5	0.5	40.00^{wxy}	53.60^{pqrs}	47.46^{tu}	17.61±0.64^{vwx}	16.82±0.30^{x}	23.43±0.41^{qr}
0.5	0.75	41.00^{vwx}	54.94^{pqr}	49.00^{stu}	9.21±0.30z	20.66±0.97^{tu}	16.60±0.86^{x}
1	0	45.33^{uv}	55.39^{pq}	50.40^{rst}	16.08±0.09^{x}	19.98±0.36^{tu}	19.54±0.62^{tu}
1	0.25	55.67^{pq}	60.75^{no}	51.66^{qrst}	22.66±0.83^{rs}	32.07±0.28^{ij}	36.66±0.77^{ef}
1	0.5	62.33^{mno}	100.00^{a}	94.08^{bc}	23.73±0.70^{pqr}	39.71±0.62^{cd}	43.44±0.5^{a}
1	0.75	58.00^{op}	64.77^{klmn}	52.08^{qrst}	17.30±0.38^{wx}	30.51±0.47^{jkl}	35.53±0.55^{fg}
1.5	0	67.33^{ijkl}	71.00^{hij}	66.67^{jklm}	19.25±0.57^{tuv}	24.99±0.35^{opq}	25.53±0.91^{op}
1.5	0.25	78.67^{f}	96.03^{ab}	90.30^{cd}	26.32±0.55^{no}	38.64±0.76^{d}	42.64±0.19^{ab}
1.5	0.5	86.00^{de}	85.51^{de}	78.96^{f}	28.98±0.35^{lm}	35.07±0.81^{fg}	41.34±0.48^{bc}
1.5	0.75	76.00^{fg}	68.34^{ijk}	61.33^{no}	26.80±0.85^{no}	32.86±0.56^{hi}	40.67±0.58^{c}
2	0	76.67^{f}	89.78^{cd}	84.42^{e}	24.36±0.81^{pqr}	26.68±0.47^{no}	31.45±0.27^{ijk}
2	0.25	71.67^{ghi}	83.97^{e}	77.98^{f}	23.89±0.29^{pqr}	37.91±0.05^{de}	34.59±137^{gh}
2	0.5	70.33^{hij}	74.59^{fgh}	70.14^{hij}	21.13±0.76st	36.25±0.73^{efg}	31.86±0.35^{ij}
2	0.75	63.00^{lmn}	70.00^{hij}	64.26^{klmn}	18.76±0.52^{uvw}	29.88±1.01^{kl}	27.62±0.75^{mn}
LSD		**4.92**	**4.92**	**4.92**	**1.9**	**1.9**	**1.9**
CV (%)		**4.7**	**4.7**	**4.7**	**4.55**	**4.55**	**4.55**

Note; the values assigned by the same letter are not significantly different ($p \leq 0.001$).

Average shoot length

Average shoot length was significantly (P<0.001) affected by genotype, levels of BAP, levels of IBA and their interaction. It ranged from 1.42±0.06 to 6.38±0.08 cm. Among the three sugarcane genotypes, C86-56 gave the maximum shoot length (6.38±0.08 cm) where cultured on MS medium supplemented with 2 mg/l BAP in combination with 0.25 mg/l IBA followed by C132-81 (6.14±0.23 cm) cultured on MS medium supplemented with 1.5 mg/l BAP in combination with 0.5 mg/l IBA (Table 4). Whereas the minimum shoot length (1.42±0.07cm) in C132-81 genotype, (1.44±0.06cm) in C86-56 genotype, were recorded in both genotypes cultured on MS medium supplemented with 0.5mg/l BAP in the absence of IBA (Table 4).

Shoot length increased when BAP was used in combination with IBA, this might be due to the ability of auxins to induce apical dominance and increase shoot elongation. Similar to this result, Behera *et al*. (2009) reported maximum shoot length of 6.2 ± 0.37 and 4.0 ± 0.61 under BAP (2.0 mg/l) + IBA (0.5 mg/l) and BAP (2.0 mg/l) + IBA (1.0 mg/l) for two sugarcane genotypes namely B52-298 and NCO334, respectively. On the other hand, other researchers observed that shoot length was found to decline with the increase in the concentration of BAP beyond optimum (2.0 mg/l BAP).

Average number of leaves/shoot

Average number of leaves/shoot was also significantly (P<0.001) affected by genotype, levels of BAP, levels of IBA and their interaction. The maximum number of leaves (8.24±0.06) for C132-81, (7.84±0.29) for C86-56 and (6.10±0.24) for SP70-1284 genotype were recorded on MS medium supplemented with 2 mg/l BAP in combination with 0.25 mg/l IBA. Leave numbers were maximum at high concentration of BAP in combination with low concentration of IBA. This is because the application of BAP at high level in combination with a low level of IBA increases cell division and increases the number of leaves. Auxins play a critical role in plant cell growth and are involved in a wide genotype of developmental processes, including initiation of leaf primordial (Ludwig-Muller, 2000).

Table 4. Effect of BAP and IBA on shoot multiplication

Hormone combination		Average length of shoot (cm) mean±SD Genotypes			Average number of leaves/shoot mean±SD Genotypes		
BAP mg/l	IBA mg/l	C86-56	C132-81	Sp70-1284	C86-56	C132-81	Sp70-1284
0.5	0	1.44±0.06^{wx}	1.42±0.07x	1.55±0009^{wx}	1.95±0.14^{t}	2.24±0.07st	1.53±0.15^{u}
0.5	0.25	2.52±0.07st	1.50±0.17wx	1.59±0.04^{w}	2.41±0.17^{s}	2.35±0.11^{s}	1.86±0.09^{tu}
0.5	0.5	2.21±0.13^{u}	2.19±0.05^{u}	1.77±0.06^{v}	2.53±0.40^{s}	3.26±0.16^{r}	3.17±0.11^{r}
0.5	0.75	2.59±0.10st	2.26±0.08^{u}	2.62±0.10st	3.51±0.31^{pqr}	3.50±0.07^{pqr}	3.31±0.10^{qr}
1	0	2.90±0.09^{qr}	2.47±0.09^{t}	2.63±0.06^{s}	3.69±0.33^{opq}	3.71±0.15^{op}	3.70±0.07^{op}
1	0.25	4.28±0.07^{hi}	3.15±0.09^{no}	3.69±0.2^{k}	6.00±0.26^{ef}	6.65±0.06^{d}	5.16±0.12^{h}
1	0.5	3.83±0.05^{k}	3.50±0.08^{l}	3.84±0.08^{k}	6.07±0.43^{ef}	7.32±0.03^{c}	4.34±0.12^{jkl}
1	0.75	3.43±0.06^{lm}	4.07±0.09^{j}	4.13±0.05^{ij}	3.95±0.45^{mno}	4.03±0.15l^{mno}	3.73±0.08^{op}
1.5	0	3.19±0.03^{no}	4.20±0.08^{ij}	4.43±0.09^{h}	4.39±0.30^{jkl}	4.42±0.10^{ikl}	4.03±0.08l^{mno}
1.5	0.25	5.72±0.3^{c}	5.37±0.06^{de}	5.26±0.09^{e}	7.37±0.29^{c}	7.60±0007^{bc}	5.83±0.11^{fg}
1.5	0.5	5.50±0.3^{d}	6.14±0.23^{b}	5.95±0.23^{c}	6.32±063de	5.71±0.18^{fg}	4.95±0.14^{hi}
1.5	0.75	4.40±0.08^{h}	3.30±0.14^{mn}	3.49±0.03^{l}	3.85±0.23^{nop}	4.65±0.11^{ijk}	4.30±0.28^{lkm}
2	0	3.05±0.09^{opq}	4.64±0.10^{g}	4.92±0.07^{f}	4.73±0049^{ij}	5.92±0.07^{fg}	4.15±0.08^{lmn}
2	0.25	6.38±0.08^{a}	3.16±0.12^{no}	3.36±0.05^{lm}	7.84±0.29^{b}	8.24±0.06^{a}	6.10±0.24^{ef}
2	0.5	4.89±0.08^{f}	3.38±0.07^{lm}	3.14±0.02^{op}	5.60±0.62^{g}	6.58±0.11^{d}	4.81±0.16^{hi}
2	0.75	4.61±0.07^{g}	2.83±0.05^{r}	2.98±0.11^{pq}	4.63±0.17^{ijk}	6.06±0.12^{ef}	4.62±0.13^{ijk}
LSD		**0.14**	**0.14**	**0.14**	**0.38**	**0.38**	**0.38**
CV (%)		**2.7**	**2.7**	**2.7**	**5.15**	**5.15**	**5.15**

Table 5. ANOVA Summery for Shoot Regeneration and Multiplication

Source	DF	Shoot regeneration capacity (%)	Number of shoot/calli	Average length of shoot	Average number of leaves/shoot
		MS	MS	MS	MS
Genotype	2	956.21***	1321.67***	2.80***	13.10***
BAP	3	8360.90***	2126.34***	48.30***	75.88***
IBA	3	1185.05***	676.76***	4.88***	4.37***
Genotype*BAP	6	86.78***	70.64***	1.26***	1.18***
Genotype*IBA	6	77.75****	42.45***	2.70***	2.49***
BAP*IBA	9	914.44***	90.78***	3.60****	9.25***
Genotype*BAP*IBA	18	124.45***	30.17***	1.07***	1.02***
CV (%)		4.7	4.55	2.7	5.15

Note ***= very highly significant at P ≤ 0.0001, ** =highly significant at p≤ 0.01, *= significant at p≤ 0.05 and ns = non-significant where, P= Probabilities Value at P ≤ 0.05, MS = Mean square, DF = Degree of freedom, CV = Coefficient of Variation

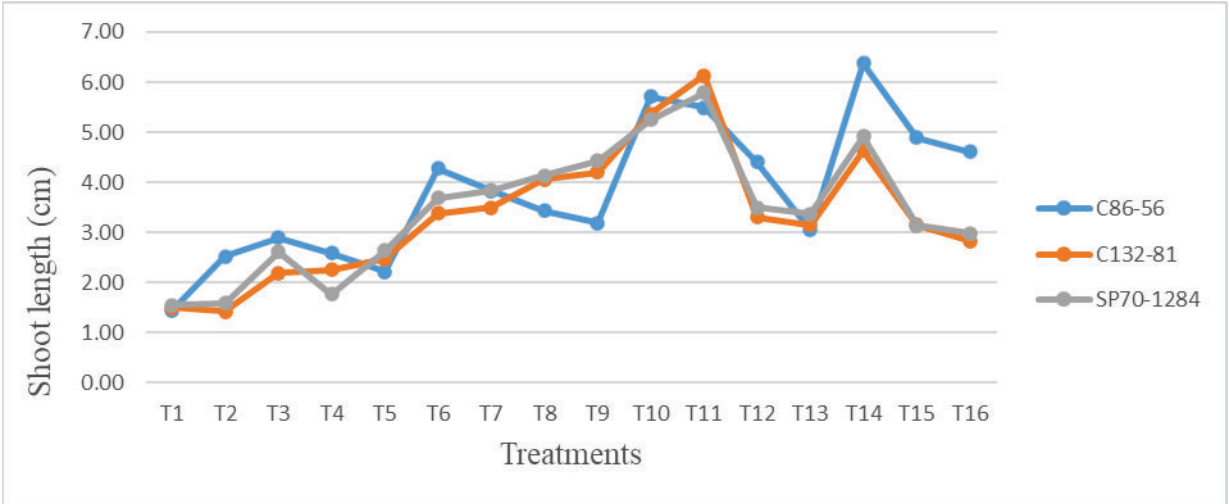


Figure 5. Effect of BAP and IBA concentration on shoot length

Genotype C132-81 was continuously increase from 0.5 BAP to 1.5 BAP with the combination of IBA.

Whereas C86-56 and SP70-1284 genotypes were discontinuously increase with the combination of BAP and IBA concentration increased. C86-56 produced highest shoot length on MS medium supplemented with 2 mg/BAP with 0.25mg/l IBA.

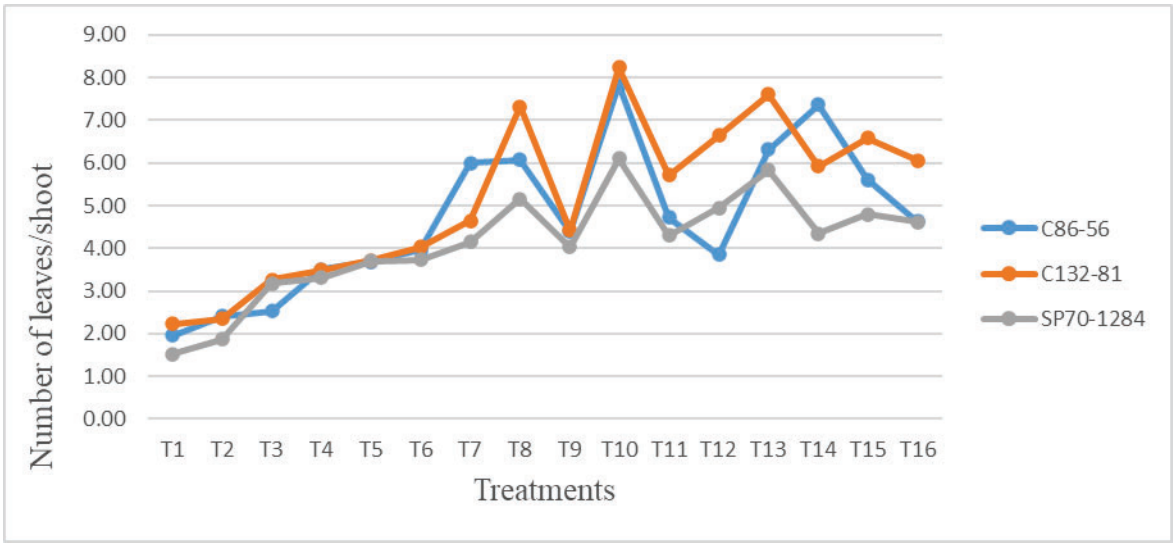


Figure 6. Effect of BAP and IBA concentration on number of leaves per shoot

All the three genotypes were continuously increased number of leaves/shoot from 0.5mg/l BAP with absence of IBA to 1mg/l BAP with 0.75mg/l IBA. C132-81 and C86-56 genotypes produced highest number of leaves on MS medium supplemented with 1.5 mg/BAP with 0.25mg/l IBA.

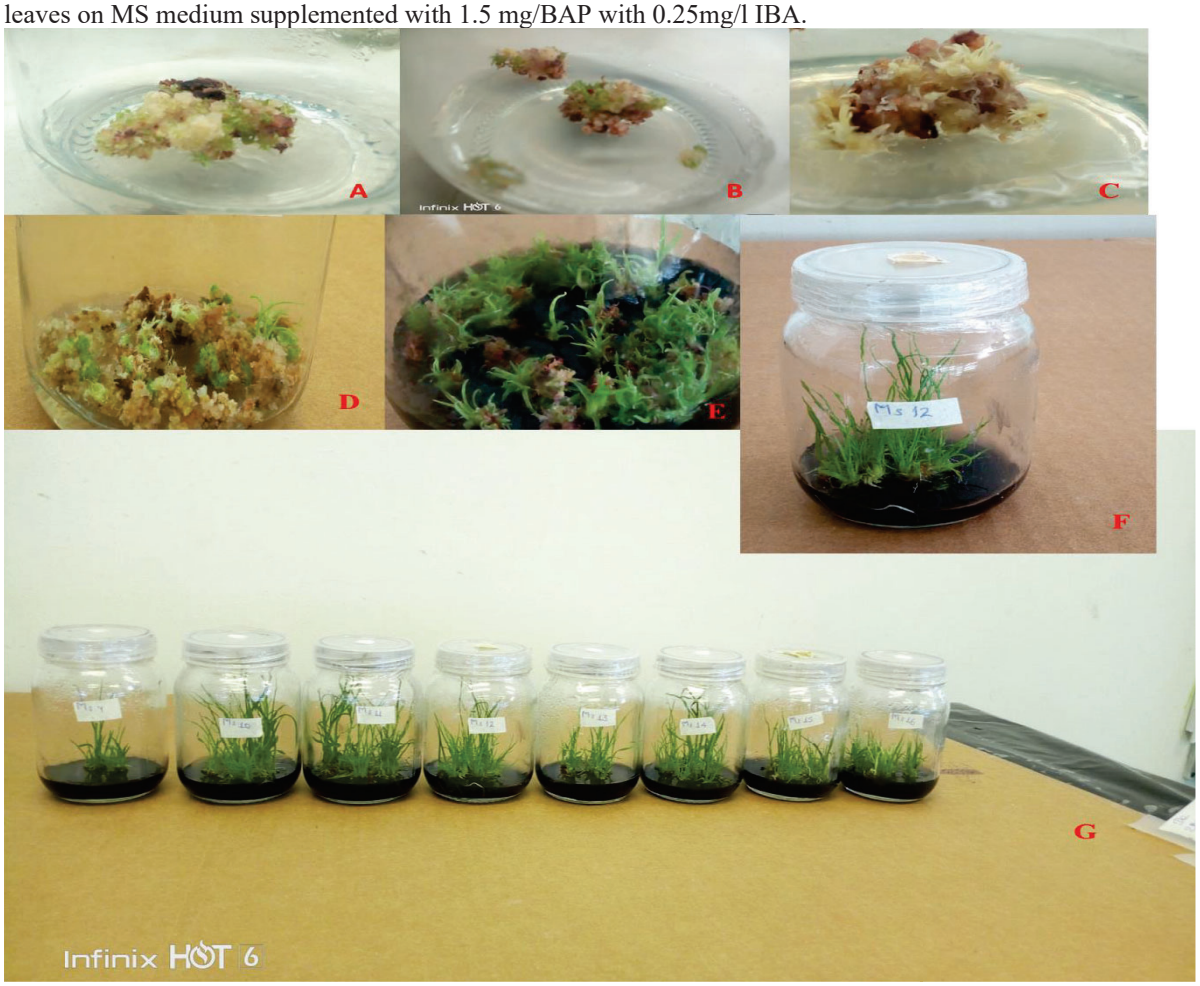


Figure 7. Shoot regeneration and multiplication

The three genotypes callus were cultured on shoot regeneration medium (A –C), shoot buds appear on the

surface of callus. A (C86-56), B (C132-81) and C (SP70-1284).Developing shoots at late stage of regeneration (D and E), Shoot multiplication (F and G)

Conclusion

In conclusion our study revealed that, C132-81 taken early days (10.5) among the three genotypes and obtained in the media supplemented with 4.0 mg/l of 2, 4-D. However, embryogenic callus formation was higher with 2, 4-D levels of in between 2.5 mg/l and 3.5 mg/l. Frequency of callus initiation also influenced by genotypes, different levels of 2, 4-D and their interaction. The highest percentage (92.31%) of callus initiation was recorded in 3.0 mg/l of 2,4-D in the genotype C132-81 followed by 85.12% in 3.5 mg/l in the genotype Sp70-1284 and 80.43% in 2.5 mg/l of 2, 4-D in the genotype C132-81, while C86-56 in 2mg/l of 2,4-D gave the lowest callus induction (44.93%). C132-81 gave higher callus weight (454.5 mg) and (430.75 mg) on MS media supplemented with 3 mg/l and 3.5 mg/l 2, 4-D, respectively. C86-56 produced minimum callus weight (114.25mg) on MS media supplemented with 4 mg/l 2,4-D. Sp70-1284 genotype gave the maximum number of shoots per callus of 43.44±0.5 with shoot regeneration capacity of (94.08%) among the three genotypes were recorded by calli cultured on the MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA.

ACKNOWLEDGEMENTS

We would like to express our gratefulness to Ethiopian Sugar Corporation for financing the study and Holeta National Agricultural Biotechnology Research Center for allowing us the tissue culture laboratory to use all laboratory facilities, chemicals and various supplies with good working environments.

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