academicJournals

Vol. 15(29), pp. 1541-1550, 20 July, 2016
DOI: 10.5897/AJB2015.14836
Article Number: BDA183159629
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Establishment of *in vitro* callus in sugarcane (*Saccharum officinarum* L.) varieties influenced by different auxins

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Received 4 July, 2015; Accepted 21 December, 2015

Sugarcane is an important perennial, polyploidy crop. Based on the growing demand, it has now attracted great attention as cash crop. Tissue culture technique, an alternative method for solving production problem and increasing production, was used in this study. The work was carried out at the Nuclear Institute of Agriculture (NIA), Tandojam, Pakistan. Standardization of protocol for proliferation of callus and induction of callus were established through *in vitro* culture using young meristem of sugarcane (*Saccharum officinarum* L.) as explants to enhance genetic variation in sugarcane varieties. Three varieties (NIA-2012, Gulabi-95 and NIA-105) were used. The shoot tips were supplemented with Murashige and Skoog (MS) medium modified with three auxins (2, 4-D, Picloram, NAA). All the auxins were applied in 0.0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}. MS basal medium was used as control free from concentration of auxins. Highly significant ($p < 0.05$) variations were observed in sugarcane varieties for all parameters of callus culture; while interactive effect of variety x treatment x concentration was non-significant for proliferation weight of callus. Among all the tested auxins 2, 4-D at 3.0 mgL^{-1} concentration proved to be the most effective auxin for callus proliferation and weight of all the sugarcane varieties. In light of the present research, it is concluded that auxins are preferable for future work in relation to *in vitro* callus induction for all varieties of sugarcane.

Key words: *Saccharum officinarum, in vitro,* callus induction, auxins, proliferation.

INTRODUCTION

Sugar cane (*Saccharum officinarum* L.) is an herbaceous agro industrial crop that belongs to the family Poaceae (Singh et al., 2003; Sharma, 2005; Cha-um et al., 2006). It is an important industrial crop of tropical and sub-tropical regions and is cultivated on 20 million hectares in more than 90 commercial countries because of its high trade value (Naz, 2003). Sugar juice is used for making sugar (Coax et al., 2000). Molasses (thick syrup residue) are used to produce ethanol (blended for motor fuel) and livestock feed. Bagasse (fibrous portion) is burned to provide heat and electricity for sugar mills, and green tops can be used as livestock feed (Mackintosh, 2000). It accounts for around 70% of the world's sugar (Khan et al., 2004). Sugarcane breeding programmes focus on the

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production of varieties with high yield, higher sucrose content, pest and disease resistance, tolerance to a biotic stress and improved rooting ability (Brumbley et al., 2008). The growing demand of newly released varieties could not be met by only conventional propagation methods as the multiplication rate set is 1:8. This restricts fast spread of superior varieties. Therefore, application of tissue culture techniques provides an alternative method for improvement of varieties (Sengar, 2011).

Tissue culture techniques have been widely used in *S. officinarum* L. for various purposes. Meristem tip or shoot tip culture has been used as a tool to produce virus-free plants (Hendre et al., 1975; Fitch et al., 2001; Tai and Miller 2001; Parmessur et al., 2002). Early efforts in sugarcane *in vitro* culture used a medium developed for efficient growth of meristem Tissue Culture150 (Thom et al., 1981; Lorenzo et al., 2001; Geijskes et al., 2003; Nieves et al., 2003; Wongkaew and Fletcher, 2004). These reports only focused on the effects of PGRs (plant growth regulators) such as BAP(6- benzylaminopurine), kinetin and coconut water on the MS medium for *in vitro* culture of commercial hybrid cultivars and *S. officinarum*. The chromosome number and ploidy levels in sugarcane plants have been reported by many authors. The type, concentration and combination of synthetic hormones of auxins caused somaclonal variation (Phillips et al., 1994). Tissue culture offers mass production (Czarinikow, 2010) of sugarcane. *In vitro* multiplication of sugarcane through callus culture, and shoot tip culture have been reported by many authors (Bakesha et al., 2002; Alam et al., 2003; Ali et al., 2008; Behara and Sahoo, 2009; Khan et al., 2012; Raza et al.,2014) to obtain regenerable type of callus. Tissue culture is efficient biotechnological tool for rapid multiplication of sugarcane plants (Kalunke et al., 2009; Kazim et al., 2015). It was also observed that callus derived from different auxins have different standardized protocols. Auxins are usually used to stimulate callus production and cell growth (Jahangir et al., 2009; Pandey et al., 2011; Shahid et al., 2012), to induce somatic embryogenesis (Edessoky et al., 2011), and stimulate growth of regenerable callus. In sugarcane tissue culture, no two genotypes give similar results within the culture conditions (Nahera et al., 1989; 1990; Smiullah et al., 2013; Mekonnen et al., 2014). This study aimed to develop genetic variability in sugarcane through callus culture, applying three auxins treatments with different concentrations. The effect of different growth regulator on the plant callus culture and extend of genetic variability induce by growth regulators among three sugarcane varieties are shown in this work.

MATERIALS AND METHODS

Explants source

Three elite hybrids of sugarcane NIA-2012 (early maturing), Gulabi-95 (mid maturing) and NIA-105 (late maturing) were used as explants source for callus culture.

Surface sterilization of explants

Apical meristem was chosen as source of explants because the cells are undifferentiated and meristematic cells actively divide. The most important reason is that there is no exposure to virus in the apical meristem and the production of virus free sugarcane explants is possible. The explants of sugarcane were taken in the laboratory. Unnecessary portion of the top was removed and the remaining was first washed with running tap water, and sterilized with 70% ethanol for 1 min and 10% sodium hypochlorite solution for 20 min (Figure 1). After sterilization, the explant materials were washed with double distilled sterilized water 2 to 3 times to remove any traces of disinfectant under aseptic conditions in laminar air flow. These sterilized leaves were cut into 2 to 3 mm apical meristem. This apical meristem was cultured aseptically into the bottles of the media. The lid of one of the bottles was removed and the mouth was flamed to avoid further chances of contamination. Explant slice was placed in the bottle with long forceps without touching the rim of the bottle; two to four sections of the explants were placed in each bottle carefully, then it was flamed lightly and tightly sealed. Finally, the name of the sugarcane variety was labeled on the bottle along with the date. All the operations were done under anemic conditions in a laminar air flow cabinet and the weight of the explants was noted.

Incubation of explants

The explants were aseptically cultured on modified MS medium with three auxins supplemented with 2, 4-dichloro phenoxy acetic acid (2, 4-D), naphthalene- acetic acid (NAA) and 4- amino-3,5,6-trichloro-picolinic acid (Picloram) for callus induction at 0.0, 0.5, 1, 2, 3 mgL^{-1}. All the cultures were incubated at 25 ± 2°C and kept under 16 h photo period of florescent tube light in the dark for 4 weeks.

Observations

Each bottle was examined to determine the callus formed from explants. The resulting calli were transferred to fresh medium for further callus proliferation. The callus materials were sub- cultured on the same medium of 2,4-D, NAA and picloram to induce callus for another 4 weeks. After 8 weeks of culture in the presence of 2,4-D, distinctions between regenerable and non- regenerable callus were examined. Regenerable callus has gross appearance, is compacted, has white to cream color and nodular structure, while non regenerable callus has wet appearance, is translucent and brownish in color. The parameters examined included numbers of explants, proliferation of callus, weight of callus, type of callus-regenerable and non- regenerable.

Data analysis

Data were analysed by analysis of variance (ANOVA) using computer software Statistics version 8.1. Complete randomized design (CRD) was used with three treatments and five different concentrations in two factorial designs. Means of callus induction including weight of explants, callus proliferation, callus weight and type of callus were compared; they were statistically significant at α = 5% probability level.

Figure 1.Explant cut into 2-3 mm cultured aseptically into the bottles of the media.

Table 1. ANOVA for callus induction of sugarcane plantlets modulated by different concentration of different auxin.

Source of variation	DF	Mean square		
		Weight of explants	Weight of callus proliferation	Weight of callus.
Varieties	2	0.46203**	1.10021**	2.29702 **
Treatment	2	0.01404 **	1.92311**	4.50119 **
Concentrations	4	0.03061**	0.68875 **	1.69259 **
V x T	4	0.02064**	0.01808 **	0.04923 **
V x C	8	0.04250**	0.02642**	0.05650 **
T x C	8	0.01637**	0.02315**	0.09987**
V x T x C	16	0.01266**	0.00267 ns	0.01152 ns
Error	88			
Total	134	CV. 3.99	CV. 3.95	CV. 4.93

In each column, means followed by common letter are not significantly different at 5% probability level. V= Variety, ns = non- significant, T= treatment, C = concentration, V= co-efficient of variance.

RESULTS AND DISCUSION

Analysis of variance showed that the different levels of auxins had highly significant effects on callus induction. The main parameters used for callus induction are weight of explants, weight of callus, proliferation of callus, as they have direct effect on final callus. The results of ANOVA for callus induction are presented in Table1. Highly significant variations were observed for all parameters of callus induction except variety x treatment x concentration which was non-significant for weight of callus proliferation and weigh of callus ($p < 0.05$).

Weight of explants

Three varieties of sugarcane were used in this experiment. Different varieties have dissimilar weight of disc of explants which also affected the callus formation and regeneration of plantlets. NIA -2012 variety has more weight compared to Gulabi- 95.

Callus proliferation

Callus proliferation was highly influenced by varying

Table 2. Effect of different concentration of 2,4-D, Picloram and NAA on weight of callus proliferation in three sugarcane varieties.

Growth regulators	Concentration (g l^{-1})	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2,4-D	0.0	0.75^{h-k}	0.70^{l-p}	0.64^{s-v}	0.70^{g}
	0.5	0.88^{c}	0.69^{n-r}	0.63^{t-w}	0.73f
	1.0	0.84^{c-f}	0.71^{k-o}	0.66^{p-t}	0.73^{ef}
	2.0	0.86^{cd}	0.75^{h-l}	0.68^{n-s}	0.76^{b-e}
	3.0	0.95^{b}	0.79^{f-h}	0.72^{j-o}	0.82^{a}
Picloram	0.0	0.70^{m-q}	0.65^{r-u}	0.60^{v-x}	0.65^{h}
	0.5	0.83^{d-f}	0.79^{f-h}	0.73^{j-n}	0.78^{bc}
	1.0	0.78^{g-i}	0.74^{i-m}	0.70^{m-q}	0.74^{d-f}
	2.0	0.80^{e-g}	0.75^{h-l}	0.67^{o-t}	0.74^{d-f}
	3.0	0.87^{cd}	0.71^{k-o}	0.65^{q-u}	0.74^{d-f}
NAA	0.0	0.81^{e-g}	1.09^{a}	0.56^{x}	0.82^{a}
	0.5	0.85^{c-e}	0.78^{g-i}	0.67^{o-t}	0.76^{b-d}
	1.0	0.88^{c}	0.76^{g-j}	0.63^{t-w}	0.76^{c-f}
	2.0	0.97^{b}	0.78^{g-i}	0.61^{u-x}	0.79^{b}
	3.0	0.99^{b}	0.77^{g-j}	0.59^{w-x}	0.78^{bc}
Mean		0.85^{a}	0.76^{b}	0.65^{c}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.0063), LSD 5%) 0.0127), Concentrations SE 0.0142), LSD 5%) 0.0283), V x C SE 0.0247), LSD 5%) 0.0490).

levels of auxins. Apical meristem was used as explants. Three different genotypes of sugarcane, NIA-2012, NIA-105 and Gulabi-95, were cultured on different MS modified media with three auxins: 2,4-dichloro phenoxy acetic acid (2,4-D), 4- amino-3,5,6-trichloro-picolinic acid (picloram) and naphthalene- acetic acid (NAA). For proliferation of callus, significant variation ($p < 0.05$) was detected for all genotypes (Table 2, Figure 2). Highest weight of proliferation was observed in NIA-2012 (1.41 g) followed by NIA-105 (1.29 g); lowest was in Gulabi-95(1.10 g). The maximum proliferation for 2,4-D was observed in NIA-105 (1.79 g), and minimum in Gulabi- 95 (1.44 g). For picloram maximum weight of proliferation was recorded in NIA-2012 (1.77 g) while minimum was observed in Gulabi-95 (1.3 g). For NAA maximum weight of proliferation was noted in NIA-2012 (1.61 g) and minimum in Gulbi-95 (1.26 g). The highest proliferation of callus was recorded at 3.0 mgL^{-1} for the entire growth regulator hormone used. An efficient and regenerable callus was formed by increasing concentration of auxins. Review of related study by other workers also supports the present results (Table 2) that weight of callus proliferation was enhanced with increase in dose of all the auxins applied. All the concentration gave best results in same combination for the weight of callus proliferation. The results are same with the finding of Khan et al. (2009), Sani (2010); Goel et al. (2010), and Abu et al. (2014). This work is quite different from that of Kenia et al. (2006) who obtained highest proliferation in low concentration of these growth hormones. (Figure. 2 A, B and C labeled on bottle was showing different hormones)

Callus induction

In callus induction different combinations of auxins were used. To obtain the highest role of NAA in callus induction of sugarcane new concentration of more than 1 mgL^{-1} was applied. In sugarcane significant variation ($p < 0.05$) in proliferation of callus was detected for all genotypes (Table 3, Figures 3, 4, 5) different varieties showed variation in callus induction and weight of calli. Highest weight of callus was observed in NIA-2012 (1.41 g) followed by NIA-105 (1.29 g) and lowest in Gulabi-95 (1.10 g). The maximum callus induction weight for 2, 4-D was observed in NIA-105 (1.79 g), and minimum in Gulabi- 95 (1.44 g). For picloram maximum weight of proliferation was recorded in NIA-2012 (1.77 g) while minimum was observed in Gulabi-95 (1.3 g). In case of NAA weight of proliferation was noted for NIA-2012 (1.61 g) and minimum in Gulbi-95 (1.26 g). The highest proliferation of callus was recorded at 3.0 mgL^{-1} for the entire growth regulator hormone used.

Naphthalene acetic acid (NAA) of 2.0 and 3.0 mg/l

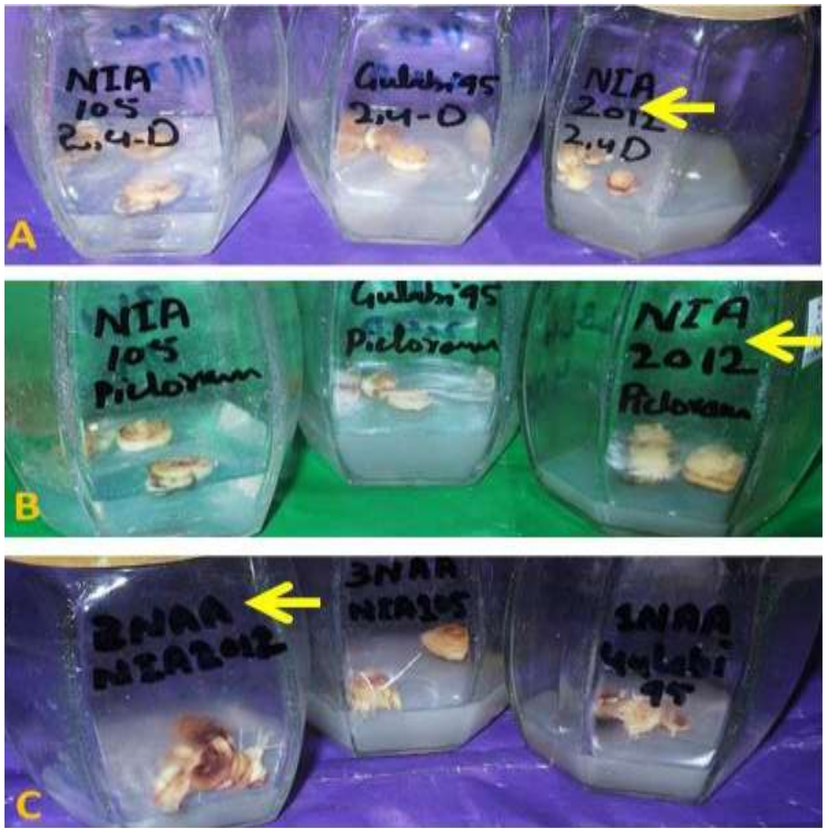

Figure 2.Callus proliferation in NIA-2012, NIA-105 and Gulabi-95 different concentrations of auxins (A= 2,4-D, B= Picloram, C=NAA).

Table 3. Effect of different concentration of 2,4-D,Picloram and NAA on callus induction in three sugarcane varieties.

Growth regulators	Concentration ($g\ l^{-1}$)	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2,4-D	0.0	1.01^{q}	$0.94^{q\text{-}s}$	0.82^{uv}	0.92^{l}
	0.5	1.44^{fg}	$1.41^{g\text{-}i}$	$1.23^{m\text{-}o}$	1.36^{de}
	1.00	1.58^{de}	1.55^{de}	$1.30^{j\text{-}m}$	1.47^{c}
	2.0	1.69^{bc}	1.67^{c}	$1.33^{i\text{-}l}$	1.56^{b}
	3.0	1.77^{a}	1.79^{a}	1.44^{g}	1.67^{a}
Picloram	0.0	$0.92^{r\text{-}t}$	0.85^{tu}	0.76^{vw}	0.84^{j}
	0.5	$1.31^{j\text{-}l}$	1.14^{p}	1.01^{q}	1.15^{g}
	1.0	1.43^{gh}	$1.34^{i\text{-}l}$	1.12^{p}	1.29^{f}
	2.0	1.58^{de}	$1.38^{g\text{-}j}$	$1.20^{n\text{-}p}$	1.39^{d}
NAA	3.0	1.7^{ab}	1.63^{cd}	$1.30^{k\text{-}m}$	1.56^{b}
	0.0	0.99^{qr}	0.76^{vw}	0.71^{w}	0.82^{j}
	0.5	$1.18^{n\text{-}p}$	$0.96^{q\text{-}s}$	$0.88^{s\text{-}u}$	1.019^{h}
	1.0	$1.33^{i\text{-}l}$	1.15^{op}	0.99^{qr}	1.16^{g}
	2.0	1.52^{ef}	$1.35^{h\text{-}k}$	1.15^{op}	1.34^{ef}
	3.0	1.61^{cd}	1.44^{g}	$1.26^{l\text{-}n}$	1.44^{c}
Mean		1.41^{a}	1.29^{b}	1.10^{c}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.0106), LSD 5%) 0.0120), Concentrations SE 0.0236), LSD 5%) 0.0469), V x C SE 0.0409), LSD 5%) 0.0813).

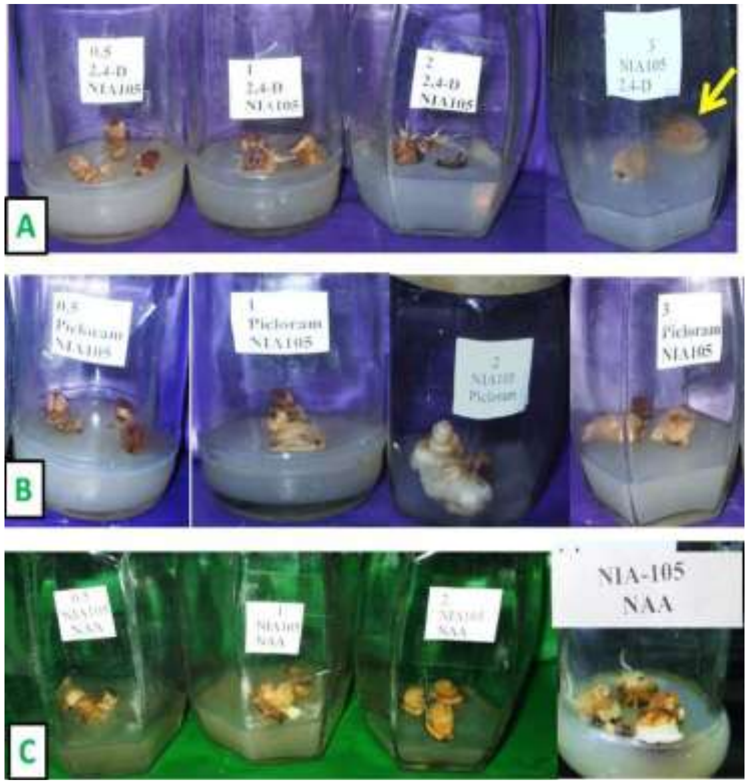

Figure 3. Callus formation in NIA-105 by different concentration of auxins (A= 2,4-D, B= Picloram, C=NAA).

Figure 4. Callus formation in NIA-2012 by different concentration of auxins (A= 2,4-D, B= Picloram, C=NAA).

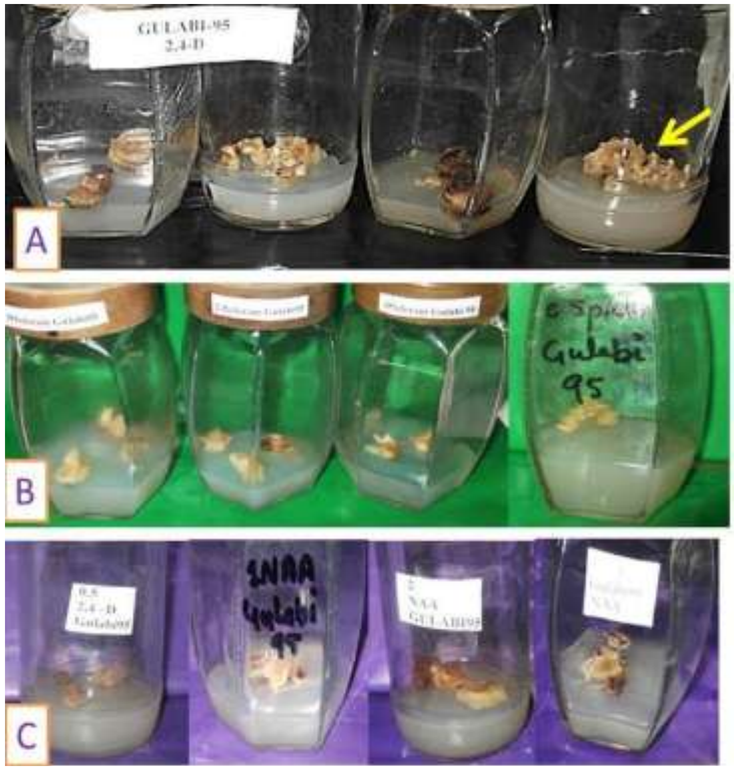

Figure 5. Callus formation in Gulabi-95 by different concentration of auxins (A= 2,4-D, B= Picloram, C=NAA).

produced small amount of non-regenerable and regenerable calli (Figure 6) (Khattak et al. (2014). High concentrations of auxin may be required for higher stages of callus formation. Weight of callus increases with increase in concentration of all the auxins applied, whereas as concentration decreases, weight of callus decreases. All the treatment gave best results at 3.0 mg/l for the weight of callus. Present results are in agreement with the finding of Omarjee et al. (2008), Ather et al. (2009), Khan et al. (2009), Raza et al. (2010), Seema et al. (2011), Khan et al. (2012) and Kazim et al. (2015).

Callus weight and type of callus

Standardized protocol is preferred for callus induction using young maristem as explants of sugarcane varieties. Highly significant variation ($p < 0.05$) in weight of callus was detected for all genotypes (Table 4). The callus induction under the influence of different growth regulators yielded maximum callus in NIA-2012 (2.54 g), followed by NIA-105 (2.34 g) and minimum in Gulabi-95(2.09 g). The maximum callus weight for 2, 4-D was detected in NIA-2012 (3.27 g), and minimum in Gulabi-95(2.67 g). In picloram maximum weight of callus was recorded in NIA-2012 (2.95 g) while minimum was observed in Gulabi-9 (2.3 g). For NAA weight of callus was outstanding for NIA-2012 (2.84 g) and minimum in Gulbi-95 (2.25 g). The highest weight of callus was recorded at 3.0 mgL^{-1} for the entire growth regulator hormone used. Many authors reported best effect of auxins when the callus remained on increased concentration of 2, 4-D applied for prolonged period. Ali et al. (2007) suggested the process of differentiation of regenerable callus and non regenerable callus (Figure.6) based on type of auxin and concentration of auxins. Present work is quite different from that of Gopitha et al. (2010), who found best result of callus induction at lower concentration of NAA, and 2,4-D. Many scientists have used different auxins for callus formation. They found (Table 5) the type of callus depends upon the auxins applied. All the treatment gave best effects at 3.0 mgL^{-1} for the regenerable type of callus. Present results are the same with the finding of Lakshmanan et al. (2006), Valentine et al. (2010), Ijaz et al. (2012), Samiullah et al. (2013), Alcantara et al. (2014). However, this work is different from those of Gandonou et al. (2005), Xing et al. (2010) and Zamir et al. (2012) who obtained regenerable callus at lower concentration of auxins.

Conclusion

This work revealed that the calli obtained from 2, 4-D and picloram produced more genetic variability compared to the calli of NAA. Callus was observed on the basis of

Figure 6. Types of callus obtained by application of different auxins (A= Regenerable. B= Non-regenerable)

Table 4. Effect of different concentration of 2,4-D, Picloram and NAA on callus weight induction in three sugarcane varieties.

Growth regulators	Concentration ($g\ l^{-1}$)	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2,4-D	0.0	2.23^{k-o}	2.06^{o-s}	1.92^{r-t}	2.07^{hi}
	0.5	2.23^{k-o}	2.00^{p-t}	2.07^{n-r}	2.10^{g-i}
	1.0	2.78^{c-f}	2.63^{f-i}	2.38^{jk}	2.60^{de}
	2.0	2.97^{b}	2.84^{b-e}	2.52^{h-j}	2.77^{b}
	3.0	3.27^{a}	2.96^{bc}	2.67^{e-h}	2.97^{a}
Picloram	0.0	1.82^{tu}	1.72^{uv}	1.58^{vw}	1.70^{j}
	0.5	2.34^{j-l}	2.27^{k-m}	1.88^{s-u}	2.16^{gh}
	1.0	2.75^{d-g}	2.58^{g-i}	2.16^{l-p}	2.49^{e}
	2.0	2.87^{b-d}	2.77^{d-f}	2.27^{k-m}	2.64^{cd}
	3.0	2.97^{b}	2.85^{b-e}	2.39^{jk}	2.74^{bc}
NAA	0.0	1.58^{vw}	1.43^{wx}	1.32^{x}	1.44^{k}
	0.5	2.24^{k-n}	1.96^{q-t}	1.85^{tu}	2.01^{i}
	1.0	2.47^{ij}	2.17^{l-p}	1.95^{q-t}	2.20^{g}
	2.0	2.70^{d-g}	2.28^{k-m}	2.11^{m-q}	2.36^{f}
	3.0	2.84^{b-e}	2.60^{f-i}	2.25^{k-n}	2.56^{de}
Mean		2.54^{a}	2.34^{b}	2.09^{c}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.0242), LSD 5%) 0.0481), Concentrations SE 0.541), LSD 5%) 0.1074), V x C SE 0.0936), LSD 5%) 0.1861).

Table 5. Effect of different concentration of 2,4-D,Picloram and NAA type of callus induction in three Sugarcane varieties.

Growth regulators	Concentration (g l^{-1})	Varieties NIA-2012	NIA-105	Gulabi-95
2,4-D	0.0	Non –regenerable	Non- regenerable	Non- regenrable
	0.5	Regenrable	Regenrable	Non – regenrable
	1.0	Regenrable glossed, white	Regenrable glossed,	Regenrable cream color
	2.0	Regenrable compact,	Regenrable nodular	Regenrable, white
	3.0	Regenrable compact, nodular cream color	Regenrable glossed aspect, white	Regenrable compact, nodular
Picloram	0.0	Non- regenrable	Non – regenrable	Non – regenrable
	0.5	Regenrable	Non – regenrable	Non – regenrable brown
	1.0	Regenrable compact, nodular	Regenrable compact	Regenrable,nodular
	2.0	Regenrable	cream color	, white
	3.0	Regenrable compact, white	Regenrable compact, nodular	Regenrable , glossed, white
NAA	0.0	Non – regenrable	Non- regenrable	Non – regenrable
	0.5	Non – regenrable	Non - regenrable translucent	Regenrable, cream color
	1.0	Regenrable	Regenrable	Regenrable, nodular
	2.0	Non – regenrable	Regenrable nodular	Non - regenrable translucent
	3.0	Regenrable nodular	Regenrable white	Regenrable white

external appearance (regenrable, non- regenrable). The capacity to produce regenerable callus depends on growth hormone.

Conflict of interests

The authors have not declared any conflict of interests.

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