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An efficient protocol optimization for callus induction, regeneration and acclimatization in sugarcane *cv*. CO 94012

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Abstract
Sugarcane's vast and complex genome, photosensitivity, low seed setting, prolonged crop duration, and complex environmental effect make it difficult to create diverse genetic variability by conventional breeding. However, the *in vitro* culture system can play a vital role in boosting yield, quality, and making the crop climate smart through *in vitro* approaches. In the present study *in vitro* culture using immature leaf sheath of Sugarcane (*Saccharum officinarum* L. *cv*- CO 94012) as an ex plant was used to standardize the protocol for callus induction and whole plant regeneration. MS medium supplemented with 2.0 mg/l, 2-4 D, recorded the highest percentage of callus induction. The highest number of multiple shoots was produced on MS medium fortified with KIN 1.0 mg/l. Rooting was more profuse when *in vitro* regenerated shoots were inoculated on MS basal medium supplemented with 3.0 mg/l NAA. Rooted shoots were hardened in the greenhouse before being transplanted to the field, where 90% survival rate.

Keywords: Sugarcane, genetic variability, callus induction, regeneration

Introduction
Sugarcane (*Saccharum officinarum* L.) is a major agricultural cash crop in the world's tropical and subtropical regions, and it is the primary source of sugar in many underdeveloped nations, accounting for more than 60% of global sugar output (Guimarces and Sobral, 1998) [16]. Conventional crop breeding in sugarcane appears to be confined due to the high heterozygous genetic architecture, rare flowering, poor reproductive status, a wide genome size, a long breeding cycle, and significant environmental interaction. Plant tissue culture techniques have emerged as a potent tool for exploring and solving basic and applied plant biotechnology challenges. Plant tissue culture is a technique for generating clones of a plant by propagating it from cells or tissues grown in sterile conditions. When disease-free material is used as the source of explants or the explants are heat-treated to eliminate pathogens, the resulting micro propagated plants are disease-free and healthy. An efficient regeneration system is a priori to micro-propagation, the creation of in vitro induced genetic variants (somaclonal variation) and successful genetic transformation. Using immature leaf whorl explants, several researchers have studied *in vitro* genetic manipulation of sugarcane to improve yield, sugar content, and resistance to biotic and abiotic challenges (Vickers *et al.*, 2005, Gilbert *et al*., 2005, Gilbert *et al*., 2009, Gao *et al.*, 2016, Kumar *et al*., 2013 and Reis *et al*., 2014) [31, 11, 10]. However, plant genotype and hormonal supplementation in the medium to sustain calli growth, subsequent plant regeneration, and plant survival limit its application. Embryogenic calli are increasingly being employed to recover genotypically stable regenerants and to genetically alter valuable genes. Genotype (s) with effective callus induction and plant regeneration abilities must be chosen to accomplish this. This improved methodology through callus mediated regeneration will aid in the development of useful somaclones, mutants and an effective system for genetic transformation of an essential sugarcane variety Co 94012 in developing red rot-resistant cultivars.

Materials and Methods
Ex plant and surface sterilization: Healthy young immature leaf whorls of 6-7 months old top shoots were collected by removing the leaf sheath from field-grown plants of sugarcane

(*Saccharum officinarum* L. cv- CO 94012) maintained in the Sugarcane Breeding Institute, Coimbatore, and brought to the tissue culture laboratory. The *in vitro* studies were carried out in the Department of Plant Biotechnology, Centre for Plant Molecular Biology & Biotechnology (CPMB & B) Tamil Nadu Agricultural University Coimbatore, during 2019-21. The young immature leaf rolls were cut into thin smaller pieces of 4.0 to 5.0 cm in length, washed thoroughly under running tap water for 5 minutes followed by Bavistin (0.1%) along with two drops of tween-20 for 10 minutes and then thoroughly rinsed with sterile distilled water and taken to the laminar airflow cabinet. The explants were treated with 0.1% (w/v) mercuric chloride (HgCl2) for another 3 minutes. Finally, the young immature leaf rolls were washed thoroughly 3 to 5 times with sterile distilled water. Then the leaf rolls were placed on sterile filter paper for absorption of excess water on the surface and cut into $2mm^2$ and inoculated in the callus induction medium.

Culture medium: The surface sterilized young immature leaf bits were inoculated on sterilized semisolid basal MS medium (Murashige and Skoog's, 1962) supplemented with different concentrations and combinations of auxins and cytokinins.

Callus induction: For callus induction different concentrations of either 2,4-D alone (0.5, 1.0, 1.5, 2.5, 3.5 mg/l) or 2,4-D together with BAP 1.0 + 0.5, 2.0 + 0.5, 3.0 + 0.5, 4.0 + 0.5, 1.0 + 1.0, 2.0 + 1.0, 3.0 + 1.0 and 4.0 + 1.0) are tried individually and together such as 2,4-D (0.5,1.0,1.5,2.0,2.5,3.0 and 3.5 mg/l, 2,4-D and BAP respectively) were supplemented to the MS basal medium.

Shoot regeneration Medium: Eight-week-old white friable calli were sub cultured on MS medium supplemented with different concentrations of either BAP or Kinetin at the concentrations of 1.0, 2.0, 3.0, 4.0 mg/l for shoot regeneration.

Rooting medium: Elongated micro shoots measuring about 5-6 cm in length were excised from the culture tube and transferred to full-strength MS medium supplemented with different concentrations of IBA or NAA at a concentration of 1.0, 2.0, 3.0, 4.0 mg/l for *in vitro* rooting

Culture condition: MS Powder (Himidia) 4.1g, 30g/l sucrose and different concentrations of 2, 4-D, BAP, Kin, NAA, and IBA (Sigma Grade) were used for callus induction, plant regeneration and *in vitro* rooting studies (Table 1). The pH of the medium was adjusted to 5.8 before adding the gelling agent Agar-agar type I (GR M666-500 G- Himedia) 8g/l. The medium was autoclaved for 20 min at121ºC and at 15 psi pressure. The molten medium of 20 ml was dispensed into the 90 x 60 mm Petri plates and wrapped with thin foil to maintain aseptic conditions. All the cultures were incubated in a growth room with a 16h light (3000 lux) and 8 hours dark and the temperature was maintained at 25 ± 1ºC in the culture room. Each treatment was repeated thrice with 10 explants per replication. After four weeks of inoculation, the best treatment was adjudged based on days taken to first callus initiation, callus induction frequency after thirty days, and callus fresh weight after sixty days. The subculturing to a fresh medium was carried out every second week. The Data recorded during regeneration were days taken for shoot initiation; numbers of shoots regenerated per calli and mean shoot length after four weeks of the culture period. Regenerated plantlets were then transferred to a root induction medium containing different combinations of NAA and IBA (Table 1). Days taken for root initiation, the number of roots per shoot, and mean root length were recorded after four weeks during *in vitro* rooting experiment.

Acclimatization and transfer of plantlets to soil: Well-rooted plantlets were transferred to disposable cups filled with coco peat, sand, clay (1:1:1) and kept in the green house, under high humidity (> 90%) for hardening. Well-grown plants with 15 cm height were planted under field conditions for establishment.

Statistical Analysis: Experiments were set up in a Completely Randomized Design (CRD) with three replications and 10 explants per replication. The treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% probability level according to Gomez and Gomez (1976) [13].

Results and Discussion

An efficient *in vitro* protocol for callus induction, shoot regeneration, *in vitro* rooting and successful hardening of sugarcane cultivar CO 94012 was standardized and the results obtained in the current experiments are summarized here.

Callus Induction

In the sugarcane cultivar CO 94012, immature leaf bits were used as explants and 15 distinct medium compositions were tried with varied doses of 2, 4-D either alone or in combination with BAP to optimize the callus induction process (Table 1). After one week of culture, callus initiation could be seen from the cut surface of young leaves in all the combinations including the control at different durations (Table 2). The number of days required for the callus initiation was varying from 6.333 (MS+ 2 mg/l 2,4-D) to 18.333 (MS basal). The callus induction rate was 100 percent (Fig: 1, Table-2). Khan *et al*. (1998) and Khatri *et al*. (2002) have also reported the induction of callus of same type. Begum *et al.* (1995) discovered that 3.5 mg/l of 2, 4-D induced the maximum percentage of callus induction from leaf base explants in the Bangladesh Nagabari sugarcane variety. Islam *et al*. (1982) similarly found that 0.5-5.0 mg/l 2,4-D induced callus formation in leaf tissue on MS medium. Abu *et al*. (2014) reported callus induction in two weeks after inoculating the explants, specifically young leaves and spindles, on MS media with three different concentrations of 2, 4-D. (2.0, 3.0 and 4.0 mg l-1). Whereas, Dash *et al.* (2011) reported callus formation from shoot tip within 10 days at the concentration of 3 mg l-1 of 2,4-D. Leaf-sheath explants showed callus initiation at the cut margins of the explants 10-12 days after inoculation (Goel *et al.*, 2010). Dash *et al.* (2011) reported callus induction was observed within 10 days of inoculation at 3 mg l-1 2, 4-D.

The frequency of callus induction ranged from 33.33 percent to 100.00 percent (Table 2). MS basal medium had the lowest response to callus induction (33.33%) of all treatments, with the highest response (100.00%) obtained in treatment T4 (MS+ 2 mg/l 2,4-D), followed by T5 (MS+ 2.5 mg/l 2,4-D) (86.66%) and T14 (MS+ 2 mg/l 2,4-D + 1 mg/l BAP) (83.66 per cent). Karim *et al* (2002) [3] reported that at 3mg/l 2, 4-D,

the callus induction frequency was 90 percent in Isd0-28 and 100 percent in Isd-16. At 3 mg l-1 of 2, 4-D, Dash *et al.* (2011) recorded the maximum callus induction frequency of 95% from the shoot tip explants. Gemechu *et al.* (2011) documented, the high callus percentage (92.5%) with 3 mg/l of 2, 4-D for variety B52-298 and a callus percentage of 90.83% in NCO-334 variety on MS medium supplemented with 2 mg/l of 2, 4-D. Tiwari *et al*. (2013) found that 2, 4-D at 3.0 mg l-1 was ideal for maximum callus induction and proliferation in all types of explants from different varieties of sugarcane and that at this concentration, the rate of callus formation in CoS 8820 was 100% with leaf, 90% with meristem, and 60% with pith. While in CoS 767, it was 100%, 90%, and 70% with leaf, meristem, and pith, respectively. Abu *et al.* (2014) observed the best callus percentage (90.83) from the explants *viz.*, young leaves and spindle on MS medium supplemented with 2 mg/l of 2, 4-D for NCO-334 variety, in which 3 mg/l of 2, 4-D produced the least callus percentage. Vu Anh Tuan *et al* (2015) observed 63.7 per cent, 88.2 per cent, 91.8 per cent, and 62.3 per cent callus induction in ROC22, My5514, LS1, and QD94 respectively at 3 mg/l 2, 4-D. The results revealed that callogenesis is genotype-dependent.

For all treatments, callus fresh weight was found to be highly significant ($p< 0.05$) (table 2). The callus fresh weight ranged from 3.03 gm to 18.50 gm per 10 calli, with a considerable variability observed in the experiments. The callus fresh weight achieved in 2,4-D @ 2 mg/L was the highest. The best plant growth regulator for callus fresh weight was 2,-4-D, either alone at 2 and 3 mg/L or 2, 4-D in combination with BAP. The average fresh weight from the medium fortified with 2.0 mg/l with 0.5 mg/l BAP and 2,4-D 2 and 3 mg/l with BAP 1.0 mg/l was 13.4-16.5 mg/l was within the range of 13.4-16.5 gm/10 callli (Table. 2). The callus cultures were maintained through frequent subculturing (up to 3) after every 10-15 days interval on to fresh medium for further proliferation and the proliferated calli was transferred to shoot regeneration medium.

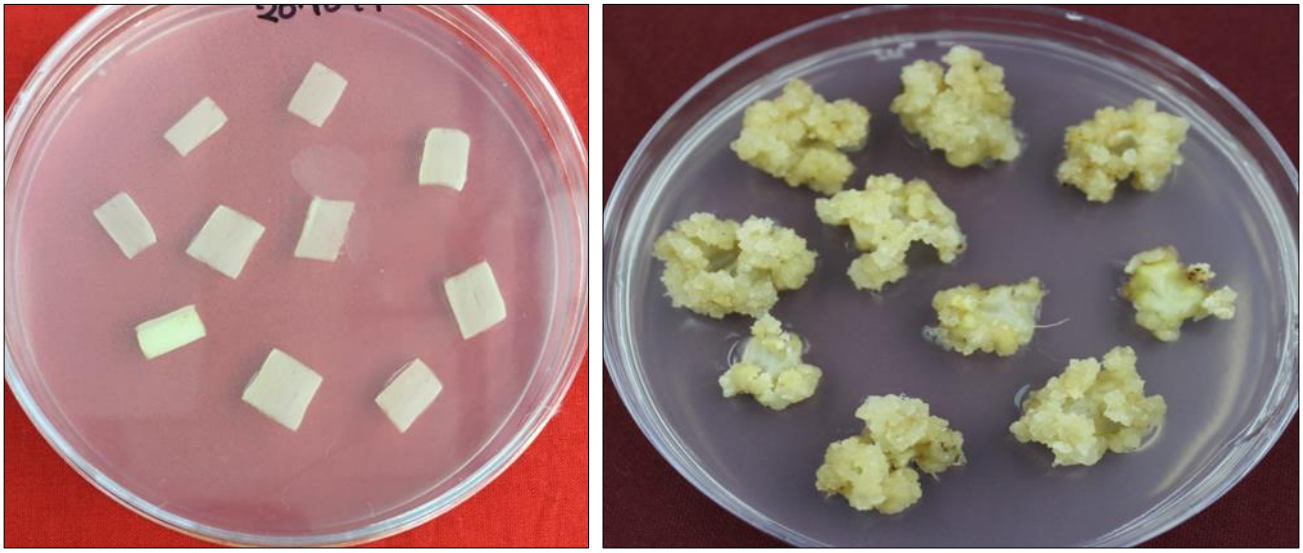

Fig 1: Callus induction on MS medium supplemented with 2mg/L 2, 4-D

Table 1: Different callus induction and regeneration media composition

Sr. No	Callus induction media				Sr. No	Regeneration media				Sr. No	Rooting media			
	MS salt (g/L)	Sucrose (g/L)	2,4-D (mg/L)	BAP (mg/L)		MS salt (g/L)	Sucrose (g/L)	BAP (mg/L)	Kin (mg/L)		MS salt (g/L)	Sucrose (g/L)	NAA (mg/L)	IBA (mg/L)
T1	4.10	30.00	0.50	0.00	M1	4.10	30.00	1.00	0.00	R1	4.10	30.00	0.50	0.00
T2	4.10	30.00	1.00	0.00	M2	4.10	30.00	2.00	0.00	R2	4.10	30.00	1.00	0.00
T3	4.10	30.00	1.50	0.00	M3	4.10	30.00	3.00	0.00	R3	4.10	30.00	2.00	0.00
T4	4.10	30.00	2.00	0.00	M4	4.10	30.00	4.00	0.00	R4	4.10	30.00	3.00	0.00
T5	4.10	30.00	2.50	0.00	M5	4.10	30.00	0.00	1.00	R5	4.10	30.00	0.00	0.50
T6	4.10	30.00	3.00	0.00	M6	4.10	30.00	0.00	2.00	R6	4.10	30.00	0.00	1.00
T7	4.10	30.00	3.50	0.00	M7	4.10	30.00	0.00	3.00	R7	4.10	30.00	0.00	2.00
T8	4.10	30.00	1.00	0.50	M8	4.10	30.00	0.00	4.00	R8	4.10	30.00	0.00	3.00
T9	4.10	30.00	2.00	0.50										
T10	4.10	30.00	3.00	0.50										
T11	4.10	30.00	4.00	0.50										
T12	4.10	30.00	1.00	1.00										
T13	4.10	30.00	2.00	1.00										
T14	4.10	30.00	3.00	1.00										
T15	4.10	30.00	4.00	1.00										

50 ml $CaCl_2$ (prepared by adding 4.4 g of anhydrous $CaCl_2$ in 500 ml of distilled water) and 8 g Agar-Agar type I also added in above media pH 5.8

Table 2: Effects of 2,4-D alone or 2,4-D in combination with BAP on callus induction from immature leaves of *cv* CO 94012

Code	Medium with Plant growth regulators (mg/L)	Days to Callus Initiation	Callus Induction Frequency (%)	Callus Fresh Weight (gm/ 10 calli)
T0	Control (T0)	18.333±0.333^{l}	33.333±3.333^{h}	3.033±0.333^{i}
T1	MS +2,4-D (0.5)	7.666±0.333^{b}	66.666±3.333^{fed}	5.413± 0.331^{h}
T2	MS +2,4-D(1.0)	9.333±0.333^{cd}	73.333±3.333^{edc}	13.853±0.342^{dc}
T3	MS +2,4-D(1.5)	10.666±0.333^{ef}	73.666±3.333^{edc}	14.533±0.415^{dc}
T4	MS +2,4-D(2.0)	6.333±0.333^{a}	100.000±0.000^{a}	18.500±0.601^{a}
T5	MS+ 2,4-D(2.5)	11.666±0.881^{fg}	86.666±6.666^{b}	11.940±1.788^{fe}
T6	MS +2,4-D(3.0)	14.667±0.577^{i}	76.666±6.666^{dcb}	9.880±0.477^{g}
T7	MS +2,4-D (3.5)	15.333±0.333^{j}	66.333±3.333^{fed}	6.820±0.352^{h}
T8	MS +2,4-D (1.0)+ BAP(0.5)	16.666±0.333^{k}	53.666±6.666^{g}	5.066±0.120^{h}
T9	MS +2,4-D (2.0)+BAP(0.5)	11.333±0.333^{ef}	76.000±10.000^{dcb}	16.846±0.336^{ba}
T10	MS +2,4-D (3.0)+ BAP(0.5)	12.666±0.333^{gh}	80.666±3.333^{cd}	15.466±0.696^{cb}
T11	MS+ 2,4-D (4.0) +BAP(0.5)	13.666±1.000^{hi}	73.333±6.666^{edc}	11.080±0.532 ^{gf}
T12	MS +2,4-D (1.0)+ BAP(1.0)	8.666.00±0.667^{bc}	63.333±3.333^{gfe}	12.753±0.221 ^{fed}
T13	MS +2,4-D (2.0)+ BAP (1.0)	10.333±0.667^{de}	56.333±3.333^{gf}	13.476±0.234 ^{ed}
T14	MS +2,4-D (3.0)+ BAP (1.0)	12.666±0.667^{gh}	83.666±6.666^{cb}	14.220±0.376 ^{dc}
T15	MS +2,4-D (4.0)+ BAP(1.0)	16.333±0.667^{jk}	66.666±3.333^{fed}	14.673±0.339 ^{dc}
	Mean	12.000	70.620	11.720
	CD	1.176	10.736	1.750
	S.Em(+)	0.408	3.720	0.608
	SED	0.577	5.270	0.859
	C.V.%	5.78	9.140	8.998

Each value represents the mean ± SE of three replicates and 10 explants/ replication. Different letters in the same column indicated the significant differences at $p \leq 0.05$. (Duncan's multiple range test)

Shoot regeneration

In the present study regeneration of shoots occurred in MS media supplemented with BAP and Kinetin at different concentrations. The results related to shoot regeneration are discussed below.

The number of days required for shoot initiation ranged from (6 days to 16 days). The minimum number of days (6 days) for shoot induction was reported on MS medium supplemented with 1mg/l KIN and the longer days (16 days) for shoot induction on MS basal medium were reported (Fig. 2, Table 3). Baksha *et al.* (2002) [3], Pathak *et al.* (2009) and Goel *et al.* (2015) observed shoot induction after 2-3 weeks of culture. The estimation of findings of Sharma *et al* (2005) showed that it took 5-8 days for 80% of freshly induced explants to become autotrophic. Garcia *et al.* (2007) observed shoot regeneration after 30 days of inoculation. Biradar *et al.* (2009) and Godheja *et al.* (2014) reported the shoot induction within 7-10 days of culture. Sughra *et al.* (2014) [22] observed shoot induction after 11 days in BL-4, 13 days in Thatta-10, and 14 days in Larkana-2001. Abu *et al.* (2014) observed shoot induction within a week from the explants *viz.*, young leaves, and spindle.

The number of shoots generated per explant varied between 9.0 and 23.0. (Table 3). The highest number of shoots per explant (23.0) was recorded on MS medium supplemented with 1 mg/l KIN, whereas the lowest number of shoots per explant (8.0) was observed on MS basal medium. Yadav *et al.* (2012) reported 23.52 ± 6 and 22.6 ± 2.4 average number of shoots per culture in CoSe 01235 and CoS 99259 respectively with BAP, Kinetin and NAA (0.5 mg/l each). Abu *et al.* (2014) observed highest shoot number on MS + 2 mg/l BA + 0.5 mg/l IBA (25.17) and 2 mg/l BA (23.33) in variety B52-298 and for NCO-334, the maximum number of shoots were recorded on 1 mg/l BA + 0.5 mg/l NAA (20.5) and 1.0 mg/l BA (19.33). Sharma (2005) also observed an average number of shoots as 3.75 were in CoS 8436 and 3.5 in CoJ 83. On contrary, Ali *et al.* (2008) also obtained a maximum of 1.8 shoots per explant with 1.5 mg/l BAP in sugarcane variety, CP 77400 and 0.5 mg/l BAP with 0.25 mg /l Kinetin in BL-4. An average number of shoot produced per explants was 8.30 ± 0.60 in Isd-32 (Roy and Kabir, 2007) [26].

The average length of a shoot ranged from 5.93 cm to 12.50 cm. On MS medium supplemented with 1 mg/l Kin, the maximum average shoot length (12.5 cm) was observed, whereas the shortest length (5.93 cm) was reported on MS basal medium (Table 3). Behara *et al.* (2009) measured mean shoot length as 6.2 cm and 4.0 cm for the two highest-performing hormone combinations, BAP (2.0 mg/l) + IBA (0.5 mg/l) and BAP (2.0 mg/l) + IBA (1.0 mg/l). Tarique *et al.* (2010) [30] found that MS medium supplemented with 1.0 mg/l BA + 0.5 mg/l NAA and 1.0 mg/l BA + 0.5 mg/l IBA resulted in shoot lengths of 4.9 and 4.7 cm, respectively. Abu *et al.* (2014) observed maximum (4.0 cm) shoot length on media supplemented with 2.0 mg/l of BAP and 2 mg/l of BA + 0.5 mg/l of IBA in B52-298 variety while, NCO-334variety, 1 mg/l of BA + 0.5 mg/l of NAA recorded maximum shoot length and moderate on media containing 1.0 mg/l of BA and 2.0 mg l-1 of Kinetin. Roy and Kabir (2007) [26] observed an average shoot length of 6.50 cm in Isd-32. Sughra *et al.* (2014) [22] observed an average shoot length of 5.50, 4.50 and 3.70 cm in BL-4, Thatta-10 and Larkana-2001 respectively.

Fig 2: Regeneration of embryo genic calli on MS medium with 1mg/L KIN

Root Induction

Auxins are the primary plant growth regulators that promote roots in the majority of plant species. Elongated micro shoots with three to four leaves and a length of about 6-7 cm were excised from the culture tube and transferred to full-strength MS medium supplemented with either NAA or IBA (1-4 mg l/1) + sucrose (30 g l-1). After micro shoots were transferred to root induction medium for 7 to 19 days, root induction was observed.

The time taken for root induction ranged from 8 to 19 days (Fig. 3, Table 4). Maximum number of days (19) for root induction was recorded on MS basal medium and a minimum number of days was observed on MS medium supplemented with 3 mg/l NAA. Sood *et al.* (2000) reported root induction in 10 -15 days on MS medium + 7 mg/l NAA and the results were also supported by Singh *et al.* (2001) who observed the best rooting in 7-15 days on MS medium with 5 mg/l NAA. Singh (2003) observed root induction in 8-10 days on MS medium supplemented with 5 mg/l NAA. On contrary, Ali *et al.* (2008) reported root induction in 8-10 days on MS medium with 1 mg/l NAA + 2 mg/l IBA. Whereas, Karim *et al*. (2002) [18] observed root induction in 10-15 days at 3 mg l-1 IBA. Abu *et al.* (2014) observed roots regeneration started 10 to 15 days after micro shoots were transferred to root induction medium for both varieties. Sughra *et al.* (2014) [22] observed root induction in 9 days, 10 and 11.50 days in BL-4, Thatta-10 and Larkana-2001 respectively with NAA (3 mg /l)

For the effective establishment and survival of the plantlets to soil transfer, abundant and healthy root induction of micro-shoots is a crucial juncture. The strongest auxin for root induction is said to be naphthalene acetic acid (NAA). Among all the treatments tested, full-strength MS Medium with NAA (3 mg/l) proved to be the most effective. Differential responses were observed in all of the treatments utilized in this experiment. The number of roots generated ranged from 14 to 5. (Table 4). A maximum number of roots per shoot (14) was recorded on MS medium supplemented with 3mg/l NAA and a minimum number of roots (5) was reported on MS basal medium. Our results were strongly supported by previous reports by Sughra *et al.* (2014) [22] recorded average number of 6.80, 2.5 and 4.90 roots per shoot in BL-4, Thatta-10 and Larkana-2001 respectively with 3 mg l-1 NAA. Yadav *et al.* (2012) observed 5.8 and 5.7 roots per shoot in CoSe 01235 and CoS 99259 respectively with 5 mg/l NAA and 50 g/l

sucrose. Behera and Sahoo, (2009) [4] recorded the highest number roots per micro shoots (13.4) at 2.5mg/l NAA. Rashid *et al*. (2009) [25] obtained maximum number roots (3.6) on half strength MS medium for 1.0 5mg/l IBA. Ali and Afghan (2001) observed 6-7 roots after 3 weeks on MS medium containing 2.0 mg/l IBA and 6 per cent sucrose. Gopitha *et al.* (2010) [14] observed the highest number of average roots per micro shoots (15) in half MS medium supplemented with 3 mg/l of NAA. Tarique *et al.* (2010) [30] recorded the highest number of roots (13.47) at 5.0 mg/l of NAA.

Root lengths ranged from 1.90 cm to 4.33 cm on average (Table 4). The maximum average root length (4.33 cm) was observed on MS medium supplemented with 3 mg/l NAA, whereas the shortest root length was observed on MS basal medium. With 3 mg/l NAA, Sughra *et al.* (2014) [22] achieved an average root length of 2.50, 1.50, and 1.30 cm in BL-4, Thatta-10, and Larkana-2001, respectively. In B41-227 and N14, Tolera (2016) [5] found an average root length of 2.92 cm and 2.58 cm, respectively. Khan *et al.* (2008) observed a maximum root length of 2.5 cm on MS medium enriched with 2.5 mg/l IBA + 2.5 mg/l NAA and 1.5 mg/l IBA respectively. Behera and Sahoo (2009) [4] obtained an average root length of 4.0 cm for the variety Nayana on MS medium supplemented with 2.5 mg/l of NAA. Rashid *et al.* (2009) [25] observed root length 3.5 cm when grown at half MS medium supplemented with 1.0 mg/l of IBA. Gopitha *et al.* (2010) [14] recorded an average length of root 4.9 cm in half MS medium supplemented with 3mg /l of NAA.

Fig 3: *In vitro* rooting on MS medium supplemented with 3mg/L NAA

Fig 4: Primary hardening at the greenhouse

Table 3: Effect of different concentrations of BAP or Kinetin on shoot regeneration

Code	Medium + Plant growth regulators (mg/L)	Days to Shoot Initiation	No of Shoots per Calli	Shoot Length(cm)
M0	Control	16.666±0.333^{g}	8.666±0.333^{e}	5.93±0.666^{f}
M1	MS +BAP (1.0)	10.333±0.333^{b}	16.333±0.333^{d}	6.881±0.195 ^{e}
M2	MS+ BAP (2.0)	12.666±0.333^{de}	19.000±0.577^{cb}	7.903±0.880^{d}
M3	MS +BAP (3.0)	13.666±0.333^{ef}	16.666±0.333^{d}	10.960±0170^{b}
M4	MS +BAP (4.0)	14.666±0.333^{f}	16.000±0.577^{d}	6.580±0.170^{e}

M5	MS +Kin (1.0)	6.333±0.333[a]	23.000±0.577[a]	12.500±0.288[a]
M6	MS+ Kin (2.0)	11.333±0.333[bcd]	20.333±0.333[b]	7.646±0.081[d]
M7	MS+ Kin (3.0)	11.000±1.000[bc]	19.333±0.333[cb]	8.890±0.162[c]
M8	MS+ Kin (4.0)	12.000±0.577[cd]	18.333±0.333[c]	7.463±0.164[d]
	Mean	12.07	17.51	8.30
	CD	1.26	1.12	0.43
	S.Em (+)	0.41	0.37	0.14
	SED	0.59	0.52	0.20
	C.V.%	6.94	4.25	3.48

Each value represents the mean ± SE of three replicates. 10 calli /replication
Different letters in the same column indicated the significant differences at $p \leq 0.05$. (Duncan's multiple range tests

Table 4: Effect of different concentrations of NAA and IBA on *in vitro* root induction

Code	Medium with Plant growth regulators (mg/L)	Days to Root Initiation	No of Roots per Shoot	Root Length(cm)
R0	Control	19.333±0.666[f]	5.333±0.333[f]	1.900±0.057[e]
R1	MS +NAA (1.0)	10.666±0.333 [de]	9.333±0.333 [ed]	1.733±0.240[e]
R2	MS +NAA (2.0)	9.667±0.881 [cd]	11.333±0.333 [c]	2.666±0.176[cd]
R3	MS +NAA (3.0)	8.333±0.333 [ab]	14.333±0.333 [a]	4.333±0.176[a]
R4	MS +NAA (4.0)	11.666±0.333 [e]	11.000±0.577 [c]	2.900±0.057[b]
R5	MS+ IBA (1.0)	9.333±0.333 [bc]	10.333±0.333 [dc]	1.166±0.166[f]
R6	MS +IBA (2.0)	10.666±0.333 [de]	10.666±0.333 [c]	2.000±0.115[ed]
R7	MS +IBA (3.0)	7.333±0.333 [a]	12.666±0.333 [b]	2.400±0.200[dc]
R8	MS +IBA (4.0)	10.814±0.333 [cd]	10.814±0.333 [e]	2.325±0.120[e]
	Mean	12.14	11.39	2.70
	CD	1.00	0.96	0.41
	S.Em(+)	0.33	0.31	0.13
	SED	0.47	0.45	0.41
	C.V.%	6.16	6.13	11.70

Each value represents the mean ± SE of three replicates. 10 shoots/replication.
Different letters in the same column indicated the significant differences at $p \leq 0.05$. (Duncan's multiple range test)

Conclusion

In vitro culture is not only viable, but also be employed as a useful tool for rapidly multiplying disease-free, high-yielding, and premium-quality planting material of highly adapted, genetically stable, and freshly released sugarcane types, according to the study. The traditional breeding technique for sugarcane genetic makeup makes it tough to introduce a new variety. Furthermore, releasing a viable variety takes a long period. The diverse and well-established protocols for in vitro modulation of sugarcane morphogenesis have a wide range of applications. The potential for creating new traits through induced somaclonal variation and screening in vitro for pest disease tolerance has yet to be fully realised. In the case of Co 94012, the callus culture methodology can be utilised to produce somaclonal variations in sugarcane for improving features that are difficult to improve through the reproductive cycle.

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