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An Efficient Micropropagation Protocol for Sugarcane viz. Isd 33

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Abstract

Six months' shoot tips of sugarcane variety Isd 33 were cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators and coconut water for callus induction. The best (100%) callus formation was obtained on MS medium containing 3.5 mg/l 2, 4-D (2, 4-Dichlorophenoxyacetic acid) with 10% coconut water. Excellent multiple shooting with 100% shoot induction was observed when medium was supplemented with 1.5 mg/l BAP (6-benzylaminopurine) along with the combination of 1 mg/l Kn (Kinetin). However, the highest (6.86 cm) shoot length was found in 1 mg/l BAP with 1 mg/l Kn. The maximum number (18.83) of roots/shoot was recorded in 5 mg/l NAA (1-Naphthaleneacetic acid). The highest (4.42 cm) root length and maximum root initiation percentage (96.67%) were performed on rooting medium with 1 mg/l NAA in combination with 2 mg/l IBA (Indole-3-butyric acid).

Introduction

Sugarcane (*Saccharum officinarum* L.) is a sugar-producing plant from the Poaceae family, grown worldwide as a significant industrial crop and accounts for 85-87% of the total sugar production globally (Miranda et al. 2020). Over 20 million hectares of land are allocated for the cultivation of sugarcane across approximately 90 countries globally (Naz 2003). It ranks as the second principal cash crop and the third among essential field crops, particularly in the northern and southern regions of Bangladesh. (Rahman et al. 2016).

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In Bangladesh, sugarcane is cultivated on approximately 0.13 million hectares of land (Rahman et al. 2016). Bangladesh produces an annual 5.79 million metric tons (MMT) of sugarcane (Rahman et al. 2016). Of the total, 1.56 MMT is utilized by sugar mills to generate 0.10 MMT of sugar, while 3.50 MMT is employed to manufacture 0.35 MMT of jaggery; the remaining 0.87 MMT is allocated for seed and chewing applications. (Anonymous 2015b). The traditional breeding of sugarcane is quite difficult due to its restricted gene pool, high ploidy (2n=100-120) (Ingelbrecht et al. 1999), rare flowering, poor fertility, large genome size, lengthy breeding cycle and complex environmental interactions (Manickavasagam et al. 2004). Consequently, it necessitates 10-15 years to properly recognize and release a new clone as a variety (Budeguer et al. 2021). Despite various challenges, *in vitro* micropropagation techniques are the preferred option for researchers aiming to enhance sugarcane. Barba and Nickel (1969) first generated plantlets from sugarcane callus cultures, Liu 1993 demonstrated that any section of the sugarcane plant may produce callus. In the realm of plant genetic modification, callus induction is the exclusive option for sugarcane among the research community (Matsuoka et al. 2001, Kaur and Gosal 2009, Ramgareeb et al. 2010, Rao and Jabeen 2013, Soares et al. 2014). Callus-based recuperation will be essential as *in vitro* mutations and somaclonal variants may be generated and subsequently employed for crop improvement. Numerous researchers have created various in vitro propagation techniques for sugarcane; nonetheless, each genotype or variety necessitates a distinct protocol for efficient and effective propagation based on its genotype explants type, the position of explant, age, plant growth regulator and it's interactions (Roy and Kabir 2007, Deole et al. 2022, Arjun and Srinath 2015, Baksha et al. 2002). Kabir et al. 2024, Amente and Feyissa 2022, Ullah 2016a, Cheema and Hussain 2004 and Khan et al. 2008 established the flexible implementation of the protocols. Scientists have also being explored *in vitro* genetic modification of sugarcane to enhance production, sugar content, and tolerance to biotic and abiotic stressors (Mahmud 2021, Abdelsalam et al. 2021, Fernando et al. 2017, Vickers et al. 2005, Gilbert et al. 2005 and Gilbert et al. 2009). By considering the above-mentioned facts, this investigation aimed to establish a standardized tissue culture techniques for the sugarcane variety Isd 33. This variety of sugarcane is high yielding, stress tolerant and comprises approximately 20-25% of total cultivated varieties in Bangladesh to meet the rising need for short-duration, high sugar-content, disease-free sugarcane germplasm and to support future research activities including the development of transgenic sugarcane plants.

Materials and Methods

Six month old top shoots of highly acceptable sugarcane variety Isd 33 were gathered from the research plot of Bangladesh Sugarcane Research Institute, Ishurdi, Pabna. Immature innermost leaf sheaths were utilized as explants for callus induction. The top shoot was harvested from the field and rinsed under running tap water for

approximately 30 min. The outer whorls were removed and the apical part of the shoots were trimmed and cut into 7 cm length. The explant was gently cleaned with 2% detergent sodium hypocloride (NaClO) and subsequently treated with 1% Chlorhexidine Gluconate + Cetrimide (savlon product of ACI Ltd.) give for five min with continuous agitation and then rinsed 3-4 times with distilled water for surface sterilization. The explants were subsequently subjected to a 5 min treatment with 70% alcohol. Surface disinfection was performed using 0.1% $HgCl_2$ for duration of 12 min (Tarique et al. 2010). The explant was thereafter rinsed 3 to 5 times with distilled water. Following sterilization, explants were aseptically sectioned into 0.5-1.0 cm weighted ± 0.2 g in a laminar airflow cabinet. The slice of explants was cultured on MS basal salt media with varying concentrations of 2,4-D (0.0-4.0 mg/l) individually, or in combination with 2,4-D (2.0-3.0 mg/l) and Kn (0.2-0.6 mg/l), as well as 2,4-D (2.0-3.5 mg/l) and coconut water (10%-15%), 0.6-0.8% agar, 3.0% sucrose was used and 5.7-5.8 p^H was maintained. All medium was autoclaved at 121°C for 20 min at 15 psi. Explants were inoculated in each test tube and cultures were maintained in darkness at 25 ± 1.0°C. The explant was subcultured every 2-3 weeks and maintained in dark conditions. Following callus induction, each callus was transferred to shooting media. The shooting medium utilized contained MS basal salt medium having different concentrations of BAP (0.0-3.0 mg/l) and different combination of BAP (1.0-2.0 mg/l) and Kn (0.25-1.0 mg/l) with 3.0% sucrose, 0.6% agar and having 5.7-5.8 pH. For successive shootings, the explant was sub cultured every three weeks and cultures were maintained in the culture room at 25 ± 1.0°C under 16 hrs of light (3000 lux) and 8 hrs of darkness. In this study, thirty one (T_1-T_{31}) treatments for callus induction, eighteen (T_1-T_{18}) treatments for shoot initiation and thirty two (T_1-T_{32}) treatments were employed with different concentration and combination of growth hormon. The regenerated plantlets were put to a rooting medium consisting of half strength MS with various concentration of NAA (0.0-6.0 mg/l) and combination of NAA (0.5-1.0 mg/l) and IBA (1.0-4.0 mg/l). Established plantlets were relocated to trays containing a blend of coco peat, sand, and clay in a 1:1:1 ratio under high humidity conditions (> 90%) for hardening (Maruprolu et al. 2022) at green house condition. Well-developed plants were transferred to polybags and subsequently planted in the field for establishment.

The experiment utilized a Completely Randomized Design (CRD) with six replications, and all the data were statistically analyzed by Statistix 10 (Tallahassee, FL 32312, USA). An LSD test was conducted when significant differences ($P < 0.05$) were detected.

Results and Discussion

Six-month-old top shoots of sugarcane variety Isd 33 were used as explants. Surface-sterilized leaf sheaths were inoculated on autoclaved MS medium with varying concentrations of 2,4-D (1.0-4.0 mg/l) individually, or in combination with 2,4-D (2.0-3.0 mg/l) and Kn (0.2-0.6 mg/l), as well as 2,4-D (2.0-3.5 mg/l) and coconut water (10%-15%).

The duration for callus initiation varied from 7 to 20 days (Maruprolu et al. 2022). Among thirty-one treatments, the fastest callus initiation occurred at T_8 (MS + 4.0 mg/l 2,4-D), T_{27} (MS + 3.5 mg/l 2,4-D + 10% CW), T_{30} (MS + 3.0 mg/l 2,4-D + 15% CW) and T_{31} (MS + 3.5 mg/l 2,4-D + 15% CW) in minimum duration 7 days and 8 days required to complete callus initiation at $T_{27,}$ T_{30} and $T_{31.}$ The maximum 15 days required to callus initiation and 20 days to complete on MS medium supplemented with 2,4-D (1.0 mg/l) (Table 1). No callus initiation was observed on only MS medium. Here, the observation was, when lower concentrations of 2,4-D took the maximum time (15-20 days) and gradually increased concentrations took short times (7-10 days) to initiate callus. Many of the researchers worked on 2,4-D with different concentrations of callus initiation days. 3 mg/l and 4 mg/l 2,4-D concentrations took minimum days for callus initiation (Zahra 2010, Patel 2015 and Ullah 2016a). Effects of 2,4-D and coconut water on callus initiation day were also described by Alam et al. 2003, Rahulbaksha et al. 2003, Gopitha et al. 2010 and Arjun and Srinath, 2015. From Table 1, it was observed that callus induction percentage varies with the increasing concentration of 2,4-D. The highest callus induction (85.88 %) was found at 4 mg/l 2,4-D alone. Jamil et al. 2022 also found the highest callus induction percentage (84.5%) at 4 mg/l 2,4-D alone. Arjun and Srinath (2015) reported best response of callus induction (100%) at 3.5 mg/l and 4.0 mg/l. From this experiment, it was also observed that combination of 2,4-D + Kn and 2,4-D + coconut water had tremendous effects on callus induction and 3 mg/l 2,4-D + 0.6 mg/l Kn showed better callus induction (93.0%). It was found that 3.5 mg/l 2,4-D + 10% coconut water showed best (100%) then that of 2,4-D alone. Callus growth also influence by increasing dose of 2,4-D. The highest callus weight (1.71 g) was observed at 4 mg/l 2,4-D and minimum was recorded (0.97 g) 1 mg/l 2,4-D alone. The combined effects of 2,4-D + Kn at 2.5 + 0.2 doses showed better (2.01 g) than 2,4-D alone and 3.5 mg/l 2,4-D + 10% coconut water showed best (2.36 g) result. From table 1, we found that 3.5 mg/l 2,4-D + 10% coconut water performed best in all aspects like callus initiation, callus induction, callus growth (weight & diameter). The data was recorded 30 days after explant placed on medium and, explant was sub cultured every 2 weeks intervals. It was reported that BAP and Kn play a vital role on shoot induction, multiplication and shoot development from callus. In this experiment, It was noticed the effects of BAP and Kn on shoot regeneration.

BAP showed the best performance on shoot induction percentage (100%), no. of multiple shoots (20.83) and shoot length (6.23 cm) at 1.5 mg/l. The lowest shoot induction percentage (43.33%), no. of multiple shoot (5.50) and shoot length (1.55 cm) result was found at 3.0 mg/l BAP (Table 2). From the study it was observed that high dose of BAP decrease shoot induction percentage, no. of multiple shoot and shoot length. Lowest result was performed without BAP in all parameters. The combined effects of BAP and Kn also performed better on shoot multiplication (33.5) @ 1.5 + 1.0 than that of BAP alone. Udhutha et al. 2016 also observed that 1.5 mg/l BAP showed the highest result on shoot formation (100%) and number of shoot/explant (1.8). Some others scientist also explained

Table 1. Effects of 2,4-D, Kn and coconut water on callus of sugarcane variety Isd 33.

Treatments		Callus initiation days	Callus induction (%)	Callus weight (g)	Callus diameter (mm)
MS + 2,4-D (mg/l)					
T_1	00	00	0.00	$0.00^{k} \pm 0$	$0.00^{k} \pm 0$
T_2	1.0	15-20	65.50	$0.97^{j} \pm 0.41$	$10.08^{i} \pm 2.91$
T_3	1.5	14-18	66.50	$1.14^{h-j} \pm 0.35$	$11.84^{h-j} \pm 3.29$
T_4	2.0	12-14	70.50	$1.48^{d-g} \pm 0.32$	$15.49^{a-f} \pm 2.22$
T_5	2.5	12-14	72.50	$1.47^{d-h} \pm 0.28$	$15.14^{a-g} \pm 3.46$
T_6	3.0	10-12	75.16	$1.34^{e-l} \pm 0.39$	$12.95^{g-in} \pm 2.19$
T_7	3.5	10-12	84.67	$1.25^{f-j} \pm 0.21$	$12.82^{g-l} \pm 1.71$
T_8	4.0	7-10	85.33	$1.71^{b-d} \pm 0.21$	$14.95^{a-g} \pm 1.98$
MS + 2,4-D (mg/l) + Kn (mg/l)					
T_9	2.0 + 0.2	12-14	75.17	$1.54^{d-g} \pm 0.39$	$14.86^{a-g} \pm 3.27$
T_{10}	2.0 + 0.3	12-14	77.67	$1.73^{b-d} \pm 0.35$	$16.20^{a-e} \pm 2.16$
T_{11}	2.0 + 0.4	10-12	78.83	$1.58^{c-f} \pm 0.34$	$15.15^{a-g} \pm 1.1$
T_{12}	2.0 + 0.5	10-12	81.00	$1.54^{d-g} \pm 0.45$	$14.72^{b-g} \pm 2.75$
T_{13}	2.0 + 0.6	12-16	83.17	$1.32^{e-l} \pm 0.38$	$12.86^{g-l} \pm 2.95$
T_{14}	2.5 + 0.2	10-14	84.83	$2.01^{b} \pm 0.35$	$15.89^{a-f} \pm 2.00$
T_{15}	2.5 + 0.3	10-14	84.83	$1.49^{d-g} \pm 0.51$	$15.98^{a-f} \pm 1.91$
T_{16}	2.5 + 0.4	10-12	86.17	$1.36^{e-l} \pm 0.51$	$14.03^{e-h} \pm 3.88$
T_{17}	2.5 + 0.5	10-12	86.00	$1.08^{ij} \pm 0.33$	$11.51^{ij} \pm 1.79$
T_{18}	2.5 + 0.6	10-12	86.33	$1.04^{ij} \pm 0.40$	$11.59^{ij} \pm 3.24$
T_{19}	3.0 + 0.2	12-16	89.50	$1.24^{g-j} \pm 0.23$	$13.78^{f-l} \pm 1.95$
T_{20}	3.0 + 0.3	12-16	89.33	$1.33^{e-l} \pm 0.33$	$14.30^{d-g} \pm 2.88$
T_{21}	3.0 + 0.4	10-12	91.67	$1.28^{f-j} \pm 0.29$	$12.84^{g-l} \pm 1.4$
T_{22}	3.0 + 0.5	10-12	92.50	$1.36^{e-l} \pm 0.46$	$14.42^{c-g} \pm 2.7$
T_{23}	3.0 + 0.6	10-12	93.00	$1.45^{d-h} \pm 0.39$	$14.56^{c-g} \pm 2.12$
MS + 2,4-D (mg/l) + Coconut water					
T_{24}	2.0 + 10%	8-12	76.83	$1.77^{b-d} \pm 0.68$	$17.03^{ab} \pm 1.88$
T_{25}	2.5 + 10%	8-12	79.83	$1.77^{b-d} \pm 0.30$	$15.69^{a-f} \pm 1.67$
T_{26}	3.0 + 10%	8-10	95.00	$1.62^{c-e} \pm 0.57$	$16.71^{a-c} \pm 3.84$
T_{27}	3.5 + 10%	7-8	100.00	$2.36^{a} \pm 0.44$	$17.18^{a} \pm 3.6$
T_{28}	2.0 + 15%	8-12	79.33	$1.71^{b-d} \pm 0.22$	$16.65^{a-d} \pm 1.64$
T_{29}	2.5 + 15%	8-12	81.83^{j}	$1.23^{g-j} \pm 0.20$	$13.63^{f-l} \pm 1.72$
T_{30}	3.0 + 15%	7-8	95.00	$1.76^{b-d} \pm 0.31$	$16.75^{a-c} \pm 2.89$
T_{31}	3.5 + 15%	7-8	98.33	$1.88^{bc} \pm 0.51$	$16.67^{a-d} \pm 3.15$
LSD (0.05)			4.28	0.34	2.37

Values with the same letter in the same column are not Statistixally different at $p < 0.05$.

the effects of BAP and BAP + Kn both on shoot formations (Goel et al. 2010 and Tolera et al. 2014)). The data was recorded 60 days after inoculation and sub-culture was done every three weeks intervals.

Table 2. Effects of different PGRs on shoot initiation and multiplication of sugarcane var. Isd 33.

Treatments		Shoot initiation %	No. of multiple shoot/callus	Average shooth length
MS + BAP				
T_1	00	15.00	$1.17^{l} \pm 1.0$	$0.5633^{k} \pm 0.44$
T_2	1.0	95.00	$17.67^{f} \pm 2.5$	$3.88e^{f} \pm 0.70$
T_3	1.5	100.00	$20.83^{de} \pm 1.0$	$6.26^{a} \pm 0.36$
T_4	2.0	60.00	$8.83^{ij} \pm 2.0$	$2.93^{ghi} \pm 0.40$
T_5	2.5	53.33	$7.83^{jk} \pm 1.5$	$2.33^{i} \pm 0.25$
T_6	3.0	43.33	$5.50^{k} \pm 1.5$	$1.55^{j} \pm 0.32$
MS + BAP + Kn				
T_7	1.0 + 0.25	81.67	$19.67^{ef} \pm 2.0$	$4.01^{def} \pm 0.70$
T_8	1.5 + 0.25	85.00	$22.83^{d} \pm 3.0$	$4.51^{cde} \pm 0.64$
T_9	2.0 + 0.25	63.33	$10.50^{hi} \pm 1.5$	$3.37^{fgh} \pm 0.90$
T_{10}	1.0 + 0.5	75.00	$20.83^{de} \pm 4.0$	$3.85^{ef} \pm 1.04$
T_{11}	1.5 + 0.5	90.00	$25.33^{c} \pm 2.0$	$4.77^{bc} \pm 1.20$
T_{12}	2.0 + 0.5	68.33	$11.17^{hi} \pm 2.0$	$3.60^{fg} \pm 0.90$
T_{13}	1.0 + 0.75	81.66	$20.67^{de} \pm 1.0$	$4.68^{cd} \pm 0.80$
T_{14}	1.5 + 0.75	95.00	$29.17^{b} \pm 3.5$	$4.84^{bc} \pm 1.60$
T_{15}	2.0 + 0.75	76.67	$13.83^{g} \pm 1.5$	$3.51^{fgh} \pm 0.80$
T_{16}	1.0 + 1.0	90.00	$25.33^{c} \pm 3.5$	$6.86^{a} \pm 0.69$
T_{17}	1.5 + 1.0	100.00	$33.50^{a} \pm 5.0$	$5.46^{b} \pm 0.90$
T_{18}	2.0 + 1.0	65.00	$12.67^{gh} \pm 2.5$	$2.84^{hi} \pm 0.63$
LSD (0.05)			2.47	0.74

Values with the same letter in the same column are not Statistixally different at $p < 0.05$.

Different types of auxins influenced primordial root formation from well-developed root. In this experiment, rooting was significantly affected by varying concentrations of NAA and IBA. The highest number of root (18.83), root length (3.80 cm) and percent of root induction (88.16%) was observed on 5 mg/l NAA. and root initiation started 10-15 days after shoot placed on medium containing 3 mg/l IBA showed the highest result on number of root (12.83), root length (3.30 cm) and percent of root induction (83.66%) among other treatments (Table 3). NAA and IBA combinedly performed significant effects on root formation (Table 3). The minimum response was found on medium without hormonal supplements. A lots of researchers found better rooting response on ½MS with 3 mg/l IBA (Arjun and Srinath 2015, Maruprolu et al. 2022, Gopitha et al. 2010). Tarique et al. 2010 found the best root formation on ½MS with 5 mg/l NAA. Kumari et al. 2016 also found the highest number of root and root length on MS with 5 mg/l NAA. The data was recorded 40 days after placed on rooting medium.

Table 3. Effects of NAA and IBA on root formation of sugarcane variety Isd 33.

Treatments		Root initiation days	Root induction (%)	No. of root/shoot	Root length (cm)
MS + NAA (mg/l)					
T_1	0.00	00	16.66	$3.17^{r} \pm 2.2$	$0.61^{P} \pm 0.22$
T_2	2.0	20-25	55.33	$7.50^{k-o} \pm 1.5$	$0.97^{n-p} \pm 0.23$
T_3	2.5	10-15	63.33	$8.83^{i-m} \pm 2.2$	$1.48^{i-m} \pm 0.43$
T_4	3.0	10-15	71.33	$10.50^{d-j} \pm 2.0$	$1.66^{h-l} \pm 0.44$
T_5	3.5	10-14	75.67	$11.33^{c-h} \pm 4.0$	$1.86^{f-j} \pm 0.26$
T_6	4.0	7-10	79.66	$11.50^{c-g} \pm 4.0$	$2.22^{e-g} \pm 0.83$
T_7	4.5	8-10	85.17	$14.16^{b} \pm 2.0$	$3.34^{BC} \pm 0.73$
T_8	5.0	10-12	88.16	$18.83^{a} \pm 1.5$	$3.80^{b} \pm 0.25$
T_9	5.5	12-14	55.66	$9.50^{f-l} \pm 1.5$	$1.35^{k-n} \pm 0.64$
T_{10}	6.0	15-20	51.67	$4.16^{qr} \pm 1.0$	$0.78^{op} \pm 0.27$
MS + IBA (mg/l)					
T_{11}	0.5	15-20	43.50	$5.16^{o-r} \pm 3.5$	$0.80^{op} \pm 0.13$
T_{12}	1.0	15-20	45.83	$7.17^{l-p} \pm 5.0$	$1.0350^{m-p} \pm 0.14$
T_{13}	1.5	15-18	52.16	$8.33^{j-n} \pm 3.5$	$1.18^{l-o} \pm 0.36$
T_{14}	2.0	12-14	65.67	$10.16^{e-j} \pm 3.5$	$2.25^{ef} \pm 1.32$
T_{15}	2.5	10-12	75.66	$11.83^{b-f} \pm 1.5$	$3.00^{cd} \pm 0.69$
T_{16}	3.0	10-12	83.66	$12.83^{b-d} \pm 2.0$	$3.30^{C} \pm 0.92$
T_{17}	3.5	12-16	65.33	$9.83^{e-k} \pm 2.0$	$1.56^{h-l} \pm 0.50$
T_{18}	4.0	16-20	43.16	$8.50^{j-n} \pm 3.0$	$1.35^{k-n} \pm 0.34$
MS + NAA + IBA					
T_{19}	0.5 + 1.0	18-20	58.00	$9.00^{h-l} \pm 3.0$	$1.75^{g-k} \pm 0.53$
T_{20}	0.5 + 1.5	15-20	61.33	$11.17^{c-i} \pm 3.0$	$1.99^{f-h} \pm 0.67$
T_{21}	0.5 + 2.0	12-15	75.33	$12.00^{b-e} \pm 6.5$	$2.32^{ef} \pm 0.89$
T_{22}	0.5 + 2.5	12-14	76.33	$13.50^{bc} \pm 1.5$	$3.42^{bc} \pm 0.15$
T_{23}	0.5 + 3.0	10-12	70.33	$10.50^{d-j} \pm 3.5$	$1.74^{g-k} \pm 0.46$
T_{24}	0.5 + 3.5	10-12	66.00	$10.17^{e-j} \pm 3.5$	$1.96^{f-i} \pm 0.45$
T_{25}	0.5 + 4.0	15-20	44.66	$5.16^{o-r} \pm 1.0$	$1.42^{j-n} \pm 0.19$
T_{26}	1.0 + 1.0	15-20	78.16	$10.50^{d-j} \pm 1.5$	$2.32^{ef} \pm 0.25$
T_{27}	1.0 + 1.5	12-14	80.00	$9.33^{g-l} \pm 5.5$	$2.60^{de} \pm 1.1$
T_{28}	1.0 + 2.0	10-12	96.66	$14.00^{b} \pm 1.5$	$4.42^{a} \pm 0.10$
T_{29}	1.0 + 2.5	10-12	90.00	$12.83^{b-d} \pm 1.0$	$2.31^{ef} \pm 0.55$
T_{30}	1.0 + 3.0	14-18	66.66	$6.16^{n-q} \pm 1.0$	$1.97^{f-l} \pm 0.48$
T_{31}	1.0 + 3.5	15-20	50.83	$6.50^{m-q} \pm 1.5$	$1.41^{j-n} \pm 0.28$
T_{32}	1.0 + 4.0	15-20	42.50	$4.83^{p-r} \pm 1.0$	$1.01^{m-p} \pm 0.19$
LSD (0.05)			4.29	2.49	0.49

Values with the same letter in the same column are not Statistixally different at $p < 0.05$.

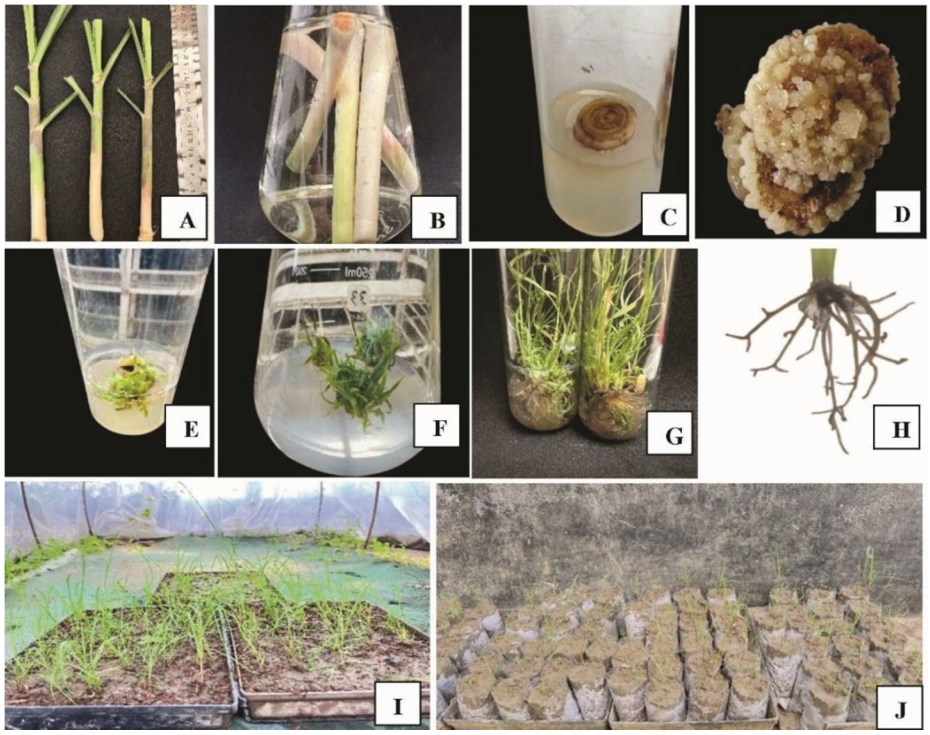

Fig. 1 (A-J). *In vitro* regeneration of sugarcane variety Isd 33: (A) Collection of healthy top shoot of from the field, (B) After sterilization of leaf sheath, (C) Placement of explant on callus media, (D) Callus from explant, (E) Shoot initiation from callus, (F) Shoot multiplication, (G, H) Shoot develop root at rooting media, (I) Hardening of plantlets on tray, (J) Transferred healthy plants to the polybag.

Well-shooted and rooted plantlets were transferred to try filled with coco peat, sand, clay (1 : 1 : 1), under high humidity (> 90%) for hardening at green house condition (Fig. 1). Well grown plants were shifted in polybag and then planted under field conditions for field establishment.

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