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High-Frequency Direct Organogenesis of Sugarcane (*Saccharum officinarum* L.) varieties of Bangladesh

Sheikh Fahmida Tasnim[1], G M Al Amin[1], Shawon Ghosh, Ripa Akter Sharmin[1] and Tahmina Islam*

Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

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Abstract

An efficient and reproducible *in vitro* regeneration protocol was developed for sugarcane varieties, Isd 33, Isd 37 and Isd 40 using five-month-old leaf sheath explants with direct shoot regeneration on MS medium supplemented with NAA. In this study, the highest shoot regeneration in Isd 33 and Isd 40 was obtained on MS medium supplemented with 10.0 mg/l NAA, producing 29.17 and 33.33 shoots per explant with 90 and 93.3% response, respectively. In Isd 37, maximum shoot induction was observed at 9.0 mg/l NAA with 25.49 shoots per explant and 90% response. Successful shoot regeneration was achieved within 65 days of culture. Rooting was observed subsequently, where in Isd 33, 100% rooting produced 33.83 roots per shoot with root length of 5.08 cm at 2.5 mg/l NAA, with root initiation occurring within 3 to 7 days, while in Isd 37, 100% rooting produced 31.83 roots per shoot with root length of 5.40 cm at 2.5 mg/l NAA, and in Isd 40, 100% rooting produced 28.33 roots per shoot with root length of 6.12 cm at 2.0 mg/l NAA, with full root development completed within 40 days of transfer to rooting medium. Plantlets that were acclimatized displayed 100% survival in Isd 33 and Isd 37 and 88.89% survival in Isd 40. Overall, this direct NAA-dependent protocol supported efficient shoot regeneration, rooting and acclimatization in all three varieties, with variety-specific optimal NAA concentrations indicating that regeneration response in sugarcane is genotype-dependent rather than attributable to a single superior variety.

Introduction

Sugarcane (*Saccharum officinarum* L.) is a tall perennial grass of the Poaceae family (Amalraj and Balasundaram 2006) that can accumulate crystallizable sugar in its stalk. Sugarcane is the main source for the sugar industry (Velvizhi et al. 2022), gur and juice in the tropical and subtropical regions, apart from which other valuable byproducts like

*Author for correspondence: <t.islam@du.ac.bd>. [1]Department of Botany, Jagannath University, Dhaka-1100, Bangladesh.

molasses, animal feed, alcoholic drinks and cane wax are made (Yadav and Solomon 2006). Among globally successful biofuel production methods, the conversion of sugarcane into bioethanol stands out due to its significant positive energy balance and very reasonable cost (Huang et al. 2020). In Bangladesh, it is one of the most economically important cash crops. Approximately 70-80% of the world's sugar is produced from sugarcane, which plays an important role in agro-industrial economies (Karamchandani et al. 2022). It has a critical role as an export commodity for many developing countries (Mabeta and Smutka 2023).

Sugarcane displays high heterozygosis and complex polyploidy, rare flowers, poor fertility, large genome size, long breeding cycle and high genotype and environment interaction (Maruprolu et al. 2022). As a result, the development and release of a new improved variety through conventional breeding usually takes 10-15 years (Budeguer et al. 2021). The prolonged propagation cycle further hampers large-scale multiplication and breeding programs, making it difficult to meet the increasing demand for improved varieties (Singh et al. 2006). Studies have shown that traditional methods of producing sugarcane are limited by low multiplication rates, high labour costs, time-consuming processes, and transfer of systemic pathogens via seed cane, thereby limiting its effectiveness (Lakshmanan et al. 2006, Rajib et al. 2025). This method also allows the persistence and accumulation of systemic pathogens like fungi, bacteria, viruses and mycoplasma, causing a drop in yield up to 70% and limiting productivity and sustainability of the system for the long run (Bhavan and Gautam 2002, Imtiaz et al. 2006).

Over the past 30 years, *in vitro* propagation has proven to be a rapid and effective technique for obtaining disease-free and genetically uniform seedlings and is essential for increasing productivity and sustainability. Different regeneration systems, such as callus culture (Patel et al. 2015, Awan et al. 2019, Abdelsalam et al. 2021, Thwe et al. 2022), axillary bud culture (Zamir et al. 2012), shoot tip culture (Biradar et al. 2010, Sughra et al. 2014, Ullah et al. 2016, Kaur and Kapoor 2017, Rajib et al. 2025) and leaf sheath (Tarique et al. 2010, Kabir et al. 2024, Rebina et al. 2025, Su et al. 2026) culture have been utilized in sugarcane improvement programs (Lakshmanan et al. 2006, Pathak et al. 2009). Previously, Tarique et al. (2010) and Kabir et al. (2024) reported organogenesis from leaf sheath with different approaches such as leaf-sheath culture but with a multistep sterilization, leaf-sheath/genotype regeneration with auxin–cytokinin combinations and/or longer or unspecified culture periods, respectively. To the best of our knowledge, no previous study has reported a simplified, single-auxin (NAA-only), callus-free, high-frequency direct shoot regeneration protocol validated within a short window (62-65 days) across these three economically important Bangladeshi varieties. The present study therefore focused on the establishment of an efficient, single-auxin (NAA-only) induced direct shoot regeneration method for three commercially important Bangladeshi sugarcane varieties (Isd 33, Isd 37 and Isd 40) with the aim of shortening the explant-to-plantlet cycle relative to earlier indirect approaches.

Materials and Methods

Three sugarcane varieties, Isd 33, Isd 37, and Isd 40 were developed at the Bangladesh Sugar Crop Research Institute (BSRI), Ishurdi, Bangladesh. The materials were subsequently cultivated in the field of the Botanical Garden of University of Dhaka and maintained under controlled conditions in the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Dhaka. Five-month-old leaf sheath tissues were collected and used as explants. Before culture, the explants were surface sterilized under aseptic conditions in a laminar airflow cabinet by rubbing with 70% (v/v) ethanol for 20 seconds. MS medium, the most widely used plant tissue culture medium, was employed throughout the experiment.

The present investigation adopted a comparatively simple and time-efficient sterilization method, where explants were treated only with 70% ethanol under aseptic conditions with a 95% survival rate. This approach required minimal handling time and avoided the use of multiple chemical disinfectants. In contrast, the protocols of Tarique et al. (2010) and Rajib et al. (2025) involved several steps using 2% NaClO, 1% Savlon (chlorhexidine gluconate + cetrimide), 70% ethanol (5 min), and 0.1% $HgCl_2$ (12 min), making them more time-consuming and chemically intensive. Similarly, explants were treated with 3% Savlon (5 min), 70% ethanol (1 min), and 0.1% $HgCl_2$ (10 min), which also required multiple steps and chemicals (Rebina et al. 2025).

Explants measuring approximately 1.0 × 0.2 cm were excised and inoculated onto MS medium supplemented with varying concentrations of NAA alone for multiple shoot induction and enhanced shoot proliferation. For root induction, well-developed shoots were excised individually and transferred to half-strength MS medium supplemented with different concentrations of IBA, IAA, and NAA. The pH of the medium was adjusted to 5.8 before autoclaving, and 3% (w/v) sucrose was used as the carbon source. Cultures were incubated at 25 ± 2°C under controlled laboratory conditions. Regenerated plantlets were carefully removed from culture vessels, washed thoroughly to eliminate residual medium, transferred to plastic pots, and acclimatized under *ex vitro* conditions. Observations on shoot induction after 25-30 days of inoculation and multiple shoot formation were recorded after 65 days of culture, while root induction was assessed after 3-7 days of inoculation and multiple root formation after 40 days of inoculation. Cultural observations were made at regular two-day intervals and subcultures were done every two weeks.

Rooted plantlets were covered with transparent polyethylene sheets for seven days and maintained in the growth room under shaded conditions with regular misting (25 ± 2°C temperature with 16h photoperiod) for acclimatization. Further, they were maintained in the greenhouse (approximately 28°C, 80-85% humidity). Subsequently, the hardened plants were transferred to field conditions.

Each treatment was conducted in three independent replications arranged in a completely randomized design. For every variety (Isd33, Isd37 and Isd40), the effect of

NAA concentration on shoot length and root length was tested by one-way analysis of variance (ANOVA). Shoot number per explant and root number per shoot were analyzed in the same way by one-way ANOVA followed by Tukey's HSD test ($P < 0.05$). Where the F-test was significant, treatment means were separated using Tukey's honestly significant difference (HSD) test at the 5% probability level ($P < 0.05$). Data are expressed as means ± standard error of three independent replications (n = 3 replications per treatment), and all analyses were conducted in IBM SPSS Statistics version 32.0.0.0 (134).

Results and Discussion

The present study was carried out to establish the regeneration system for sugarcane varieties using leaf sheath explants with direct shoot regeneration on MS medium supplemented with NAA. MS media with different concentrations of NAA induced shoot regeneration within 25-30 days after inoculation (Fig. 1a, 1b and 1c). Maximum shoot regeneration was observed after 65 days of culture (Table 1, Fig. 2). In the present study, NAA alone appeared sufficient to induce shoot organogenesis without exogenous cytokinins, which may be consistent with its reported role in reducing developmental and genotypic constraints in sugarcane leaf culture (Lakshmanan et al. 2006). By contrast, 2,4-D tends to favor callus formation over direct organogenesis in sugarcane (Mamun et al. 2004, Thwe et al. 2022). The precise mechanism underlying this NAA-mediated response in the present varieties, however, remains to be elucidated. The cultures were monitored at two-day intervals. Without an intervening callus phase, shoot buds directly developed from the explants, indicating direct shoot regeneration based on morphological observation. Isd 33 showed 90% response in shoot regeneration on MS supplemented with 10.0 mg/l NAA, producing 29.17 shoots per explant with 7.17 cm shoot length (Fig. 1d). In Isd 37, 90% response was obtained on MS supplemented with 9.0 mg/l NAA with 25.49 shoots per explant and 7.50 cm shoot length (Fig. 1e). Isd 40 achieved 93.3% response on MS supplemented with 10.0 mg/l NAA, producing 33.33 shoots per explant with 7.37 cm shoot length (Fig. 1f). Numerous studies have reported indirect regeneration through a callus-mediated pathway using combinations of 2,4-D and cytokinins in sugarcane (Mamun et al. 2004, Tiel et al. 2006, Rajib et al. 2025). Indirect regeneration was reported in Isd 37 and Isd 40, respectively, with relatively reduced shoot production and unclear culture periods (Mahmud et al. 2013, Rebina et al. 2025). The regeneration of callus was obtained using 2.5 mg/l 2,4-D, followed by 2.0 mg/l BAP and 0.5 mg/l NAA for shoot regeneration with a lower number of shoots (Thwe et al. 2022). Shoot proliferation on 0.3 mg/l BAP + 0.03 mg/l NAA is reported by Ajadi et al. (2018) with a reduced number of shoots. However, these protocols required extra steps of culture and resulted in a smaller number of shoots.

Gill et al. (2006) and Khan et al. (2009) reported direct shoot regeneration in sugarcane from young leaf and spindle leaf roll segments without a callus phase, achieving regeneration frequencies up to 83.12% and a maximum of 7.68 shoots per explant using NAA-based media within 6 weeks. The current study successfully

established a regeneration system using only NAA, producing higher shoot numbers directly from explants within a culture period of 65 days. It is worthy to mention that the optimum concentration of NAA for shoot regeneration was genotype-dependent. It was observed that Isd 33 and Isd 40 responded best at 10.0 mg/l, whereas Isd 37 responded best at 9.0 mg/l. This observation suggests that shoot regeneration of sugarcane is governed by genotype. This interpretation is supported statistically, i.e., NAA concentration significantly affected shoot length within each variety, with the genotype-specific optimum grouped as significantly superior by Tukey's HSD test (Fig. 2).

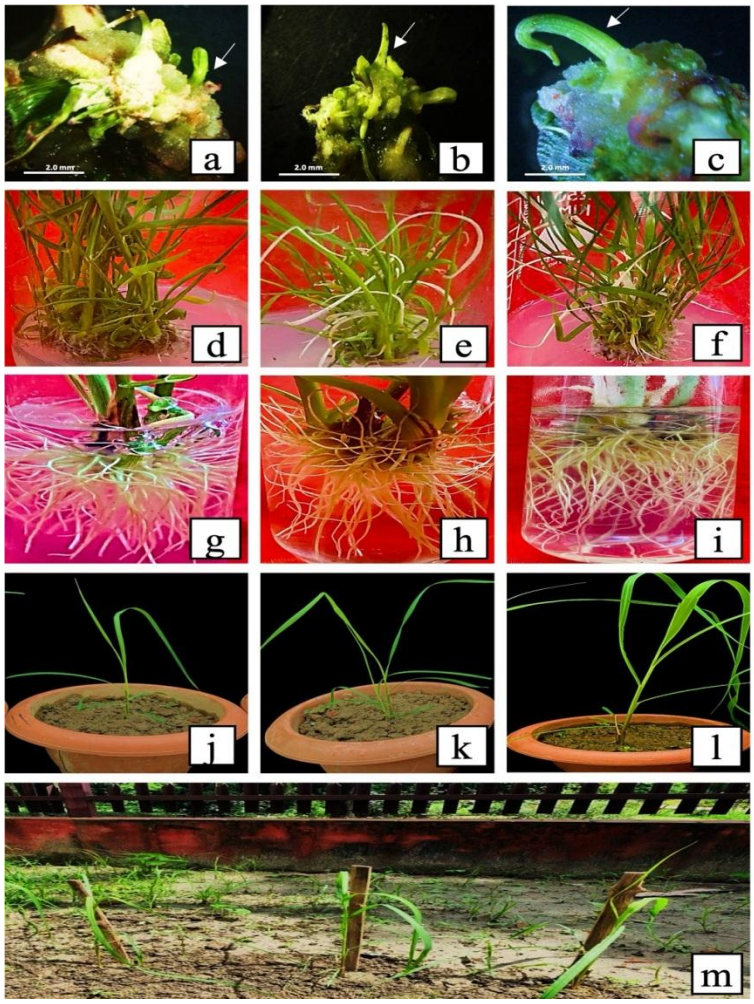

Fig. 1(a-m). Different stages of direct *in vitro* plant regeneration of Sugarcane (*Saccharum officinarum* L.) var. Isd 33, Isd 37 and Isd 40, respectively using leaf sheath explant: (a-c) shoot initiation of Isd 33, Isd 37 and Isd 40, respectively, (d-f) multiple shoots regeneration and elongation of Isd 33, Isd 37 and Isd 40, respectively, (g-i) development of roots from the excised regenerated shoots of Isd 33, Isd 37 and Isd 40 varieties, (j-l) Established plantlets of all three varieties in clay pots, and (m) acclimatized sugarcane varieties Isd 33, Isd 37 and Isd 40 were sown in the field condition.

Table 1. Influence of NAA in MS-supplemented medium on shoot formation from leaf sheath explants of sugarcane varieties, Isd 33, Isd 37, and Isd 40 after 65 days of culture.

Plant Growth Regulator	Sugarcane varieties	Concentrations (mg/l)	% of explants forming shoots	Average number of shoots/explants Mean ± SE
NAA	Isd 33	8.0	50.0	9.0 ± 0.73[b]
	Isd 37		65.0	8.0 ± 0.58[b]
	Isd 40		60.0	14.67 ± 1.26[a]
	Isd 33	9.0	63.3	11.33 ± 0.92[b]
	Isd 37		90.0	25.49 ± 1.40[a]
	Isd 40		66.7	12.5 ± 1.71[b]
	Isd 33	10.0	90.0	29.17 ± 1.78[a]
	Isd 37		63.33	8.67 ± 0.71[b]
	Isd 40		93.3	33.33 ± 1.54[a]
	Isd 33	11.0	70.0	16.83 ± 1.52[a]
	Isd 37		56.67	6.33 ± 0.88[b]
	Isd 40		76.7	16.83 ± 1.52[a]

Within each variety, values (means ± SE, n = 3 replications per treatment) in the same column followed by the same letter are not significantly different by Tukey's HSD test (P <0.05).

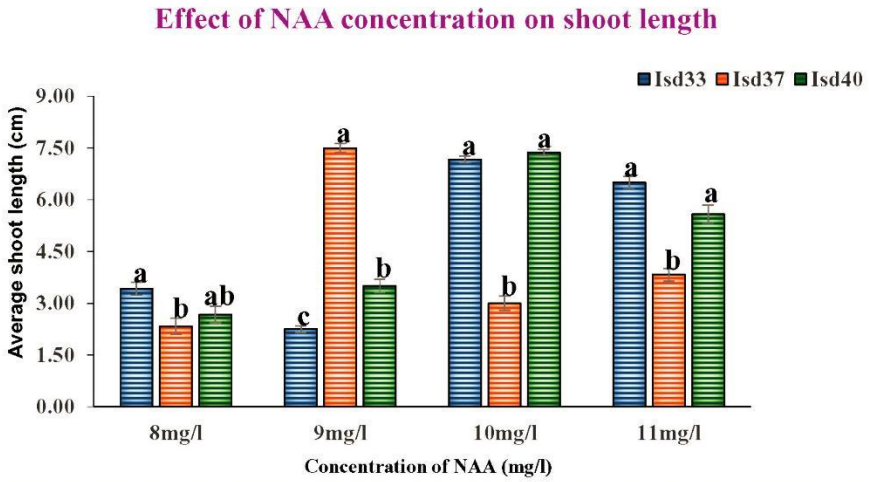

Fig. 2. Effects of NAA concentration (8-11 mg/l) on the mean shoot length (cm) of three varieties (Isd33, Isd37, Isd40). Values are means of three replicates; bar colour denotes variety. Within each variety, means bearing the same letter do not differ significantly by Tukey's HSD test (P <0.05).

Consistent with the role of auxin in overcoming developmental and genotypic constraints in sugarcane leaf culture reported by Lakshmanan et al. (2006), the present study found that NAA alone, without supplementary cytokinins, was sufficient to induce efficient shoot regeneration in the tested sugarcane varieties, indicating genotype-dependent differences in optimal NAA concentration.

Various hormonal treatments were applied to half-strength MS medium to optimize root induction (Table 2, Fig. 3). All the shoots developed roots within 3 to 7 days of the inoculation, indicating that NAA was the most responsive auxin. Although previous studies reported successful rooting using IAA and IBA (Rahman et al. 2001, Behera and Sahoo 2009, Tarique et al. 2010), in the present study, the effects of IAA and IBA were not satisfactory.

Table 2. Effects of NAA on ½ strength of MS media in root induction from *in vitro* raised shoots of sugarcane varieties Isd 33, Isd 37 and Isd 40.

Plant Growth Regulators	Sugarcane varieties	Concentrations (mg/l)	Root initiation Days	% of shoots inducing roots	Average number of roots induced/shoot Mean ± SE
NAA	Isd 33	1.0	4-7	77.78	9.67 ± 0.67[a]
	Isd 37		3-7	85.71	12.00 ± 0.73[a]
	Isd 40		4-7	66.67	11.67 ± 0.80[a]
	Isd 33	1.5	3-5	85.71	15.67 ± 1.38[a]
	Isd 37		3-7	85.71	13.00 ± 1.01[a]
	Isd 40		3-7	85.71	16.33 ± 1.35[a]
	Isd 33	2.0	3-6	100	18.33 ± 1.11[b]
	Isd 37		4-7	100	14.50 ± 1.55[b]
	Isd 40		3-7	100	28.33 ± 2.84[a]
	Isd 33	2.5	3-5	100	33.83 ± 1.40[a]
	Isd 37		3-7	100	31.83 ± 2.57[a]
	Isd 40		4-7	100	25.33 ± 1.87[a]
	Isd 33	3.0	3-7	100	25.50 ± 2.29[a]
	Isd 37		3-7	88.89	23.00 ± 3.13[a]
	Isd 40		3-5	100	19.67 ± 2.02[a]

Within each variety, values (means ± SE, n = 3 replications per treatment) in the same column followed by the same letter are not significantly different by Tukey's HSD test (P <0.05).

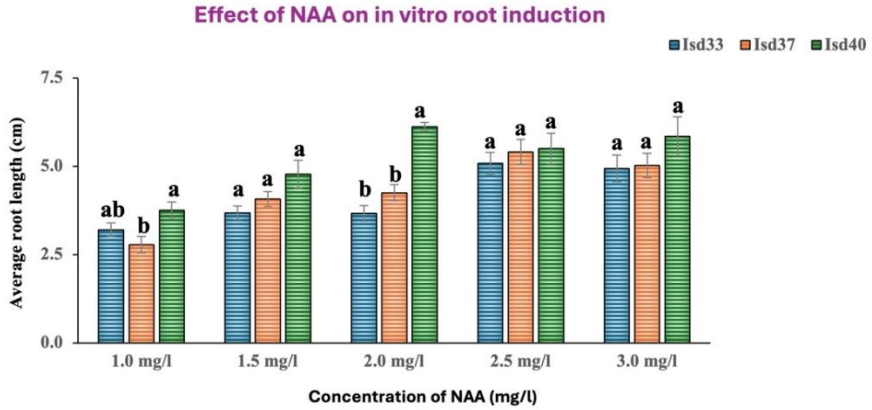

Fig. 3. Effects of NAA concentration (1-3 mg/l) on the mean root length (cm) of three varieties (Isd33, Isd37, Isd40). Values are means of three replicates; bar colour denotes variety. Within each variety, means bearing the same letter do not differ significantly by Tukey's HSD test (P <0.05).

In this study, regenerated shoots of three sugarcane varieties were transferred to ½-strength MS medium supplemented with NAA for root induction. Within each variety, NAA concentration significantly affected both root number (Table 2) and root length (Fig. 3), with the genotype-specific optimum -2.5 mg/l for Isd 33 and Isd 37 whereas 2.0 mg/l for Isd 40. Among varieties, mean root number did not differ significantly where Isd 40 produced significantly longer roots than Isd 33 and Isd 37. In preliminary trials, IAA and IBA gave weak and declining rooting responses above 2.5 mg/l (with browning at higher doses), so the effective range tested was 0.5-2.5 mg/l; NAA continued to give strong responses and was therefore extended to 3.0 mg/L to locate its optimum. Earlier studies by Rahman et al. (2001), Tarique et al. (2010), Mahmud et al. (2013), Kabir et al. (2024), Rajib et al. (2025) and Rebina et al. (2025) reported lower rooting response and longer rooting time in MS, modified MS and half-strength MS media. The present study established an efficient rooting system on ½-strength MS medium supplemented with NAA, producing higher root number and greater root length in all three sugarcane varieties than those reported by the earlier studies cited above.

Table 3. Survival and growth performance of transplanted sugarcane plantlets of Isd 33, Isd 37, and Isd 40 during the hardening stage.

Variety	No. of Replication	Number of transplanted plantlets	Number of survived plantlets	Survival rate (%)	Mean survival rate (%)
Isd 33	1	5	5	100	100
	2	6	6	100	
	3	3	3	100	
Isd 37	1	4	4	100	100
	2	3	3	100	
	3	4	4	100	
Isd 40	1	4	4	100	88.89
	2	3	2	66.67	
	3	2	2	100	

Rooted plantlets of sugarcane varieties Isd 33, Isd 37 and Isd 40 were washed under running water to remove agar and transferred to plastic pots containing sterilized soil for acclimatization. The acclimatization experiment was conducted with three independent replications as shown in Table 3. Acclimatized plantlets were maintained in a greenhouse for further growth (Fig. 1j, 1k and 1l). Subsequently, the hardened plants were transferred to field conditions (Fig. 1m). The survival rate was 100% in Isd 33 and Isd 37 and 88.89% in Isd 40 (Table 3), which is like earlier reports by Rahman et al. (2001), Tarique et al. (2010), Akter et al. (2016), Kabir et al. (2024) and Rebina et al. (2025).

In this study, a simple, reproducible and efficient direct-regeneration protocol was established for three commercial Bangladeshi sugarcane varieties (Isd 33, Isd 37 and Isd 40) from five-month-old leaf sheath explants, using NAA as the sole plant growth

regulator in the optimized protocol and without an intervening callus phase. High-frequency shoot regeneration was achieved within 65 days at genotype-specific optima - 10.0 mg/l NAA for Isd 33 and Isd 40 and 9.0 mg/l NAA for Isd 37 - followed by successful rooting on ½ strength MS medium (2.5 mg/l NAA for Isd 33 and Isd 37; 2.0 mg/l NAA for Isd 40) within 40 days, and 88.89-100% survival of acclimatized plantlets after field transfer. The consistent finding that the optimal NAA concentration differed among varieties demonstrates that regeneration in sugarcane is genotype-dependent rather than the property of a single superior cultivar, implying that such protocols should be screened and tuned per genotype rather than transferred unchanged between varieties. By eliminating callus induction, multi-step sterilization and auxin-cytokinin combinations, and by compressing the cycle from explant to field-ready plantlet into roughly three months, this protocol offers a practical, low-cost route to the rapid, large-scale production of disease-free planting material-directly addressing the slow multiplication rates and long breeding cycles that currently constrain sugarcane supply for the sugar and bioethanol industries.

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