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ORIGINAL RESEARCH

Adventitious shoot organogenesis from immature leaves of sugarcane (*Saccharum* spp. hybrid) cultivar Yuetang 03-373 improving shoot proliferation and industrial scale-up of plants

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Abstract

Sugarcane (*Saccharum* spp. hybrid) cultivar Yuetang 03-373 is a hybrid with superior agronomic characteristics that was obtained from the cross between Yuetang 92-1287 (♀) and Yuetang 93–159 (♂). In this study, transversely cut discs of immature leaves were used to induce adventitious shoots for the first time. Embryonic callus was induced from leaf explants on Murashige and Skoog (MS) medium with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l *α*-naphthaleneacetic acid (NAA). On MS medium containing 0.75 mg/l 6-benzyladenine and 0.1 mg/l NAA, 73.1% of explants from which callus was induced differentiated into adventitious shoot within 30 d and the mean number of adventitious shoot buds was 128.3 per explant. This is many times than regular axillary shoot propagation (mean 5.6 shoots) on the MS medium supplemented with 1.25 mg/l Kinetin (KIN) + 0.1 mg/l NAA. On rooting MS medium containing 0.4 mg/l indole-3-butyric acid (IBA), 0.2 mg L^{-1} NAA and 50 g/l sucrose, all shoots rooted. Well-rooted plantlets showed 100% survival in five tested substrates after 60 d. Using this efficient in vitro regeneration system, scaled-up production was achieved to mass produce sugarcane cultivar Yuetang 03-373 for national markets. The results of our genetic diversity study using DNA-based ISSR markers showed that there was no heritable variation in the induction of shoot organogenesis through immature leaves, which provided a shortcut for future vegetative propagation of sugarcane.

Keywords Histology · Large-scale production · Rooting · Shoot proliferation · Transplantation, DNA-based ISSR markers

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid;
BA	6-benzyladenine;
CIM	Callus induction medium;
IBA	Indole-3-butyric acid
KIN	Kinetin
MS medium	Murashige and Skoog (1962) medium
NAA	α-naphthaleneacetic acid;
ORM	Optimal regeneration medium
PGR	Plant growth regulator
SPE	Shoot proliferation coefficient;
TDZ	Thidiazuron

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Introduction

Sugarcane (*Saccharum officinarum* L.; Poaceae) is a perennial herb that grows in tropical and subtropical regions around the world. Globally, sugarcane is the source of approximately 80% of sugar (sucrose) and 35% of ethanol (OECD/FAO 2018), making it an important cash crop and a major sugar crop worldwide. Modern sugarcane cultivars result from interspecific crosses (Huang et al. 2022). The yield and quality of sugarcane can be improved through breeding and the promotion of new varieties with high yield and strong stress resistance (Abreu et al. 2020). Sugarcane can be efficiently converted into sugar and other renewable energy sources using solar energy, and used to make ethanol (Sanchez et al. 2020), acetate (Tondro et al. 2020), and industrial enzymes (Mason et al. 2020). The leaves can be used as animal feed and are a good raw material for sugar-related industries (de Oliveira et al. 2005; Shang et al. 2022). Yuetang 03-373 is a superior sugarcane hybrid cultivar that was obtained via a cross between Yuetang 92-1287 (♀) and Yuetang 93–159 (♂). This cultivar has several superior agronomic characteristics: early-mid maturity, high sugar content (14–16%), high and stable yield, strong perennial roots, strong stress resistance (wind, drought, disease and pest), wide adaptability, and excellent agronomic traits (Wu et al. 2011). After field trials were successfully conducted, Yuetang 03-373 is now planted in sugarcane-growing areas of China such as Guangdong, Guangxi, Guizhou and Yunnan. However, because there is no robust technology to proliferate plants, it is difficult to expand this variety to other suitable cultivation areas (Liu et al. 2020). One way to achieve plants at an industrial scale is through the use of in vitro culture, i.e., tissue culture. The tissue culture of Yuetang 03-373 was previously achieved using axillary buds as explants, but the shoot proliferation coefficient (SPC) was not so high (5.2 shoots per explant) (Chen et al. 2017). Consequently, further optimization and improvement of tissue culture of this variety is needed.

Somatic embryogenesis and shoot organogenesis as plant tissue culture techniques, can be used to solve several challenges and limitations of traditional breeding and proliferation. This technology can produce many virus-free sugarcane plantlets on a large scale (Lakshmanan et al. 2005; Kaur and Sandhu 2015; Kaur and Kapoor 2016). In sugarcane, somatic embryos (SEs) have been induced from inflorescences (Desai et al. 2004b), young leaves (Khan and Khatri 2006; Maulidiya et al. 2020; Passamani et al. 2020; Reis et al. 2021), midrib segments (Franklin et al. 2006). Callus induction, shoot organogenesis in vitro are viable pathways for clonal propagation and the preservation of germplasm, while virus-free sugarcane plantlets can be obtained via the latter two pathways (Roumagnac 2021). These techniques also allow for the large-scale proliferation of plants (Suprasanna et al. 2011) and the possibility of improving germplasm (Dorairaj et al. 2018).

Simple Sequence Repeats (SSR) labeling is a molecular labeling technology based on specific primer PCR developed in recent years, also known as microsatellite DNA (Microsatellite DNA), which is a kind of tandem repeat sequence of dozens of nucleotides composed of several nucleotides (generally 1–6) as repeat units (Tikendra et al. 2021). Since the sequences on either side of each SSR are generally relatively conserved, single-copy sequences. It has been found that there is a high degree of variation in the number of repeating units in microsatellites, which is manifested as euploidy variation in the number of microsatellites or the possibility that the sequences in the repeating unit sequence are not identical, resulting in polymorphisms at multiple loci (Saker et al. 2000). SSR marker technology has been widely used in the construction of genetic maps, the indexing of target genes, the drawing of fingerprints, breed identification, pedigree analysis, genetic distance analysis between populations, evolution and genetic diversity (Bansal et al. 2024). In the current study, the objective of this study was to induce shoots from immature leaf discs of sugarcane variety Yuetang 03-373, and evaluate of genetic integrity using ISSR markers revealed complete endomorphism and indicating their variations among the regenerants.

Materials and methods

Surface disinfection of immature leaf discs

Six month old Yuetang 03-373 plants were grown outside in the field at the breeding base of Zhanjiang Research Center at the Southern Breeding Industry Research Institute of Guangdong Academy of Sciences, in China. The plants were identified by the institutional and/or licensing committee that approved these experiments, and all experiments were performed in accordance with relevant guidelines and regulations. Shoot tips were cut, outer leaf sheaths were peeled off, and the surfaces of remaining leaf tissues were sprayed with 75% ethanol. Leaf sheath segments were disinfected in 75% ethanol for 30 s, washed three times with sterilized distilled water (SDW), soaked in 0.1% $HgCl_2$ solution for 5 min, washed 3–4 times with SDW, then dabbed dry on sterilized Kraft paper (Ertai, Dongguan, Guangdong, China). The outer leaf layer was peeled off, leaving a leaf explant with only immature leaves (circles) that were cut into slices (explants) about 1 mm thick (Fig. 1a). Leaf circles were cut into two equally sized halves that were used for subsequent experiments.

Fig. 1 Induction of callus and adventitious shoots from immature leaf discs of sugarcane cultivar Yuetang 03-373. **a**, Freshly cut immature leaf disc; **b**, from half-discs, yellow callus was induced after incubation in the dark for 25 days on ideal callus induction medium (CIM; MS with 0.5 mg/l 2,4-D and 0.1 mg/l NAA); **c**, after culture in the dark on CIM for 30 days, some callus became purple; **d**, after culture in the dark on CIM for 30 days, then transfer to light for one week, shoot buds formed on the surface of callus; **e-f**, after culture in the dark on CIM for 30 days, then transfer to MS medium with 0.75 mg/l BA and 0.1 mg/l NAA in light within 30 days, some adventitious shoots and SEs developed multiple shoot clump. Bars: a-c, 1.0 mm; d-e, 0.5 mm; f, 0.5 cm

Callus induction

Explants were plated on Murashige and Skoog (MS) medium (Chembase, Beijing, China) (Murashige and Skoog 1962) supplemented with different concentrations and combinations of plant growth regulators (PGRs): 6-benzyladenine (BA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (KIN), and thidiazuron (TDZ) (Sigma-Aldrich ®, Saint Louis, MO, USA), and cultured in the dark. Six explants were plated in each culture jar (Honghua, Xuzhou, Jiangsu, China). Each treatment had 30 culture jars. Callus induction was observed after 30 days (Table 1; Fig. 1). All media (pH 5.8) were supplemented with 30 g/l sucrose (Sinopharm, Beijing, China) and 7.5 g/l carrageenan (Aladdin Co., Shanghai, China). The temperature of the growth culture room was 28 ± 2 °C. After culture in the dark for 30 days, culture jars were transferred to light (80 µmol $m^{-2}s^{-1}$; 12-h photoperiod), and callus induction was investigated (Table 1). Somatic embryogenesis and shoot organogenesis were directly observed under a stereomicroscope (Nikon SMZ745T, Tokyo, Japan) at different culture times and photographs were taken.

Table 1 Effect of PGRs on callus induction from immature leaf half discs of sugarcane cultivar Yuetang 03-373

PGRs (mg/l)	Callus induction within 30 d (%)	Callus induction-related observations from leaf half discs
PGR-free	0 h	No callus
2,4-D 0.1	100 a	Yellowish, granular callus
2,4-D 0.5	100 a	Yellowish callus, some browning and necrosis
2,4-D 1.5	100 a	Yellowish callus, about half browned and became necrotic
2,4-D 2.5	96.0 ± 4.0 a	Yellowish callus, most became brown and die
2,4-D 3.5	91.0 ± 3.5 a	Most callus became brown and died
TDZ 0.1	29.7 ± 5.0 f	Leaves became loose, little callus, most leaves died
TDZ 0.5	46.3 ± 5.0 d	Leaves became larger and hardened, more leaves died
TDZ 1.5	24.0 ± 3.1 g	Leaves became loose, partial callus formation, leaves died
TDZ 2.5	35.0 ± 1.7 ef	Leaves became loose, partial callus formation
BA 0.1	11.0 ± 0.5 i	Leaves hyperhydric, small amount of callus died
BA 0.5	25.7 ± 2.3 fg	Leaves hyperhydric, small callus became brown and died
BA 1.5	74.3 ± 1.8 b	Leaves hyperhydric, some callus became brown and died
BA 2.5	51.7 ± 2.6 c	Leaves hyperhydric, some callus became brown and died
2,4-D 0.5 + NAA 0.1	100 a	Yellowish callus, no browning, strong growth
2,4-D 3.5 + NAA 0.1	96.3 ± 3.7 a	Yellowish callus, very few browning, strong growth
TDZ 0.5 + NAA 0.1	22.0 ± 1.7 g	Very little callus, most leaves did not induce callus
TDZ 3.5 + NAA 0.1	39.0 ± 3.6 e	Very little callus
BA 0.5 + NAA 0.1	42.7 ± 4.1 d	Leaves hyperhydric, little callus was induced
BA 3.5 + NAA 0.1	40.7 ± 0.9 de	Leaves hyperhydric, and became brown and died

Values represent means ± SD. Different letters within the second column indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$). $n = 60$ per treatment

Differentiation of adventitious shoots

Callus (about 0.5 g) that was derived from MS medium supplemented with 0.5 mg/l 2,4-D and 0.1 mg L^{-1} NAA, i.e., callus induction medium (CIM), in the dark for 30 d was inoculated onto MS medium with different combinations and concentrations of PGRs: 0.1, 0.5, and 1.0 mg/l BA (or KIN); 0.75 mg/l BA (or KIN) and 0.1 mg/l NAA (Table 2). Six callus clumps were cultured for each PGR combination, and each treatment included 10 culture jars. After culture in light for 30 days, organ differentiation from callus was investigated under a Nikon SMZ745T stereomicroscope and photographs were taken.

Table 2 Effects of PGRs on shoot organogenesis from the callus of sugarcane cultivar Yuetang 03-373

PGRs (mg/l)	Callus formation within 30 d (%)	Number of adventitious shoots
PGR-free	10.0 ± 1.5 ef	5.8 ± 1.3 g
BA 0.1	20.0 ± 2.7 d	16.6 ± 1.6 e
BA 0.5	53.3 ± 4.9 b	86.1 ± 3.6 b
BA 1.0	30.3 ± 2.9 c	63.6 ± 4.1 c
KIN 0.1	0 g	0 h
KIN 0.5	2.0 ± 0.6 fg	9.7 ± 1.1 f
KIN 1.0	7.3 ± 0.9 fg	8.0 ± 1.1 f
BA 0.5 + NAA 0.1	51.7 ± 3.5 b	86.0 ± 3.8 b
BA 0.75 + NAA 0.1	73.1 ± 0.2 a	128.3 ± 5.6 a
KIN 0.5 + NAA 0.1	16.7 ± 2.2 de	31.1 ± 2.7 d
KIN 0.75 + NAA 0.1	35.0 ± 4.0 c	29.7 ± 2.3 d

Callus induction medium consisted of MS medium supplemented with 0.5 mg L^{-1} 2,4-D and 0.1 mg L^{-1} NAA. Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$). $n = 60$ per treatment

Shoot proliferation

Callus clumps that were induced on CIM after 30 days were transferred to ORM to differentiate SEs and adventitious shoots. Both SEs and adventitious shoots generally developed shoot clusters, and multiple shoots were divided into smaller explants, each with 2–3 shoots, that were inoculated onto MS medium with different concentrations and combinations of PGRs (Table 3). Each treatment included 60 shoot clumps. SPC (i.e., the number of new shoots that formed from each original shoot) was calculated after 30 days.

Rooting

Multiple shoots were cut into clusters with 2–3 shoots and transferred to MS medium with different concentrations of IBA and/or NAA, as well as sucrose (Table 4). Each treatment included 60 shoot clusters. After 21 days, rooting percentage, root number, and root length were investigated. Rooting percentage was calculated as: number of rooted plantlets / number of inoculated shoots (or somatic embryos) × 100%.

Table 3 Effect of PGRs on shoot proliferation coefficient (SPC) and formation of multiple shoots of sugarcane cultivar Yuetang 03-373

PGRs (mg/l)	SPC within 30 d	Observations
PGR-free	4.7 ± 0.3 c	No callus, normal shoot
BA 0.25	5.1 ± 0.3 b	Multiple shoots, hyperhydric
BA 1.25	4.1 ± 0.3 cd	Multiple shoots, hyperhydric, callus
BA 2.25	3.1 ± 0.3 e	Multiple shoots, hyperhydric
KIN 0.25	4.3 ± 0.3 c	Multiple shoots
KIN 1.25	5.2 ± 0.4 b	Multiple shoots
KIN 2.25	3.9 ± 0.3 d	Multiple shoots, normal
BA 0.25 + NAA 0.1	3.7 ± 0.4 de	Less multiple shoots, hyperhydric
BA 2.25 + NAA 0.1	4.5 ± 0.4 c	Multiple shoots, hyperhydric
KIN 1.25 + NAA 0.1	5.6 ± 0.4 a	Multiple shoots
KIN 2.25 + NAA 0.1	2.7 ± 0.3 h	Multiple shoots

Callus induction medium consisted of MS medium supplemented with 0.5 mg/l 2,4-D and 0.1 mg/l NAA. Optimal regeneration medium (for shoots and somatic embryos) was MS medium supplemented with 0.75 mg/l BA and 0.1 mg/l NAA. Both adventitious shoots and somatic embryos that were induced generally developed multiple shoot clusters. Values represent means ± SD. Different letters within the second column indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$). $n = 60$ per treatment

Acclimatization and transplanting

Culture jars with well-rooted plantlets were placed in a greenhouse (temperature range: 10–35 °C; light intensity ≤ 3000 µmol m^{-2} s^{-1}; 45–95% relative humility) for 10 days and then lids were opened for 15 d to expose plants to ambient environmental conditions. Roots were carefully washed with tap water, and plantlets were soaked in 0.1% rooting solution that contained NAA and IBA (Peste Sciences Co., Zhengzhou, China) for 1–2 min. After drying roots slightly in air, plantlets were potted in seedling trays (Hongxiang Co., Dezhou, Shandong, China), at 2–3 plantlets per cell, in the following 10 substrates (ratios = *v*/*v*): peat: sand (1:1); vermiculite: sand (1:1); perlite: sand (1:1); peat: vermiculite (1:1); perlite: vermiculite (1:1); peat: perlite (1:1); yellow mud: sand (1:1); perlite: vermiculite: sand (1:1:1); perlite: peat: sand (1:1:1); peat: vermiculite: sand (1:1:1). Each treatment included 60 cells. After 60 days, survival percentage of transplanted plants was calculated as [number of surviving plants / total number of transplanted plants] ×100%.

Assessment of genetic integrity

To assess the genetic integrity of in vitro raised plantlets, inter simple sequence repeat (ISSR) markers were employed. Genomic DNA was isolated from 10 plantlets, including three randomly selected mother plants (8-week-old plantlets used as explant source) and three plantlets each obtained from shoots multiplied on media containing 0.4 mg/L IBA and 0.2 mg/L NAA, using CTAB method (Doyle and Doyle 1987). The quality and quantity of extracted DNA were estimated using NanoDrop 1000 (Thermo Fischer Scientific, USA). PCR conditions were optimized for each primer, and PCR reactions (10 µl reaction volume) containing 1X reaction mixture (One PCRTM, GeneDireX Inc. USA), 10 µM primer, and 40 ng template DNA were set up. The PCR was carried out in a thermal cycler (Gene Pro, Hangzhou Bioer Technology Co., China) with specific conditions, including initial denaturation at 94 °C for 5 min, followed by 37 cycles of denaturation at 94 °C (30 s), primer annealing at 37–56.7 °C (45 s), and primer extension at 72 °C (60 s), with a final extension at 72 °C for 5 min. PCR amplicons were resolved on a 2.5% agarose gel in 1X TAE buffer. The size of the amplified product was determined by co-electrophoresing a 100 bp DNA ladder. Amplification profiles were visualized using a Gel Documentation System (GenoSens

Table 4 Effects of different concentrations of PGRs and sucrose on rooting of sugarcane cultivar Yuetang 03-373 within 30 d

PGRs (mg L^{-1}) and sucrose (g L^{-1}) in ½MS media	Shoots forming roots (%)	Number of roots/shoot	Mean root length (cm)
Control + sucrose 30	52.7 ± 3.5 f	2.9 ± 0.2 i	2.1 ± 0.2 e
IBA 0.2 + sucrose 30	53.7 ± 4.6 f	3.3 ± 0.3 i	2.4 ± 0.2 e
IBA 0.4 + sucrose 30	67.7 ± 3.3 e	3.5 ± 0.3 h	2.7 ± 0.2 d
IBA 0.6 + sucrose 30	74.3 ± 3.0 d	4.2 ± 0.3 g	2.8 ± 0.2 d
IBA 0.8 + sucrose 30	82.0 ± 4.5 c	5.0 ± 0.4 f	3.2 ± 0.3 c
IBA 1.0 + sucrose 30	83.3 ± 4.2 c	5.7 ± 0.4 e	3.3 ± 0.3 c
IBA 0.4 + NAA 0.1 + sucrose 30	79.7 ± 2.1 cd	5.4 ± 0.4 ef	2.2 ± 0.2 e
IBA 0.6 + NAA 0.1 + sucrose 30	84.3 ± 2.3 c	5.6 ± 0.4 ef	3.1 ± 0.2 c
IBA 0.4 + NAA 0.2 + sucrose 30	96.3 ± 3.7 a	8.3 ± 0.5 c	3.4 ± 0.3 bc
IBA 0.6 + NAA 0.2 + sucrose 30	91.0 ± 4.5 b	6.4 ± 0.5 d	3.5 ± 0.3 bc
IBA 0.4 + NAA 0.2 + sucrose 10	68.0 ± 3.5 e	4.9 ± 0.4 f	2.1 ± 0.2 e
IBA 0.4 + NAA 0.2 + sucrose 20	82.0 ± 4.7 c	6.6 ± 0.5 d	2.9 ± 0.3 d
IBA 0.4 + NAA 0.2 + sucrose 30	92.3 ± 4.2 b	6.8 ± 0.2 d	3.7 ± 0.3 b
IBA 0.4 + NAA 0.2 + sucrose 40	94.0 ± 4.5 ab	8.5 ± 0.5 c	3.8 ± 0.3 b
IBA 0.4 + NAA 0.2 + sucrose 50	100 a	30.9 ± 1.9 a	5.5 ± 0.4 a
IBA 0.4 + NAA 0.2 + sucrose 60	100 a	11.9 ± 1.1 b	4.1 ± 0.3 b

Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$). $n = 60$ per treatment

2100, Clinx Science Instruments Co., China), and the data were noted.

Large-scale proliferation and cultivation

Based on in vitro results, a large-scale proliferation and regeneration system was established by replacing glass culture jars with domestically manufactured PVP plastic bags (11 cm × 13.5 cm) (Jinhongda, Shenzhen, China), which can withstand high temperatures (> 130 °C), thus allowing them to be autoclaved and used in plant tissue culture, broadly following a previously described procedure (Zheng et al. 2022). 100 leaf sheath segments were used as explants, after they were disinfected in 75% ethanol for 30 s and soaked in 0.1% $HgCl_2$ solution for 5 min, washed 3–4 times with SDW, the immature leaves (circles) that were cut into slices (explants) about 1 mm thick, they were inoculated on the MS medium supplemented with 0.5 mg/l 2,4-D and 0.1 mg/l NAA, in the dark on CIM for 30 days. Callus clumps were transferred to optimal regeneration medium (ORM), which was MS medium with 0.75 mg/l BA and 0.1 mg/l NAA, and cultured in light to induce SEs and/or adventitious shoots i.e., callus induction medium (CIM) Plantlets were produced in vitro in three steps: first on CIM in the dark for 30 days, then on ORM in the light for 30 days, followed by eight 30-d subcultures of shoots/SEs in light. Shoots were transferred to PVP plastic bags (10 shoots/bag) containing rooting medium (MS with 0.4 mg/l IBA and 0.2 mg/l NAA) and placed in light conditions for 14 days in the culture room, followed by 14 days of natural conditions in a greenhouse. After rooted plantlets elongated by 20 cm, they were transplanted to a field with an inter-plant space of 15 cm and an inter-row space of 80 cm. Plants were irrigated daily with tap water. After six months, plant growth and development were investigated.

Data analysis

Data are reported as the mean ± standard deviation (SD). Statistical analyses followed the procedure described by Xiong et al. (2019), using Duncan's multiple range test ($P \leq 0.05$) with SPSS v. 19.0 (IBM, New York, NY, USA).

Results

Shoot induction from immature leaf discs

When immature leaf discs (explants) (Fig. 1a) were cut in half and cultured on MS medium with 0.1–3.5 mg/l 2,4-D for 30 d in the dark, callus formed on cut surfaces of select treatments (Fig. 1b). Although callus was induced on 100% of explants in some treatments, such as 0.1–1.5 mg/l 2,4-D, they displayed several developmental deficiencies, including explant browning, explant necrosis, the formation of hard callus, and purple pigment formation (Fig. 1c). The ideal callus induction medium, based on induction percentage and the quality of callus formed, was MS with 0.5 mg/l 2,4-D and 0.1 mg/l NAA (Table 1), i.e., CIM. When callus was transferred to several media, shoots or SEs formed, and in several cases, they were visually indistinguishable, so the shoot and SE data was combined (Table 2). In terms of receptivity (% of explants inducing shoots or SEs), and productivity (number of shoots or SEs per explant), ORM was determined to be MS with 0.75 mg/l BA and 0.1 mg/l NAA. When callus was transferred to CIM, shoot buds formed on the surface of callus (Fig. 1d), and when these were transferred to ORM, shoots developed into shoot clusters (Fig. 1e and f).

Shoot proliferation, rooting and transplantation

Most multiple shoots (i.e., highest SPC) (Fig. 2a) were induced in MS with 1.25 mg/l KIN and 0.1 mg/l NAA. Several other treatments produced hyperhydric shoots (Table 3). The optimal rooting medium, as assessed by the number of shoots that formed roots, number of roots per shoot, and root length, was MS with two auxins (0.4 mg/l IBA and 0.2 mg/l NAA) and 50 g/l sucrose (Table 4). Resulting plantlets had well-developed leaves and root system (Fig. 2b and c). When plantlets were divided into clusters of three plantlets per tray of seedling trays with different substrates (Fig. 2d and e), the optimal substrate was, as assessed by survival, plant height, stem diameter and leaf number, perlite: peat (1:1) or perlite: sand (1:1) (Table 5).

Large-scale production, and growth characteristics and yield of plants

Using PVP plastic culture bags, the optimized in vitro culture protocols that were developed and described above were adopted (Fig. 3a-c). Culture bags were transferred to a greenhouse for 15 days (Fig. 3d), then to seedling trays (Fig. 3e), and finally to a field (Fig. 3f, g). It was possible to mass produce approximately 5 million plants per year (from callus induction until transplantable age) of sugarcane var. Yuetang 03-373. Production was approximately 40-fold higher than a previous protocol that induced shoots from shoot tips or axillary buds (Chen et al. 2017). Field-grown plants did not display any visible morphological deficiencies. Throuth successfully three test, the mean yield was 92,045 plants/ha, yield of sucrose stems was 92.88 tons/ha, cane stem yield was 100,290 kg/ha, and sugar content was 15,420 kg/ha.

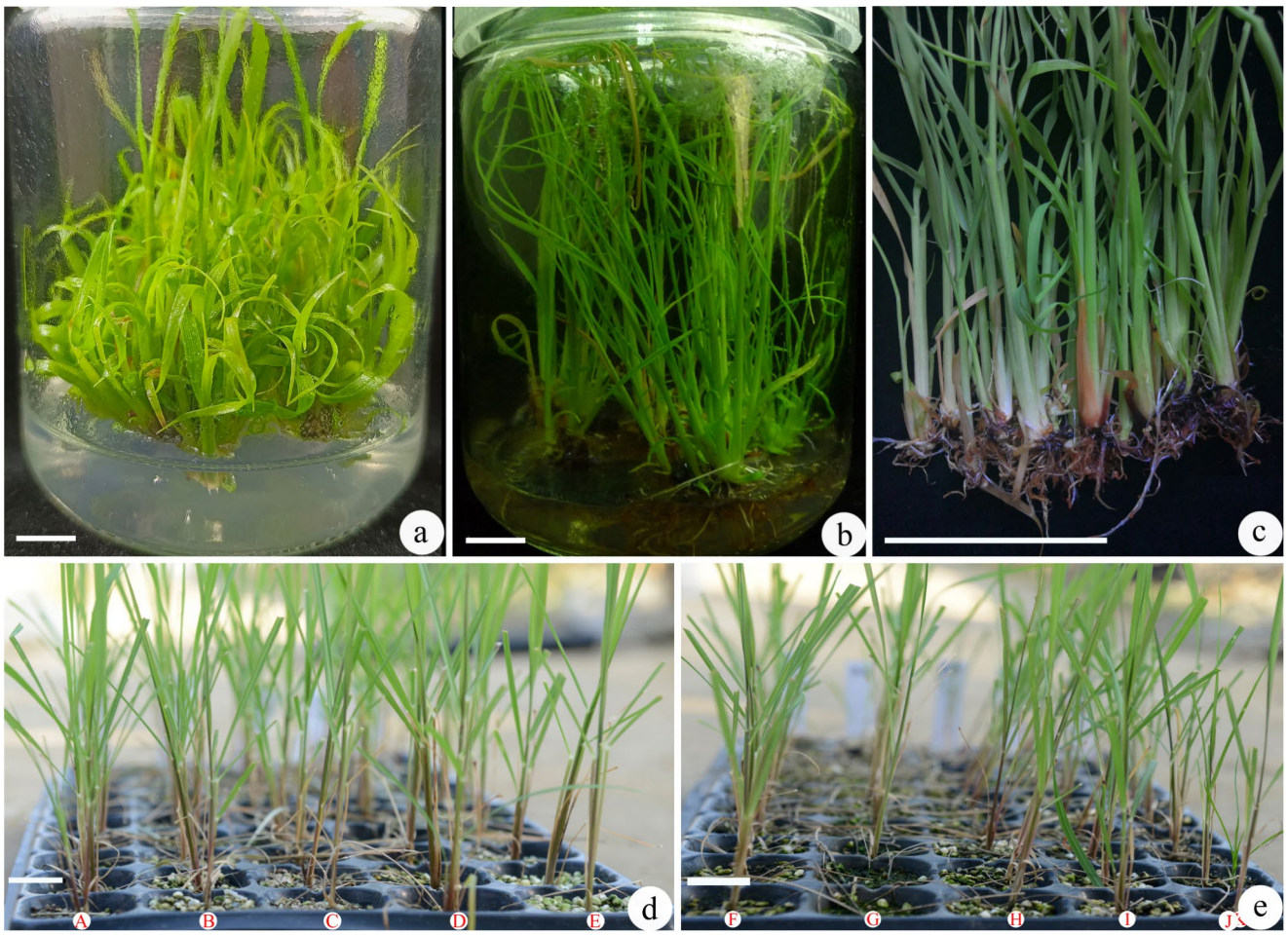

Fig. 2 Shoot proliferation, rooting and transplanting of sugarcane cultivar Yuetang 03-373 **a**, Multiple shoots were proliferated on MS medium with 1.25 mg/l KIN and 0.1 mg/l NAA for 30 days; **b**, shoots rooted on MS medium with 0.4 mg/L IBA, 0.2 mg/l NAA, and 50 g/L sucrose; **c**, rinsed plantlets from in vitro culture; **d-e**, plantlets with well-developed roots were transferred to cells (8 × 5) in seedling trays (30 cm × 50 cm) containing different substrates (ratios = *v/v*): **A**, peat: sand (1:1); **B**, vermiculite: sand (1:1); **C**, perlite: sand (1:1); **D**, peat: vermiculite (1:1); **E**, perlite: vermiculite (1:1); **F**, peat: perlite (1:1); **G**, yellow mud: sand (1:1); **H**, perlite: vermiculite: sand (1:1:1); **I**, perlite: peat: sand (1:1:1); **J**, peat: vermiculite: sand (1:1:1). Bars: **a-b**, 1.0 cm; **C**, 0.5 cm. **d-e**, 3 cm

Table 5 Effects of different substrates on the survival and growth of transplanted plantlets of sugarcane cultivar Yuetang 03-373

Substrate (volumetric ratio)	Plant survival (%)	Plant height (cm)	Stem diameter (mm)	Leaf number
peat: sand (1:1)	100 a	7.2 ± 0.4 a	2.1 ± 0.1 a	4.9 ± 0.2 cd
vermiculite: sand (1:1)	96.7 ± 3.1 ab	6.3 ± 0.5 b	2.0 ± 0.0 a	5.3 ± 0.2 c
vermiculite: sand (1:1)	100 a	8.2 ± 0.1 a	2.2 ± 0.2 a	5.8 ± 0.1 b
perlite: sand (1:1)	90.0 ± 3.2 b	8.1 ± 0.6 a	2.0 ± 0.2 a	5.7 ± 0.3 b
perlite: vermiculite (1:1)	93.3 ± 3.6 ab	7.6 ± 0.4 a	1.8 ± 0.1 ab	6.4 ± 0.2 a
peat: perlite (1:1)	100 a	7.7 ± 0.7 a	2.0 ± 0.1 a	4.6 ± 0.1 d
yellow mud: sand (1:1)	50.1 ± 5.8 c	5.3 ± 0.3 c	1.3 ± 0. e	4.6 ± 0.1 d
perlite: vermiculite: sand (1:1:1)	100 a	6.0 ± 0.2 b	1.7 ± 0.2 b	5.6 ± 0.1 ab
perlite: peat: sand (1:1:1)	96.4 ± 3.6 ab	5.4 ± 0.5 c	1.6 ± 0.1 b	4.8 ± 0.4 d
peat: vermiculite: sand (1:1:1)	100 a	5.2 ± 0.2 c	1.4 ± 0.2 c	4.4 ± 0.4 d

Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$). $n = 60$ per treatment

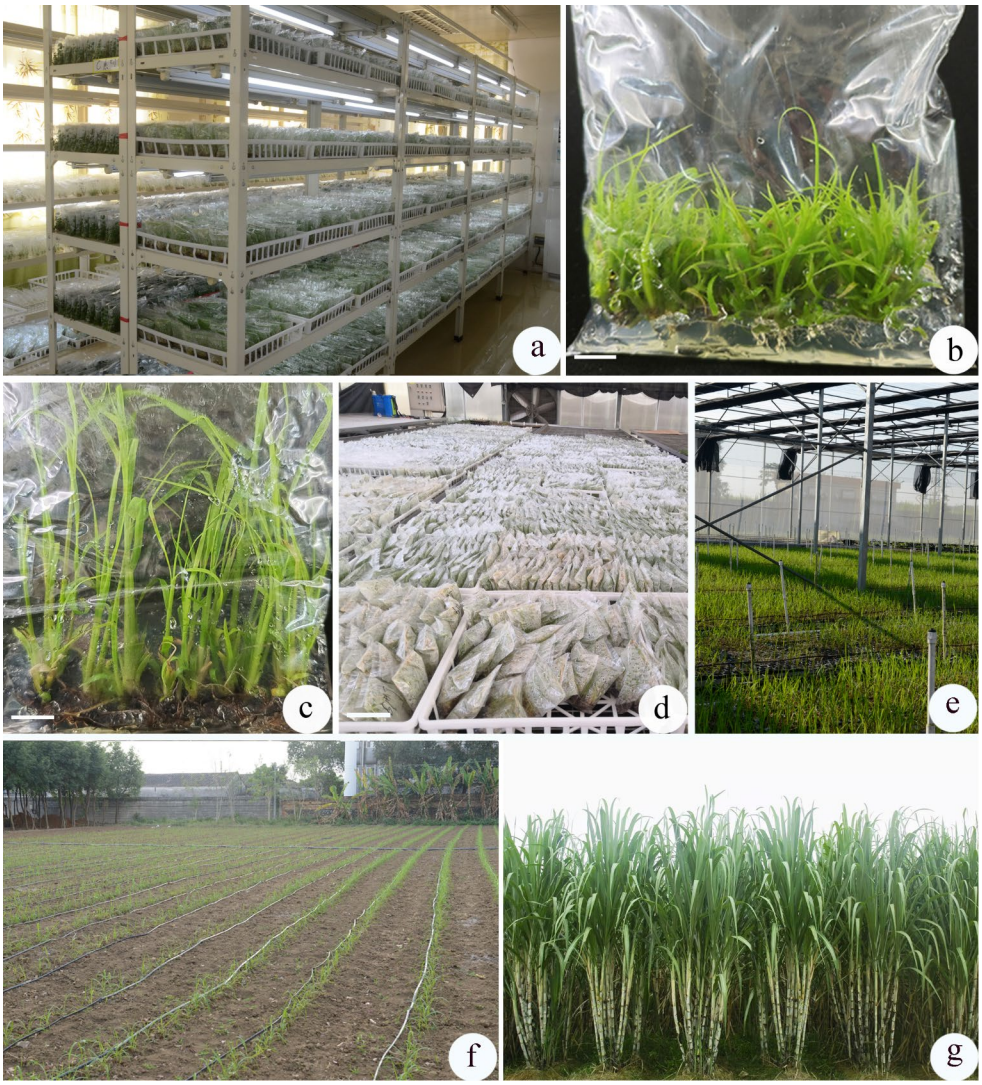

Fig. 3 Large-scale production of sugarcane cultivar Yuetang 03-373 and field cultivation **a**, Shoot proliferation and rooting were achieved in the tissue culture room in PVC plastic bags for 30 days; **b**, multiple shoots were proliferated in MS medium with 1.25 mg/l KIN and 0.1 mg/l NAA for 30 days; **c**, shoots were rooted in MS medium with 0.4 mg/l IBA, 0.2 mg/l NAA and 50 g/l sucrose for 30 days; d, rooted plantlets in plastic bags were acclimatized in a greenhouse for 15 days; **e**, acclimatized plantlets were transferred to seeding trays (30 cm × 50 cm) with 40 cells (8 × 5) in a peat: sand (1:1, *v/v*) substrate for further plant development and hardening; **f**, two-month old plants were transferred directly to the field with an inter-plant spacing of 15 cm and an inter-row spacing of 80 cm. Irrigation tubing was placed alongside all rows of field-grown plants; g, six month-old mature plants. Bars: b-c, 1 cm; d, 5 cm;

ISSR primer polymorphisms

Three pairs of primers detected a total of 50 alleles in 10 individual samples. Among them, the minimum number of alleles was 1 (mSSCIR68, mSSCIR74) (Fig. 4a and b), the maximum number of alleles was 3 (mSSCIR43), and the average number of alleles per point was 1.6667 (Fig. 4c).

Population genetic structure analysis

The total number of effective alleles (Ne, the more evenly distributed the alleles in the population, the closer Ne is to the number of alleles actually detected) was 5.0000, the numerical variation range was 1.0000, and the average number of effective alleles per locus was 1.0000. The Shannon index (I) has a range of 0.0000 (Fig. 5a). The optimal delta K value calculated in STRUCTURE HARVESTER according to the method of Evanno et al. (2005) is 2, indicating the presence of 2 gene pools out of 10 samples (Table 6). As can be seen from the graph, when K = 2, the gene composition of the 10 samples is derived from gene pools 1 and 2 (Fig. 5b).

Discussion

The success of the plant regeneration pathway depends on a variety of factors such as the sugarcane variety used (Gandonou 2005; Kaur and Kapoor 2016), the media sterilization method (Maribel et al. 2020), the type of explant (Lakshmanan et al. 2006), PGRs (Reis et al. 2021), light (Neto et al. 2020), pH (Ali et al. 2008), sucrose content (Lu et al. 2020), activated charcoal (Mittal et al. 2016), gelling agents (Manchanda and Gosal 2012), type of basal medium (Kaur and Gosal 2009), and several other factors. The literature on sugarcane tissue culture reveals wide differences in callus induction, somatic embryogenesis, and plant regeneration capacity of different genotypes or cultivars. Callus induction

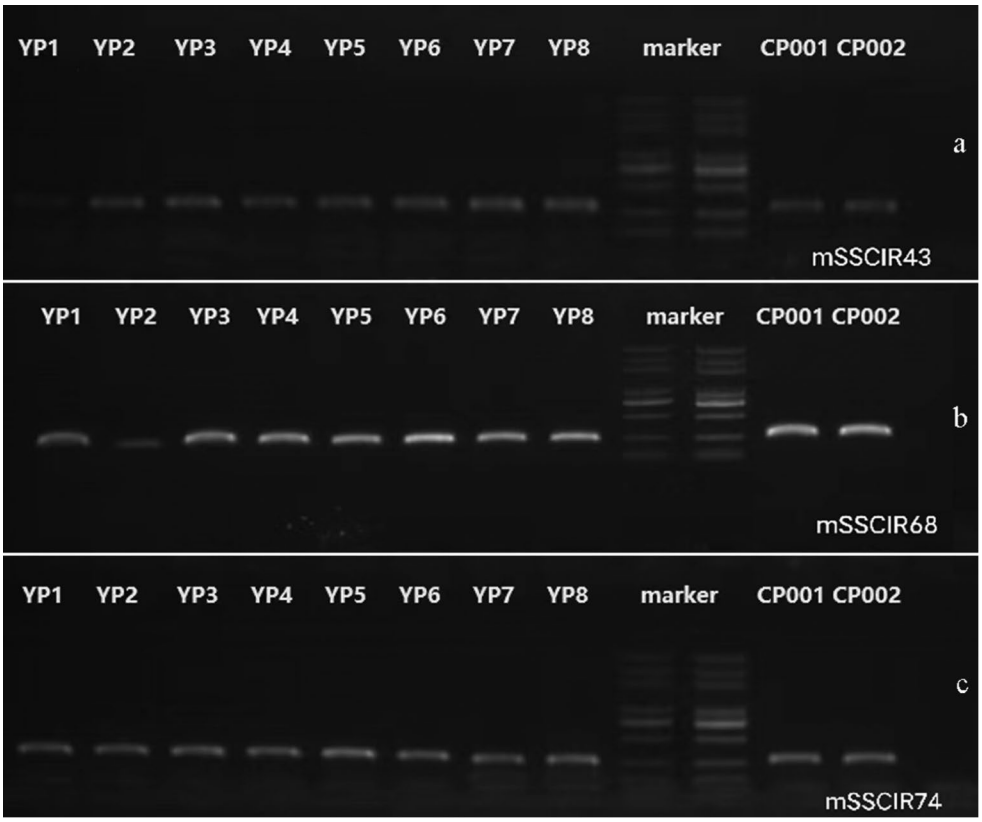

Fig. 4 SCoT analysis of in vitro regenerated plants of sugarcane cultivar Yuetang 03-373 with mother plant. Lane 1–10: In vitro regenerated plant's DNA banding patterns, Lane 11: Mother plant DNA banding pattern, Lane 12: DNA ladder – 1 kb (Thermo Scientific, USA).

and/or somatic embryogenesis have been achieved from immature inflorescences (Desai et al. 2004a), young leaves (Franklin et al. 2006; Ali et al. 2007; Joshi et al. 2013; Dorairaj et al. 2018), stem tips (Ali et al. 2008), and stem bases (Kaur and Kapoor 2016). These studies revealed that callus could be induced from several explants, although there were vast differences in callus induction, somatic embryogenesis and plant regeneration capacity. Immature leaves are a popular and commonly used explant for the induction of callus and SEs (Desai et al. 2004b; Franklin et al. 2006; Ali et al. 2007; Joshi et al. 2013; Dorairaj et al. 2018). Even though 2,4-D has been widely used to induce callus, it can inhibit the development of SE into normal plants (Reis et al. 2021). In this study on Yuetang 03-373, high concentrations of 2,4-D generally induced embryonic callus while low concentrations of 2,4-D (0.1–1.5 mg L^{-1}) directly induced SEs as well as 100% callus from leaf explants. Our histological analysis revealed that callus, SEs, and adventitious shoot buds originated exclusively from epidermal cells (Fig. 3).

Carrageenan, a natural seaweed polysaccharide, has been used in plant tissue culture as a solidifying agent for in vitro media (Hurtado and Biter 2007; Prajapati et al. 2014). Since it is very cheap and easy to obtain, compared to agar, replacing agar with carrageenan in tissue culture can reduce the costs of plant tissue culture. As before, for the industrial scale-up of *Dracaena cambodiana* (Zheng et al. 2022), we replaced glass jars with PVP plastic bags, which greatly improved work efficiency and reduced the cost of large-scale production of sugarcane plantlets.

From among 10 tested substrates for the acclimatization of in vitro-derived Yuetang 03-373 plants, five allowed for 100% transplant survival. This indicated that the cultivar was easy transplanted. In the last large-scale proliferation and transplantation, we just used peat soil: sand (1:1, *v/v*) substrate for further plant development and hardening. In the previous report, they only use pure river sand as the substrate for plantlet transplantation and transplantation survival percentage was 98% within one month (Chen et al. 2017).

Regeneration from callus may cause a certain amount of somaclonal variation (Ghosh et al. 2021). Even though we did not observe any noticeable phenotypic variation, somaclonal variation might impact plant proliferation or yield, so detailed trials that assess such variation (Rajeswari et al. 2009; Martínez-Estrada et al. 2017) would need to be conducted in the future. Here we first induce calli from the leaves, and then shoot organogenesis, so that we can reproduce a large number of small shoots in larger adventitious shoots, and their shoot induced coefficient can be as large as 128 within 30 d, through which we can produce out large-scale shoots via shoot proliferation (the reproductive coefficient is only 5.6). Through our ISSR experiment, it was found that it did not undergo hereditary mutation, so that we obtained a large number of shoots through the initial

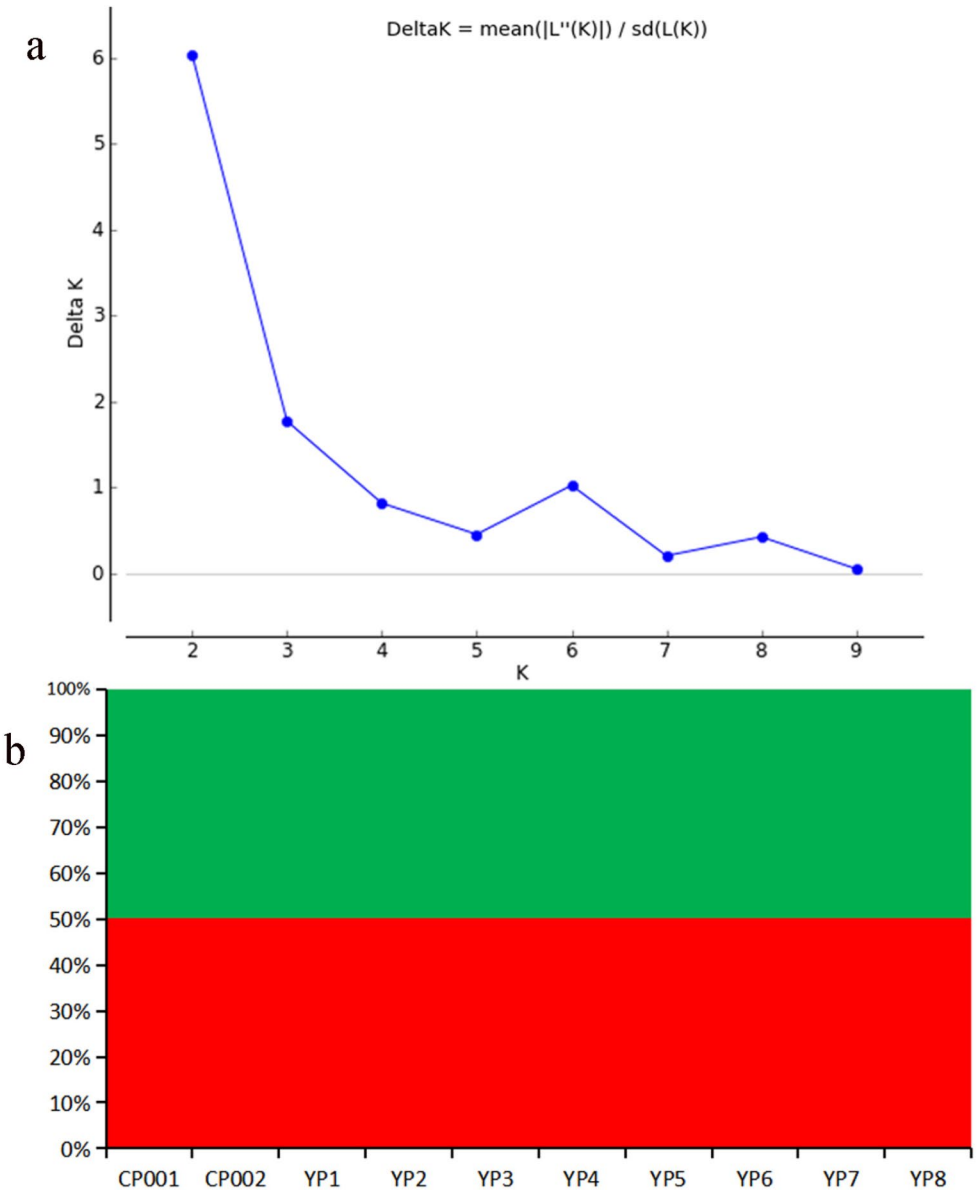

Fig. 5 Genetic structure analysis of the population
a, the change of K value plotted by the ΔK method of STRUCTURE analysis; b, STRUCTURE results for 10 samples at K = 2

Table 6 The genetic diversity of 3 selected SSR loci

Primes	Total lines	Number of polymorphic bands	PP (%)	Na	Ne	H	Shannon Index I
mSSCIR43	3	0	0%	1.0000	1.0000	0.0000	0.0000
mSSCIR68	1	0	0%	1.0000	1.0000	0.0000	0.0000
mSSCIR74	1	0	0%	1.0000	1.0000	0.0000	0.0000
Mean	1.6667	0	0%	1.0000	1.0000	0.0000	0.0000
St. Dev				0.0000	0.0000	0.0000	0.0000

Note Na: observed allele, Ne: effective allele, I: Shannon index

shoot organogenesis, and then carried out shoot propagation, which is much faster than traditional shoot propagation, and can reduce genetic variation, which is conducive to large-scale reproduction, We have mapped a fast and simple propagation model that can be used to propagate sugarcane seedlings on a large scale (Fig. 6).

Conclusion and limitations

Transversely cut discs of immature leaves were used as explants of sugarcane var. Yuetang 03-373 to induce callus, shoots for the first time. On MS medium with 0.5 mg/l 2,4-D and 0.1 mg/l NAA, 100% of leaf explants induced embryogenic callus. On MS medium with 0.75 mg/l BA and

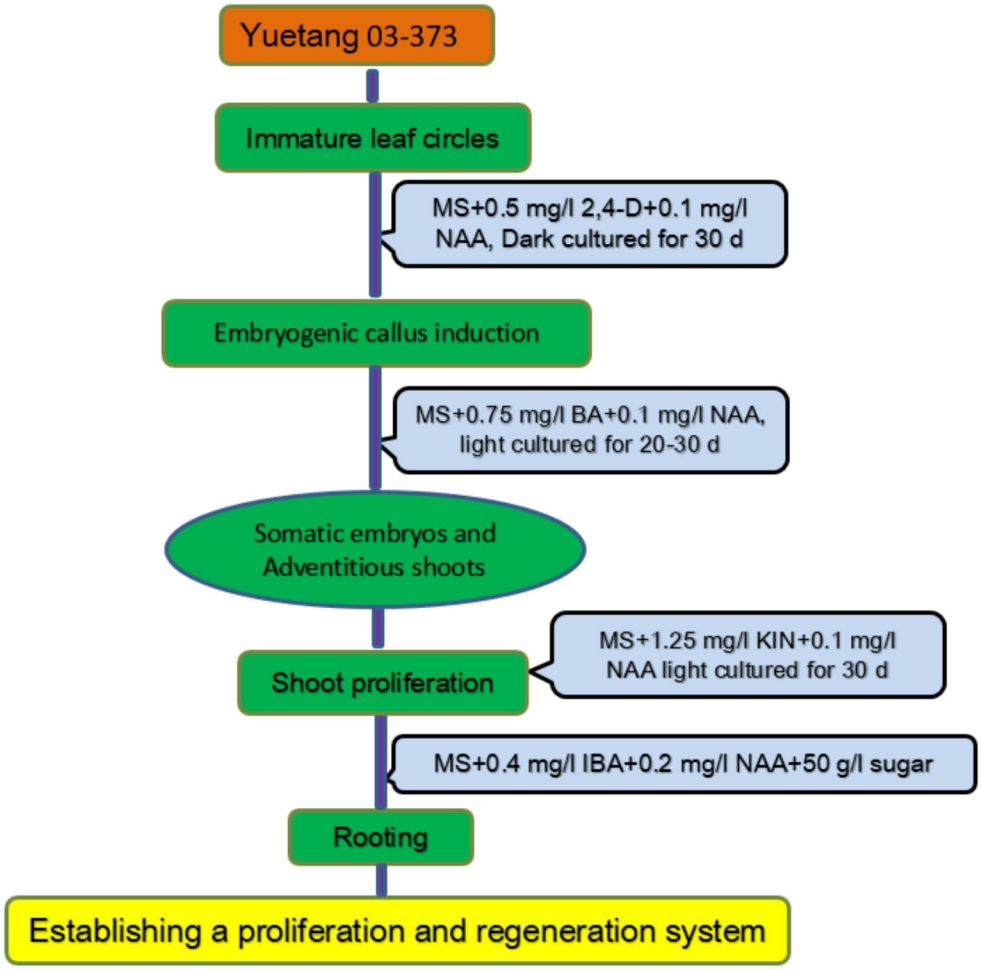

Fig. 6 An efficient plant proliferation and regeneration route of sugarcane variety Yuetang 03-373

0.1 mg/l NAA, 62% of induced callus differentiated into adventitious shoots within 30 d (coefficient highest 128). All the adventitious shoots developed multiple shoots on the shoot proliferation medium MS medium supplemented with 1.25 mg/l KIN and 0.1 mg/l NAA and SPC reached 5.6 with each 30-day subculture. Histological observations indicated that both SEs and adventitious shoot buds were derived from parenchymatous meristems in the outer or inner epidermal layers of the cut edges of leaf discs. On optimal rooting medium (MS with 0.4 mg/l IBA, 0.2 mg/l NAA and 50 g/l sucrose), 100% shoots rooted. Well-rooted plantlets showed 100% survival in five out of 10 tested substrates after 60 days. An efficient in vitro regeneration system was thus established. This study also scaled up production using large-scale technology to mass produce sugarcane var. Yuetang 03-373 for the national markets. A summary of the process for efficient plant proliferation and regeneration of sugarcane var. Yuetang 03-373 is described in Fig. 6. It was possible to mass produce approximately 5 million plants per year. Production was approximately 40-fold higher than a previous protocol, which induced shoots from shoot tips or axillary buds (Chen et al. 2017).

Author contributions SYC, JNT, JYG, JPL, YCX, MYC, RZ, YL and QWL, YFZ and GHM designed the experiment and provided guidance for the study. SYC, and JNT prepared samples for all analyses. JPL and YCX conducted statistical analyses. SYC, GHM and JATdS co-wrote the manuscript. GHM and JATdS interpreted the data. GHM assessed the experimental results. All authors read and approved the manuscript for publication.

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Data availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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