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Efficient callus-mediated system for commercial production of sugarcane (*Saccharum* spp.) planting material in Ghana

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An efficient callus-mediated regeneration system was developed for high-frequency production of planting material of sugarcane genotypes LSC and B36464. Spindle leaf segments cultured on MS basal medium supplemented with 2,4-D or picloram at 1, 2, 3 or 4 mg/L resulted in callus induction. Callus induction was higher on 2,4-D amended medium compared to picloram. Nevertheless, for both auxins, callus induction improved significantly ($p \leq 0.05$) with increasing concentration; the highest (82 and 82.5% for B36464 and LSC respectively) was achieved at 4 mg/L. For shoot induction, calli were transferred to MS medium supplemented with BAP (0.1, 0.5, 1.0 or 1.5 mg/L). The highest number of shoots (18.13 and 16.75 for B36464 and LSC respectively) was achieved at 1.5 mg/L. Serial subculture at four-week intervals on a higher concentration of BAP (2.5 mg/L), in combination with NAA (0.5 mg/L) and GA_3 (0.5 mg/L), resulted in a four-fold increase in shoot number within 16 weeks. On this medium, 40% of shoot clusters of B36464 formed well-defined shoots. On MS medium containing solely NAA (3 mg/L), 88 and 72% (B36464 and LSC respectively) formed roots. Post-flask acclimatisation of the plantlets led to 85 and 91% survival rates in LSC and B36464 respectively after which plantlets were successfully transferred to field conditions. The callus-mediated regeneration system reported in this study has the potential to sustainably provide sugarcane planting material for the emerging sugar industry in Ghana.

Key words: Sugarcane, spindle leaf explants, callus-mediated organogenesis, plantlet regeneration, 2,4-D, Picloram.

INTRODUCTION

Globally, sugarcane (*Saccharum officinarum* L.) is an industrial crop used mainly to produce sugar and bioethanol. In Ghana, its full potential is yet to be exploited as most of the country's sugarcane is used either for the production of local alcoholic drink "*akpeteshie"* by small-scale distilleries or sold fresh as a snack (Ababio and Lovatt, 2015). Recently however, the crop is gaining more prominence in the country as an industrial crop particularly with the re-commissioning of the Komenda Sugar Factory in the Central Region of Ghana.

The sugar factory is expected to trigger large demand for planting materials for the establishment of plantations which in turn will serve as feedstock. Such plantations

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may be established conventionally through vegetative propagation (planting of setts). However, setts are usually unavailable in commercial quantities and require high labour input for preparation and planting (Bello-Bello et al., 2018). Even when available, setts are often contaminated by pests and diseases resulting in low yields (Jalaja et al., 2008).

An alternative method of generating large quantities of planting material is tissue culture. Since the 1960s, the technique has been shown to be highly efficient for rapid multiplication of sugarcane (Heinz and Mee, 1969; Ho and Vasil, 1983). Plantlets have been successfully regenerated from various types of explants including immature leaf rolls (Soares et al., 2014), apical meristems (Ramgareeb et al., 2010), young leaves (Chengalrayan and Gallo-Meagher, 2001) and axillary buds (Vazquez Molina et al., 2005). Additionally, tissue culture has been shown to be advantageous in that, plants are disease-free thereby exhibiting increased field productivity and improved sugar yields (Sawant, 2014; Bello-Bello et al., 2018).

Although worldwide, significant progress has been made in refining tissue culture protocols for sugarcane, the crop has been shown to be genotype-dependent/ specific in its response to culture conditions (Jadhav et al., 2001). For instance, sugarcane genotypes can vary highly in their response to exogenous growth regulators in culture media (Gandonou et al., 2005). Consequently, efficient regeneration of specific genotypes often requires optimization of *in vitro* protocols (Tesfa and Ftwi, 2018).

Thus, in this study, we tested the response of two important local sugarcane cultivars to a simple and efficient callus-mediated *in vitro* regeneration system. This method of organogenesis not only presents a good strategy for rapid multiplication of the crop but also allows for future improvement of these cultivars via genetic transformation and *in vitro* mutagenesis (Jamil et al., 2017). For example, callus-mediated regeneration has been used to improve sugarcane for resistance to pests and diseases as well as tolerance to drought and salinity (Matsuoka et al., 2001; Eldessoky et al., 2011). Given the expected demand for large quantities of sugarcane planting material in Ghana, we envisage that the application of this technique to locally adapted and economically important cultivars has great potential to support the emerging sugar industry in the country. Additionally, it will contribute to job creation throughout-grower schemes, and improve productivity and income of smallholder farmers in sugarcane-growing regions.

MATERIALS AND METHODS

Study location

The experiment was conducted at the Biotechnology Centre of Biotechnology and Nuclear Agriculture Research Institute (BNARI), Ghana Atomic Energy Commission (GAEC) from March 2019 to September 2020.

Planting materials

Two genotypes of sugarcane, LSC and B36464 were used in the study. B36464 also locally referred to as *Alata*, is one of the main cultivars grown by smallholder farmers in the Komenda-Edina-Eguaafo-Abrem (KEEA) District of the Central Region, Ghana. It is also grown on commercial scale by farmers at Abura-Asebu-Kwamankese (AAK), Shama and Mpohor Wassa East districts in the Central Region due to their proximity to the Komenda Sugar Factory (GSS 2014; Yawson et al., 2018). The second genotype LSC is a popular landrace grown by smallholder farmers in several parts of Ghana including the Eastern, Central, Volta and Greater Accra regions. This landrace is often sold as a snack in small, peeled segments and provides income for many women in both rural and urban areas.

Explant preparation and sterilisation

Apical sections of field-grown canes (6 to 8 months old) were soaked overnight in 1% (w/v) fungicide solution (Bendazim® 50WP, active ingredient, carbendazim). Spindle leaf explants were obtained by removal of outer leaf whorls and trimming of the apical sections. The explants were then washed in mild detergent (Morning Fresh Dishwashing Liquid, Cussons®) solution for five minutes followed by soaking in 10% bleach (Clorox®, active ingredient – 6.05% sodium hypochlorite) for 30 min. Final surface sterilisation consisted of soaking explants in 0.2% (w/v) mercuric chloride for 10 min followed by thorough rinsing with three changes of sterile distilled water.

Callus induction

Thin slices (approximately 5 mm) of spindle leaf explants were inoculated on callus induction medium (CIM) which comprised Murashige and Skoog medium (1962) supplemented either with picloram or 2,4– dichlorophenoxyacetic acid (2,4 -D) at varying concentrations (0, 1, 2, 3 or 4 mg/L). The callus induction medium (CIM) also contained sucrose at 30 g/L. The pH of the culture medium was adjusted to 5.8 followed by addition of phytagel at 3.5 g/L and autoclaving at 121°C for 15 min. The cultures were maintained in the dark at 25 ± 1°C for 28 days, after which the number of explants that formed calli was scored. Fifty explants were cultured per treatment, consisting of 10 replicates.

Shoot induction from callus

For shoot induction, embryogenic calli (cream-coloured and friable) were transferred to shoot induction medium (SIM) consisting of MS medium supplemented with 6-benzylamino purine (BAP) at 0.1, 0.5, 1.0 or 1.5 mg/L, referred to hereafter as SIM1, SIM2, SIM3 and SIM4, respectively. The cultures were maintained under cool fluorescent light (16/8-h light/darkness; 2500 lux) at 25±1°C for six weeks after which the number of germinated shoots per callus clump was scored for the various treatments. Sixteen calli were cultured per treatment with each callus considered as a replicate.

Shoot proliferation and root induction

To improve shoot multiplication, micro shoot clusters were transferred to shoot proliferation media (SPM) containing BAP at 2.5 mg/L in combination with NAA (0.5 mg/L) and GA_3 (0.5 mg/L). The total number of well-differentiated shoots was scored at the end of each culture cycle (every four weeks). Multiplication rates were calculated as a ratio of the total number of shoots at each

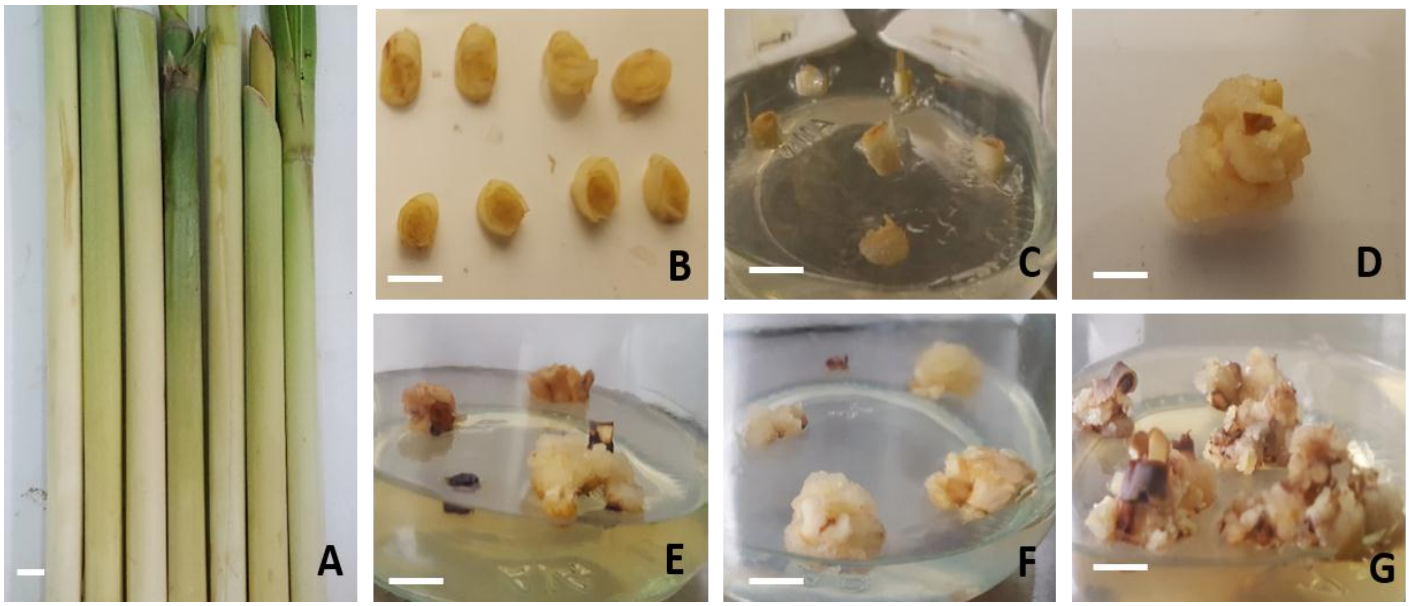

Figure 1. Callus induction from spindle leaf explants of LSC and B36464. **(A)** Spindle leaf explants **(B, C)** Sliced explants before and after inoculation **(D, E)** Callus formation in LSC explants incubated on MS media supplemented with 2,4-D and picloram respectively (**F, G)** Callus formation in B36464 incubated on MS media supplemented with 2,4-D and picloram respectively. Bar represents 1cm (**A**) and 5mm (**B - G)**.

sub-culture divided by the number of shoots at the previous subculture stage. Unrooted shoots were transferred to MS medium containing NAA at 3 mg/L (RIM) for root induction.

Plantlet acclimatization

Two hundred plantlets (100 per genotype) were washed gently with tap water followed by a five-minute rinse in 0.1% fungicide solution (Bendazim® 50WP, active ingredient, carbendazim). The plantlets were then transferred to polythene bags filled with soil, cocopeat and sawdust mixture (2:1:1) with high humidity conditions (>85%) created by covering the plantlets with transparent plastic cups for three days. The plantlets were maintained in a greenhouse at 28 ± 2°C and were irrigated every other day for six weeks. The number of plants that survived was recorded. Following successful acclimatisation, well-hardened plantlets (approximately 9 weeks old) were transferred to field conditions. Prior to transplanting, older leaves were trimmed. During transplanting, plantlets were carefully removed from polythene bags to minimise damage to roots. Plantlets were then placed in furrows two feet apart and covered with adequate amounts of soil and irrigated immediately after transplanting. All recommended intercultural operations (weeding, irrigation and pest control) were performed.

Statistical analysis

All experiments were laid out in a completely randomized design (CRD). For callus induction, each treatment was replicated 10 times. For shoot induction, each treatment was replicated 16 times. Data were analysed using Microsoft Excel 2010 and GraphPad Prism version 7 (San Diego, United States). All data were subjected to analysis of variance (ANOVA) and statistically significant results at the 5% level were compared either with Tukey's or Sidak's multiple comparisons test.

RESULTS AND DISCUSSION

Callus induction

Spindle leaf explants (Figure 1A) of sugarcane cultivars LSC and B36464 formed callus on MS media containing varying concentrations of 2,4-D or picloram by the twelfth day of incubation (Figure 1B, C). For both cultivars, the absence of 2,4-D and picloram in the culture media resulted in no callus production, indicating an auxin requirement for callus formation in sugarcane, similar to other plant species (Ikeuchi et al., 2013; Osman et al., 2016; Zang et al., 2016). Similar observations were made by Ramgareeb et al. (2010) and Alcantara et al. (2014) where various types of sugarcane explants failed to form callus on auxin-free media. Exogenous auxin supplementation is required to reprogram somatic cells to acquire pluripotency as well as initiate cell division leading to the formation of callus (Skoog and Miller, 1957; George et al., 2008; Fehér, 2019). An increase in the number and size of calli was observed after 21 days of incubation on both 2,4-D (Figure 1D, E) and picloram-supplemented media (Figure 1F, G).

Callus induction on 2,4-D supplemented media was significantly higher ($p \leq 0.05$) compared to picloram at the same concentrations (Table 1; supplementary Table 1) suggesting that 2,4-D is an effective auxin for callus

Table 1. Callus induction in LSC and B36464 sugarcane genotypes on 2,4-D or picloram-supplemented MS media after 21 days in the dark.

Auxin	Concentration (mg/l)	Mean callus production (%) ± SD	
		LSC	B36464
2,4-D	C (control)	0.0 ± 0.00[a]	0.0 ± 0.00[a]
	D1	18 ± 0.39[a]	20.0 ± 0.41[a]
	D2	44 ± 0.50[b]	72.5 ± 0.45[b]
	D3	68 ± 0.47[c]	77.5 ± 0.38[b]
	D4	82 ± 0.39[c]	82.5 ± 0.38[b]
Picloram	P1	10 ± 0.30[a]	7.5 ± 0.27[a]
	P2	16 ± 0.37[a]	22.5 ± 0.42[ac]
	P3	44 ± 0.50[bc]	27.5 ± 045[ac]
	P4	32 ± 0.47[bc]	60.0 ± 0.49[bc]

induction in sugarcane. This is similar to earlier reports(Ho and Vasil, 1983; Gill et al., 2004; Dibax et al., 2011; Alcantara et al., 2014; Arjun Srinath, 2015). However, our findings contradict those of Gallo-Meagher et al. (2012) who reported higher callus induction on picloram-supplemented medium rather than 2,4-D in sugarcane. This may be due to differences in genotypic response to auxins as reported by Gandonou et al. (2005) and Mekonnen et al. (2014).

In general, induction of embryogenic calli improved with increasing auxin concentration (Table 1). For LSC, callus induction was best achieved at the highest concentrations of 3 and 4 mg/L for 2,4-D. Similar observations have been reported by several authors including Badawy et al. (2008), Jahangir et al. (2010) and Arjun Srinath (2015). A similar trend was observed for picloram. However, as picloram concentration increased from 3 to 4 mg/L callus induction rate declined (from 44 to 34%) in LSC. The reduction in callus formation may be attributed to the presence of endogenous hormones in the explant tissues prior to culture initiation (Can et al., 2008). Wernicke et al. (1986), explain that high auxin concentrations may prevent meristematic cell divisions, resulting in decreased reactions such as callus induction. In the genotype B36464, the best concentration for callus formation is between 2- 4 mg/L 2,4-D or 4 mg/L picloram. Nonetheless, the response of the two genotypes in this study to callus induction is beneficial in that, it is a first and crucial step in *in vitro* mutagenesis as well as genetic transformation protocols for improvement of local sugarcane genotypes. Calli are ideal propagules for reception of genes since they are mostly of single cell origin (Nagmani et al., 1987) and can also be reached by either physical or chemical mutagens in mutagenesis (ref).

Mean production of embryogenic callus is the ratio of callus formed to the number of explants cultured expressed as a percentage. Data represent mean ± SD. Values in a column followed by the same letters are not significantly different from each other at $p \leq 0.05$ (Tukey's pairwise comparison test).

Shoot induction from calli

The addition of cytokinins, particularly BAP, to MS medium has been shown to be effective for inducing shoots from sugarcane callus cultures (Ather et al.. 2009; Dibax et al.. 2011; Arjun Srinath. 2015). Thus, to determine the optimum concentration of BAP for shoot induction from callus for the two cultivars, we transferred friable calli to MS medium containing BAP ranging from 0.1 to 1.5 mg/L BAP. By the second week, green microstructures were observed on the surfaces of calli of both B36464 (Figure 2A, B) and LSC (Figure 2C, D), suggesting the formation of totipotent shoot primordia. The presence of high concentration of cytokinin in the shoot induction medium induces shoot stem cell regulators from callus (Gordon et al., 2007). According to Dibax et al. (2013), the formation of such shoot primordia is triggered from both nodular and friable calli due to the presence of cytokinins which initiate a pathway redirection. The formation of shoot primordia on callus of sugarcane resulted in the development of shoots within 28 days of culture. The mean number of shoots formed per callus explants ranged from 9.69 ± 2.55 to 18.13 ± 3.07 in B36464 genotype and 8.44 ± 4.19 to 16.75 ± 3.55 in LSC genotype (Figure 3; Supplementary Table 2). The shoot induction rates obtained from callus cultures in this study are higher than those obtained from shoot tip or lateral bud culture (Danso et al., 2011; Mekonnen et al., 2014) making this protocol efficient for commercial production of sugarcane planting materials. Callus develops more shoot primordium, hence more shoots than from direct organogenesis in shoot tips. Shoot regeneration from callus is a result of localised cell division leading to differentiation of globular meristemoids which can develop into shoots (Ovečka et al., 1997). For

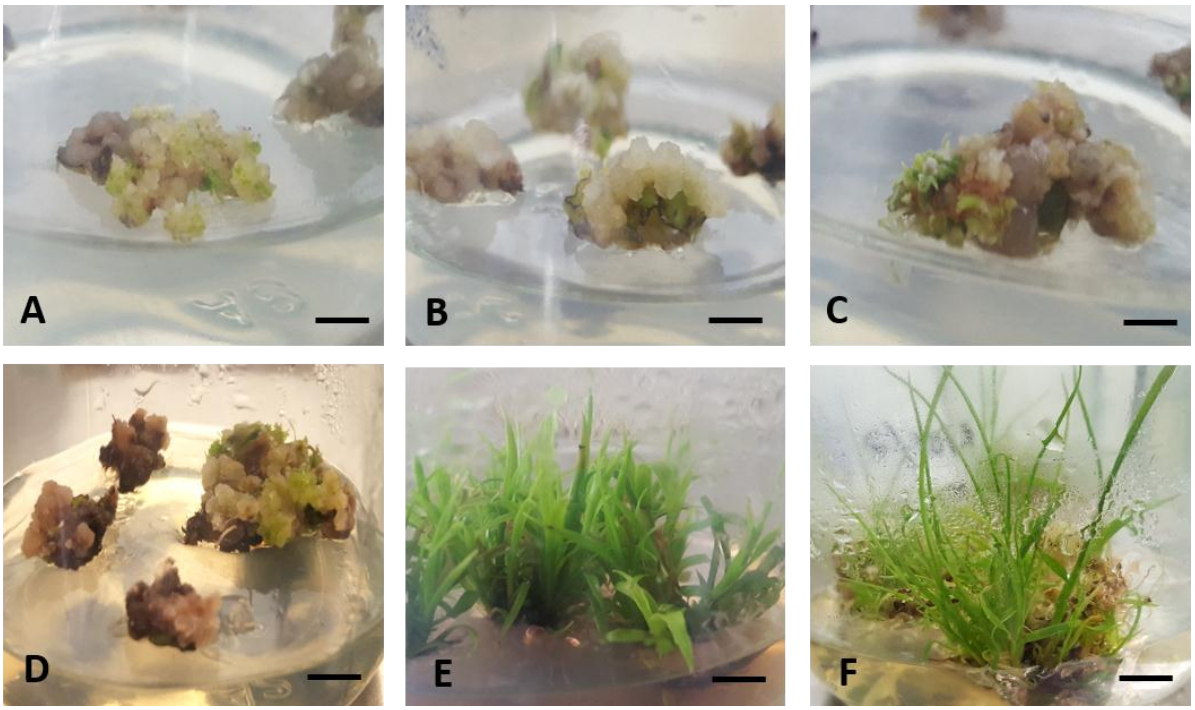

Figure 2. Shoot induction from calli of sugarcane genotypes B36464 and LSC. **(A, B)** Green micro shoots forming on embryogenic callus of B36464 and **(C, D)** LSC after three weeks of incubation. Shoot clusters of **(E)** B36464 and **(F)** LSC respectively after six weeks of incubation. Bar represents 2mm (**A - D)** and 1mm (**E, F)**.

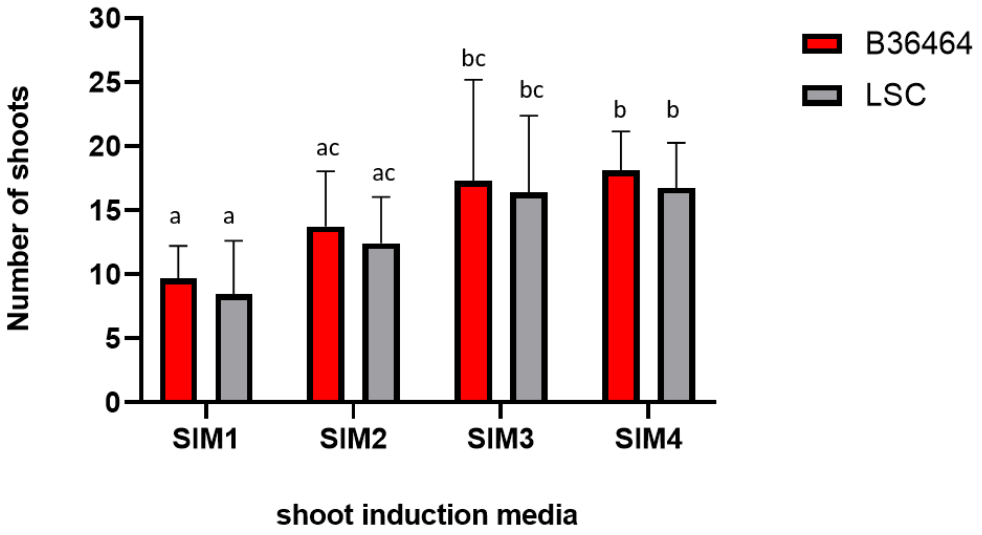

Figure 3. Shoot induction in calli of LSC and B36464 genotypes on MS media with varying concentrations of BAP after 4 weeks of culture.

example, in *Nicotiana tabacum,* the palisade cells divide to form callus which gives rise to shoot primordia and finally regeneration into shoot (Gupta et al., 1966; Zhi-hong and Gui-Yun, 1980).

The lowest concentrations of BAP (0.1 and 0.5 mg/L) induced the least number of shoots from callus in both genotypes. However, at higher BAP concentrations of 1.0 and 1.5 mg/L, we recorded a significant increase ($p \leq 0.05$) in shoot induction in both genotypes (Supplementary Table 2). An increase in shoot production

Table 2. Shoot multiplication rates in sugarcane cultivars LSC and B36464 on SPM at 4-week intervals.

Sub-culture cycle (weeks)	Total number of shoots		Multiplication rates	
	LSC	B36464	LSC	B36464
4	2902	2797		
8	6927	7209	2.39	2.58
12	9673	10724	1.40	1.49
16	12278	13707	1.27	1.28
Total	12278	13707	4.23	4.90

from sugarcane callus cultures with increasing BAP concentration has been reported by Behera and Sahoo (2009) and Hapsoro (2017). A comparison between the two genotypes showed higher shoot induction from callus of variety B36464 compared with LSC, suggesting genotype effect on shoot induction. Similarly, genotypic differences have been observed in shoot regeneration from callus cultures of different sugarcane cultivars (Gandonou et al., 2005; Kaur and Kapoor, 2016; Di Pauli et al., 2021).

Shoot proliferation and root induction

Given the increasing frequency of shoot induction (from callus) with increasing BAP concentration in the previous experiment, we evaluated the proliferation of shoots on MS medium supplemented with a higher concentration of BAP (2.5 mg/L) in combination with low concentrations of NAA (0.1 mg/L) and GA_3 (0.1 mg/L). In sugarcane, as in many plant species, a high cytokinin to auxin ratio in the culture medium has been shown to effectively stimulate shoot regeneration and differentiation (Ikeuchi et al., 2013; Fehér, 2019). In order to increase plantlet production and maximise economic gains, four serial subcultures were used to multiply the shoots in culture.

After subculture cycle 1, the total number of shoots produced in LSC genotype was higher (2902) compared to B36464 (2797) (Table 2 and Figure 4A, B). However, after 8 weeks of culture on SPM (cycle 2), shoot production was higher (7209) in B36464 compared to LSC (6927) and this trend was maintained for the next two cycles (till 16 weeks of culture). The total number of shoots formed in both genotypes increased more than two-fold by the second subculture (at 8 weeks) and four-fold by the fourth subculture (at 16 weeks) (Table 2). This high frequency of plantlet production indicates the efficiency of the callus-mediated technique for sugarcane micro-propagation.

Interestingly, shoot multiplication rates decreased with successive subcultures (second subculture onwards) likely due to the cumulative effects of BAP in succeeding subcultures which might have led to high endogenous concentration of BAP, hence the phytotoxic effect. In general, decline in shoot multiplication rates as a result of repeated *in vitro* subculture has been reported in several plant species (Norton and Norton, 1986; Hussain et al., 2007; Vujović et al., 2012). Although reducing the concentration of hormones in the culture medium can delay the decline in shoot multiplication rates (Vujović et al., 2012), regular initiation of new cultures from spindle leaf explants is necessary to maintain high shoot multiplication rates. The initiation of new cultures is important to reduce the risk of somaclonal variation which is quite common in sugarcane (Khan, 1999; Khan et al., 2008). It also ensures genetic homogeneity of plantlets and the production of true-to-type planting materials for the establishment of commercial plantations (Petolino et al., 2003). However, in this work using LSC and B36464 genotypes, we did not observe any genetic or somaclonal variation in plants transferred to the field at six months old.

With respect to root induction, 25% of shoot clusters of LSC formed well-defined roots spontaneously on BAP-containing medium (SPM) after 4 weeks of culture compared to 57% in B36464 shoot clusters (Figure 4C). This phenomenon of spontaneous root formation at the shoot maturation stage on media containing only cytokinins has been reported by Zamir et al. (2012). Nonetheless, to improve root formation from *in vitro* shoots, we supplemented MS media with 3 mg/L NAA which resulted in marked improvement in root development in both B36464 (88%) and LSC (72%) after 8 weeks of culture.

Plantlet acclimatization

Efficient weaning and hardening of *in vitro*-generated plantlets are critical for successful transfer of plantlets to field conditions. To determine the efficiency of weaning and hardening, a total of 200 plantlets (100 per genotype) were transferred to the greenhouse for weaning. Survival rates of 85 and 91% were achieved for LSC and B36464, respectively (Figure 5). The rates of acclimatization obtained for plantlets of both sugarcane genotypes in this study are similar to survival rates obtained for other commercial Indian varieties (Kaur and Kapoor, 2016) but higher compared to other reports where up to 87% survival rates were obtained (Mekonnen et al., 2014). Well-developed roots in *in vitro* plantlets prior to weaning

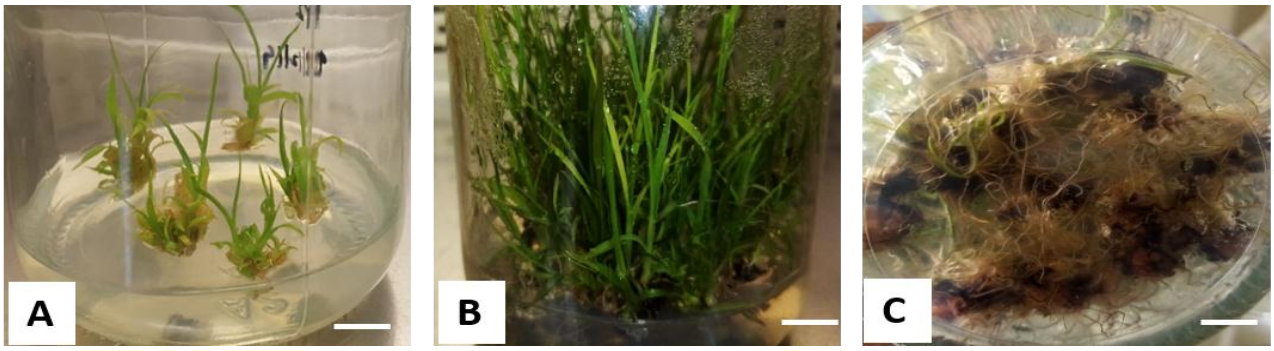

Figure 4. Shoot proliferation and root induction in B36464.
(A) Separated micro shoot clusters on SPM **(B)** Shoot proliferation and **(C)** root induction after 4 weeks of culture. Bar represents 1 mm.

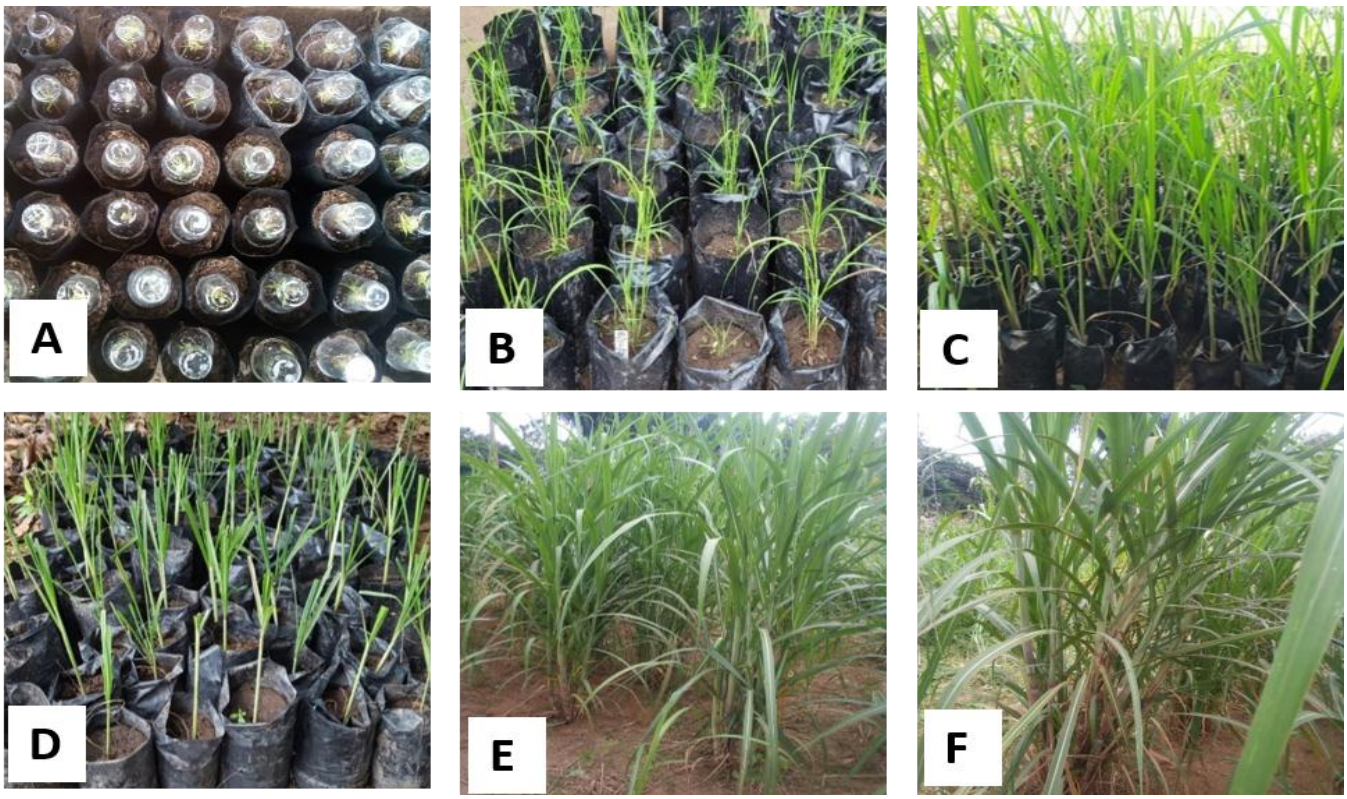

Figure 5. Plantlet acclimatization and field establishment of B36464 variety.
(A) Weaned plantlets in humidity chamber. Weaned plantlets at **(B)** 2-weeks and **(C)** 8 weeks old. **(D)** Older leaves of weaned plantlets trimmed prior to field transfer. Established plants at **(E)** three months and **(F)** six months after transfer to the field.

have been shown to enhance survival of plants at the weaning stage (Danso et al., 2011). Other factors such as genotype and the medium used for weaning also play significant role in post-flask acclimatisation: cocopeat: soil: sawdust mixture (2:1:1) could explain the differences in plantlet acclimatization compared with other studies. The weaned plantlets were successfully transferred to field after 8 weeks of hardening for further development.

Conclusion

The emerging sugar industry in Ghana and across Africa requires the availability of large quantities of sugarcane planting materials to ensure sustainability of the industry. As a strategy for meeting this demand, the response of two important locally adapted cultivars to micro-propagation via somatic embryogenesis was tested. The

successful induction of embryogenic calli from spindle leaf explants in the two cultivars followed by efficient plantlet regeneration on cytokinin-auxin amended MS medium augur well for adequate and sustainable supply of planting materials for the sugarcane industry. A four-fold increase in shoot production in 16 weeks demonstrates the efficiency of our protocol for large scale production of sugarcane planting material. Thus, this study has demonstrated that somatic embryogenesis using spindle leaf explants offers an efficient protocol for large scale production of disease-free sugarcane plantlets for the emerging sugar industries across Africa.

ABBREVIATIONS

2,4-D, 2,4-dichlorophenoxyacetic acid; **BAP,** 6 – benzylamino purine; **BNARI,** Biotechnology and Nuclear Agriculture Research Institute; **GAEC,** Ghana Atomic Energy Commission; **MS,** Murashige and Skoog Basal medium; **NAA,** 1-naphthaleneacetic acid; **Picloram,** 4-amino-3,5,6-trichloropicolinic acid.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1. Two-way ANOVA table comparing callus formation from spindle leaf explants on 2,4-D and picloram supplemented media after 21 days of incubation in the dark.

Source of variation	% of total variation	P value	P value summary	Significance	
Interaction	2.787	0.0642	ns	No	
Cultivars	67.21	<0.0001	****	Yes	
Treatment	0.3980	0.1433	ns	No	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	10.89	8	1.361	F (8, 161) = 1.894	P=0.0642
Cultivars	262.5	8	32.81	F (8, 161) = 45.67	P<0.0001
Treatment	1.554	1	1.554	F (1, 161) = 2.164	P=0.1433
Residual	115.7	161	0.7184		

* Alpha = 0.05; SS = sum of squares; DF= degrees of freedom; MS = mean squares; F (DFn, DFd) = F ratio (MS value divided by residual).

Supplementary Table 2. Shoot induction in calli of LSC and B36464 on varying concentrations of BAP after 4 weeks of culture.

Genotype	Media	Number of calli cultured	Mean number of shoots / callus
B36464	SIM1	16	9.69 ± 2.55^{a}
	SIM2	16	13.75 ± 4.31^{ac}
	SIM3	16	17.31 ± 7.90^{bc}
	SIM4	16	18.13 ± 3.07^{b}
LSC	SIM1	16	8.44 ± 4.19^{a}
	SIM2	16	12.44 ± 3.61^{ac}
	SIM3	16	16.38 ± 6.03^{bc}
	SIM4	16	16.75 ± 3.55^{b}

Mean shoot production is the ratio of the number of shoots formed to the number of calli cultured expressed as a percentage. Data represents mean ± SD. Values in a column followed by different letters are significantly different from each other at $p \leq 0.05$ (Sidak's multiple comparison test).