Plant Regeneration from Embryogenic Calli of the Wild Sugarcane (*Saccharum spontaneum* L.) Clone 'Glagah Kloet'

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Abstract

A wild sugarcane clone, *Saccharum spontaneum* 'Glagah Kloet', is utilized as breeding material for development of high-yielding sugarcane cultivars. In the present study, we established a plant regeneration system for this clone to increase its potential for use in molecular breeding of sugarcane with a transformation system in the future. Although embryogenic callus was not induced with the medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) alone that has been used commonly for callus induction of sugarcane, we obtained embryogenic calli when apical meristems aseptically isolated from shoots were cultured on callus induction medium containing both 0.01 mg L^{-1} benzyladenine (BA) and 5 mg L^{-1} 2,4-D. This finding indicates that BA is positively effective for induction of embryogenic calli of 'Glagah Kloet'. The embryogenic calli produced shoots when cultured on medium containing 0.3 mg L^{-1} gibberellic acid or on hormone-free medium supplemented with 3 g L^{-1} activated charcoal (AC). The morphology of the shoots clearly differed between the regeneration media. The shoots formed on medium containing gibberellic acid were finer and softer, and the leaf color was lighter compared with those formed on the hormone-free medium containing AC, thus more healthy shoots were regenerated.

Key words: activated charcoal, benzyladenine, plant regeneration, tissue culture, wild sugarcane

Introduction

Sugarcane is a tall perennial grass that is cultivated in tropical and subtropical regions of the world. Notably, this grass stores a high concentration of sucrose in the stem. Approximately 65–70% of global sugar production in the form of sucrose is derived from sugarcane[8]. Sugarcane belongs to the genus *Saccharum*. Although six polyploid species are recognized within *Saccharum*[11,29], modern cultivars for sugar production are mostly derived from interspecific hybridization between *S. officinarum* and *S. spontaneum*[11,22]. Of the other four species, *S. robustum*, *S. barberi*, and *S. sinense* have also provided minor contributions to the breeding of some modern sugarcane cultivars[6].

Saccharum spontaneum is an important breeding resource because of its high dry matter yield, good ratooning ability, and possession of some tolerance against biotic and abiotic stresses in spite of a relatively low sugar content. In the late nineteenth century, a spontaneous hybrid group called Kassoer derived from a cross between *S. officinarum* and Glagah— the wild Javan form of *S. spontaneum* from Indonesia—was used as breeding material, and the progenies have given rise to many cultivars, such

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as POJ2722, POJ2725, POJ2875, and POJ2878, by backcrossing with *S. officinarum*[5,16,26]. In particular, POJ2878 was an excellent clone and was called 'Java Wondercane' because it showed 35% higher sugar productivity than that of the previously best cultivars[19,26]. Glagah still has potential as an important breeding resource for development of cultivars with high biomass, high ratooning ability, and improved stress tolerance for production of sugar and/or bioenergy in the future.

In Japan, sugarcane breeders also employed the wild sugarcane species to introduce the above-mentioned agronomically important traits to their breeding system. They have succeeded in developing the two most recent distinctive forage cultivars 'KRFo93-1'[24, 27] and 'Shimanoushie'[27] (as of November 8th, 2012, an application for seedling registration in Japan is pending as application number 25824). In addition, Japanese breeders have developed a prominent cultivar, 'KY01-2044', with 1.5 times the total biomass yield and 1.3 times the total sugar yield than the major Japanese sugar-producing cultivars[2,10,27,30]. Interestingly, the above-mentioned three cultivars were derived from a common clone, 'Glagah Kloet', which was the male parent of 'KRFo93-1' and a grandparent of 'Shimanoushie' and 'KY01-2044'. 'Glagah Kloet' is one of the clones belonging to the wild species group Glagah.

Thus, we focused on 'Glagah Kloet' and decided to further expand the potential of this clone for use in molecular breeding of sugarcane with a transformation system in the future. A transformation system requires plant regeneration via callus culture, which is indispensable for selection of transformed cells. To the best of our knowledge, only two previous reports have focused on development of a plant regeneration system for *S. spontaneum*. Sobhakumari and Mathew[25] reported hybrid vigor in their plant regeneration system and found that the hybrid parents, *S. spontaneum* and *S. officinarum*, have lower potential for tissue culture compared with that of the hybrids. Fitch and Moore[9] succeeded in achieving plant regeneration via green organogenic calli with picloram, but did not obtain embryogenic callus of *S. spontaneum*, and quantitative data on the regeneration system were not presented.

In contrast, numerous reports of a plant regeneration system for many hybrid sugarcane cultivars have been published, of which most have focused on the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), but sometimes other synthetic auxins, such as picloram and 3,6-dichloro-2-methoxybenzoic acid (dicamba), were used, with or without the cytokinins benzyladenine (BA), 1-phenyl-3-(1,2,3-thidiazol-5-yl) urea, or kinetin[12,13,28,29]. In addition to the plant hormones, a combination of carbon sources composed of 1.5% (w v^{-1}) each of sucrose and sorbitol, and addition of casein hydrolysate to the regeneration medium were reported to have a positive effect on plant regeneration from protoplast-derived calli[14,15]. Furthermore, effects of exogenous amino acids such as glycine, arginine, and cysteine on embryogenic callus formation have been observed. Incorporation of these amino acids in culture media significantly induced somatic embryogenesis and promoted plant regeneration[1,18]. Genotypic differences in responses to tissue culture conditions were observed in these studies reflecting the outcrossing reproductive system of sugarcane, which indicated that tissue culture conditions must be optimized for individual cultivars and genotypes[29].

In the present study, we aimed to optimize tissue culture conditions for callus induction and plant regeneration from callus of 'Glagah Kloet'. We observed the effect of BA on callus induction in the presence of 2,4-D, and the effect of different media on the manner of plant regeneration and morphology of the regenerants. We report here the development of a plant regeneration system from embryogenic calli induced by a low concentration of BA in the presence of 2,4-D for 'Glagah Kloet'.

Materials and Methods

Plant materials

A wild sugarcane clone, *S. spontaneum* L. 'Glagah Kloet', registered as JP 172015 in the National Institute of Agrobiological Sciences (NIAS) GeneBank, Japan, was used in this study. In addition, a Japanese commercial hybrid cultivar, 'KRFo93-1' (a

Saccharum spp. hybrid), was used as a reference. Tillers of the plants were transplanted into soil in pots and grown in a glasshouse maintained at 30°C. For clonal propagation, axillary buds on cut stem sections were planted in soil in pots and cultured in the glasshouse maintained at 30°C. Shoots that sprouted from the buds were used as donors for provision of explants for tissue culture.

Culture media

Components of the culture media are listed in Table 1. In callus induction medium (CIM) 2 and CIM3, 750 mg L^{-1} of additional $MgCl_2$ was added, whereas other macro- and micro-nutrients of all media used were based on MS medium[17] containing 3% (w v^{-1}) sucrose, adjusted to pH 5.8, and solidified with 0.25% (w v^{-1}) Gelrite (Wako, Osaka, Japan). Vitamins of CIM1 were the same components as those of N6 medium[7], whereas vitamins of CIM2, CIM3, regeneration medium (RM) 1, and RM2 were the same components as those of MS medium.

Callus induction

Leaf sheaths of shoots each including an apical meristem were washed in 70% ethanol for 1 min, surface-sterilized in 30% (v v^{-1}) sodium hypochlorite solution (3% available chlorine) for 20 min, and rinsed twice in sterile distilled water. Apical meristems each covered with one or two small leaves were aseptically isolated from shoots under a stereomicroscope. The isolated tissues were placed on callus induction media (Table 1) in Petri dishes and were cultured in the dark at 25°C. For 'Glagah Kloet', meristems were cultured on CIM1, CIM2, and CIM3, whereas those of 'KRFo93-1' were cultured on CIM1 as a reference. The induced calli were subcultured every month onto the same fresh medium prior to plant regeneration experiments. In the present study, each culture was derived from one explant and was maintained as a single culture line.

Plant regeneration from calli

For plant regeneration, calli were divided into small pieces (5 mm in diameter) and transferred onto plant regeneration media (Table 1) and were cultured under continuous fluorescent light (40 µmol m^{-2} s^{-1}) at 25°C. For 'Glagah Kloet', independently induced calli were cultured on both regeneration media, RM1 and RM2, whereas calli of 'KRFo93-1' were cultured on RM2 as a reference.

Statistical analysis

Data on the culture responses (percentage of non-callused explants and callus formation frequency) of 'Glagah Kloet' were obtained in each experiment, and mean values were analyzed by analysis of

Table 1. Components of culture media for callus induction and plant regeneration

Media	Components[a]
Callus induction medium (CIM)	
CIM1	0.25 mg L^{-1} benzyladenine (BA) 4 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) N6 vitamins
CIM2	5 mg L^{-1} 2,4-D 25 mM L^{-1} Proline 750 mg L^{-1} $MgCl_2 \cdot 6H_2O$ MS vitamins
CIM3	CIM2 medium + 0.01 mg L^{-1} BA
Regeneration medium (RM)	
RM1	0.3 mg L^{-1} gibberellic acid MS vitamins
RM2	3 g L^{-1} activated charcoal MS vitamins

a) Other macro- and micro-nutrients in all media not shown here were based on MS medium containing 3% (w v^{-1}) sucrose, adjusted to pH 5.8, and solidified with 0.25% (w v^{-1}) Gelrite (Wako, Osaka, Japan) (see Materials and Methods).

variance (ANOVA) and Tukey contrasts in R v. 2.15.2 software[23] using plant hormones, proline, $MgCl_2$, and vitamins as factors.

Results

Callus induction

A summary of the callus induction data is presented in Table 2. Soon after culture initiation, most meristems were expanded in both 'KRFo93-1' and 'Glagah Kloet'. In 'KRFo93-1', although most cultured tissues exuded a black substance that stained the culture medium around the tissues, callus appeared from 1 month after culture initiation (Fig. 1A), and on average 87.9% of cultured explants produced embryogenic calli and underwent somatic embryogenesis (Table 2, Fig. 1B). In 'Glagah Kloet', most tissues cultured on CIM1 died without forming callus (Fig. 2A) or turned brown soon after callus formation (Fig 2B) during 1 month of culture (Table 2). However, some tissues cultured on CIM2 and CIM3 continued to grow and we observed two types of calli—watery and embryogenic—on their surface (Fig. 2C–F). The watery calli were induced on both

Table 2. Effects of culture media on induction of different types of calli

Clone/Cultivar	Culture medium	Experiment	No. of explants	Non-callused explants (%)	Formation of each callus type (%)		
					Browning*	Watery	Embryogenic
Glagah Kloet	CIM1	1	2	100.0	0.0	0.0	0.0
		2	5	80.0	20.0	0.0	0.0
		3	3	100.0	0.0	0.0	0.0
			Mean ± SD†, ‡	93.3 ± 11.5a	6.7 ± 11.5a	0.0a	0.0a
	CIM2	1	3	0.0	0.0	100.0	0.0
		2	5	40.0	20.0	40.0	0.0
		3	3	0.0	0.0	100.0	0.0
		4	3	0.0	33.3	66.7	0.0
			Mean ± SD	10.0 ± 20.0b	13.3 ± 16.3a	76.7 ± 29.1b	0.0a
	CIM3	1	7	0.0	28.6	14.3	57.1 (42.9)§
		2	3	33.3	33.3	0.0	33.3
			Mean ± SD	16.7 ± 23.6b	31.0 ± 3.4a	7.1 ± 10.1a	45.2 ± 16.8b
KRFo93-1	CIM1	1	14	7.1	7.1	0.0	85.7
		2	10	10.0	0.0	0.0	90.0
			Mean ± SD	8.6 ± 2.0	3.6 ± 5.1	0.0	87.9 ± 3.0

* Callused explants that died after callus formation.
† SD: Standard deviation.
‡ Mean values for 'Glagah Kloet' in each column followed by the same letter are not significantly different at $P < 0.01$ based on Tukey contrasts.
§ Value in parentheses is the frequency of embryogenic calli that partially contained watery callus.

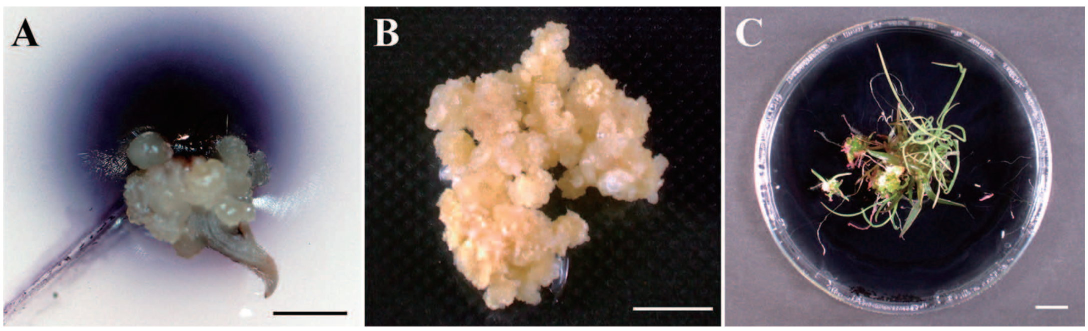

Fig. 1. Embryogenic callus formation and plant regeneration from callus in 'KRFo93-1'.
(A) Black substance surrounding a callused meristematic tissue cultured on CIM1, (B) embryogenic callus induced on CIM1, (C) shoot formation from embryogenic callus on RM2. Bar = 0.5 mm in (A), and 1 cm in (B) and (C).

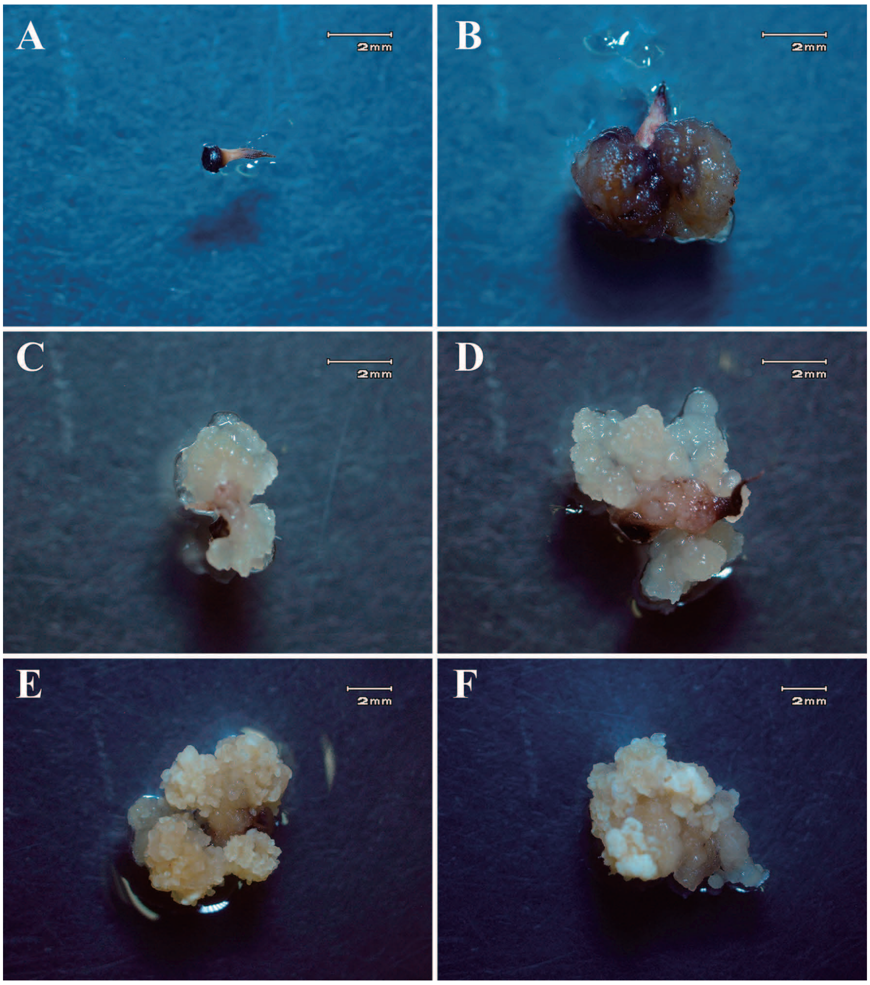

Fig. 2. Types of calli induced by different culture media in 'Glagah Kloet'.
(A) Non-callused explant cultured on CIM1, (B) browning callus induced on CIM1, (C, D) watery callus induced on CIM2, (E) embryogenic callus induced on CIM3, (F) embryogenic callus also containing watery callus. Bar = 2 mm.

CIM2 and CIM3, but their growth was extremely slow even after subculturing (Table 2, Fig. 2C and D). Five embryogenic calli were independently induced from on average 45.2% of cultured explants only on CIM3 in 'Glagah Kloet' (Table 2, Fig. 2E and F). In three of the five instances embryogenic callus induction was accompanied by watery callus induction but the embryogenic parts of the calli grew well during tissue culture (Fig. 2F). ANOVA revealed that for 'Glagah Kloet' BA concentration significantly influenced the frequencies of watery callus and embryogenic callus formation ($P < 0.01$, Table 2). We used the embryogenic calli for the following plant regeneration experiments.

Plant regeneration

After 3 months of callus culture initiation, embryogenic calli were subjected to plant regeneration experiments. In 'KRFo93-1', five randomly selected calli were cultured on RM2. We observed shoot formation from all of the transferred calli 1 month after the transfer (Fig. 1C). In 'Glagah Kloet', three of the five independently induced calli were cultured on both RM1 and RM2. Growth of the calli after transfer to RM1 was vigorous and subsequent formation of shoot primordia occurred 12 days after transplanting, whereas shoots formed from calli on RM2 after about 1 month. However, during culture for shoot formation, calli cultured on RM1 partially turned brown and occasionally died (#2 callus in Fig. 3). The morphology of the shoots clearly differed between regeneration media. The shoots formed on RM1 were finer and softer, and their leaf color was lighter compared with

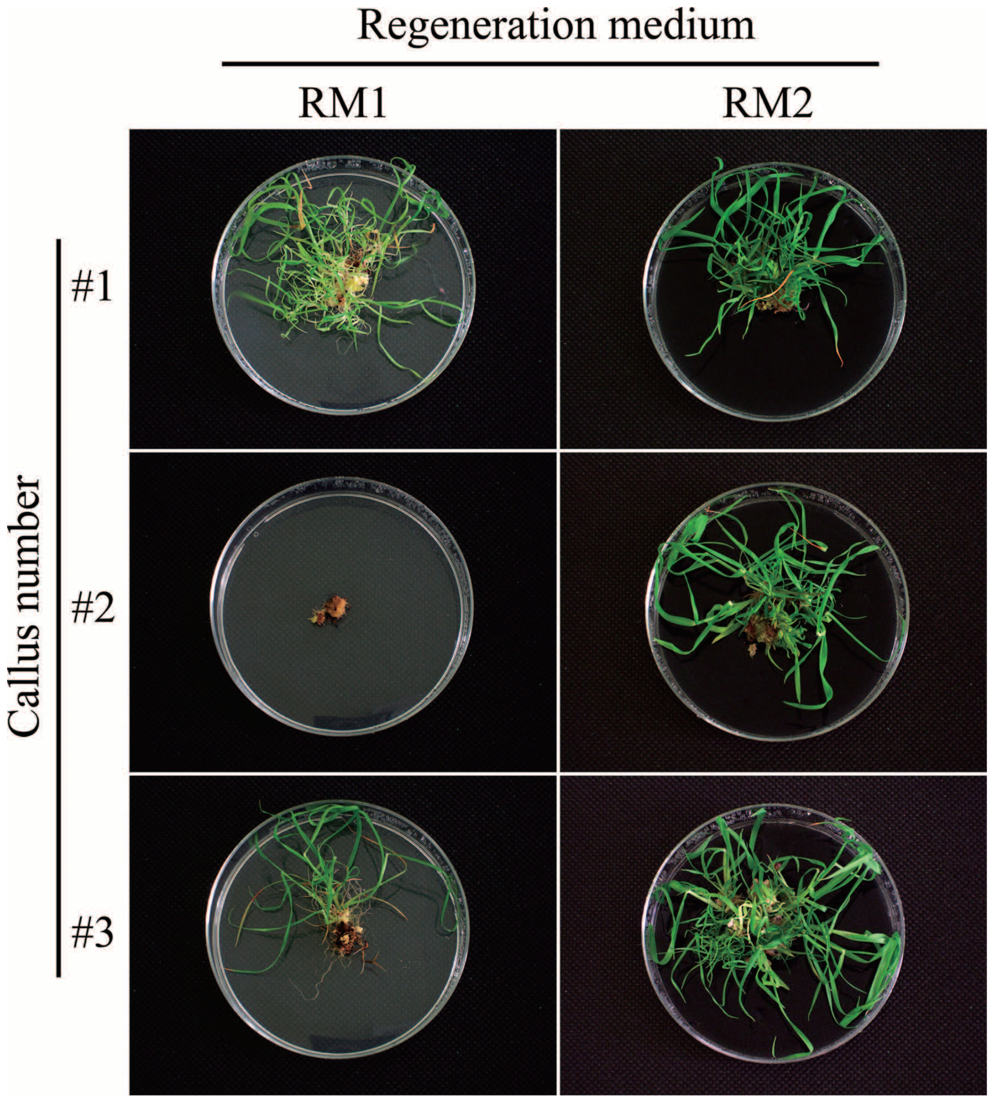

Fig. 3. Morphological differences of shoots regenerated on RM1 and RM2 in 'Glagah Kloet'.
Shoots regenerated on RM1 were finer and softer, and the leaf color was lighter than in those formed on RM2. Results of three independently induced calli are shown.

those formed on RM2 (Fig. 3). Each of the three calli produced shoots on RM2 (Fig. 3). All regenerated shoots on RM1 and RM2 were established in soil, after washing their roots to remove culture media, without the need for acclimatization treatment.

Discussion

In the experiment with the commercial hybrid cultivar 'KRFo93-1', which was used as a reference, we obtained embryogenic calli from apical meristems on CIM1 with a high frequency (87.9% on average; Table 2, Fig. 1B). In 'Glagah Kloet' severe tissue-browning of explants was observed during tissue culture, and most cultured tissues (93.3% on average) did not produce any callus on CIM1 (Fig. 2A and B). However, the problem of tissue browning was almost eliminated with CIM2 and CIM3, and calli were induced on both media (Fig. 2C–F). Although direct evidence was not obtained because of differences in the vitamin components and the different concentration of $MgCl_2$ between CIM1 and the other two media, the presence of proline in CIM2 and CIM3 might be a major factor in preventing tissue-browning because proline is well known to prevent tissue-browning caused by oxidized phenolic compounds that result from polyphenol oxidase activity in cultured cells[20].

Most published research on induction of

regenerable embryogenic callus in sugarcane has focused on auxins such as 2,4-D and picloram[9,13]. In the present study, however, a low concentration of BA in the CIM3 medium promoted callus growth, whereas calli induced on the CIM2 medium without BA were watery and did not grow well. Eventually, embryogenic calli were significantly induced on CIM3 ($P < 0.01$, Table 2), which indicated that a low concentration of BA (0.01 mg L^{-1}) was effective for induction of embryogenic calli in 'Glagah Kloet', although a somewhat higher concentration of BA (0.25 mg L^{-1}) in the CIM1 medium possibly prevented callus induction. To the best of our knowledge, in sugarcane tissue culture this response to BA of callus growth might be specific to this clone. Overall, embryogenic calli with regeneration potential were only induced on the CIM3 medium (Table 2, Fig. 2E and F). In *Miscanthus sinensis*, a relative of sugarcane, a low concentration of BA was recently reported to have a positive effect on induction of embryogenic calli, although a high concentration of BA caused tissue browning[31]. These findings are consistent with a previous report on *Ranunculus asiaticus* in which addition of kinetin—like BA one of the most important cytokinins—to a culture medium containing 2,4-D promoted the formation and growth of regenerable callus[4].

For plant regeneration from embryogenic calli, we prepared two types of media (Table 1). Subsequently, we obtained shoots from calli with both RM1 and RM2 in 'Glagah Kloet', but some obvious differences in shoot morphology were observed (Fig. 3). These differences might reflect the presence of gibberellic acid in RM1 and activated charcoal (AC) in RM2. Gibberellic acid in RM1 might promote fine shoot elongation and callus browning, which seems to be detrimental for further growth of developed shoots on the calli. A similar effect of gibberellic acid on shoot elongation has been reported for one other sugarcane cultivar[3]. The presence of AC in RM2 medium might prevent callus browning by absorbing oxidized phenolic compounds exuded from the calli because AC in culture media adsorbs aromatic compounds such as phenolics and their oxidates[21]. This allowed the calli to grow well and promoted subsequent healthy shoot formation from the calli. On the basis of these results, the RM2 medium is considered to be suitable for plant regeneration in 'Glagah Kloet', although both RM1 and RM2 media can be used for plant regeneration in 'Glagah Kloet' because shoots obtained on both media were readily established in soil in pots.

In conclusion, we established a plant regeneration system for the wild sugarcane clone 'Glagah Kloet'. This clone clearly requires a low concentration (0.01 mg L^{-1}) of BA for induction of regenerable embryogenic calli in the presence of 2,4-D. This distinctive response might be specific to this clone. The optimal conditions for plant regeneration in 'Glagah Kloet' comprised use of CIM3 and RM2 (Table 1) for induction of embryogenic calli and plant regeneration from the calli, respectively. It remains to be examined whether the present system is applicable to other sugarcane cultivars and wild relatives. Given that 'Glagah Kloet' might be a valuable genetic resource for development of high-yielding cultivars for forage and bioethanol production as well as sugar production, the present plant regeneration system is applicable for genetic transformation of 'Glagah Kloet', and is likely to contribute to the molecular breeding of high-yielding sugarcane in the future.

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サトウキビ野生種（*Saccharum spontaneum* L.）系統“Glagah Kloet”のカルスからの植物体再生

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摘　要

サトウキビ野生種（*Saccharum spontaneum* L.）系統“Glagah Kloet”はサトウキビの高収量品種開発のための育種材料としてその利用が期待される。本研究では，遺伝子組換え技術を軸とした分子育種において本系統の利用を図るため，本系統のカルス培養からの再分化系確立を試みた。当初，これまでに多くのサトウキビ品種・系統のカルス培養系で利用されてきた2,4- ジクロロフェノキシ酢酸（2,4-D）5 mg L^{-1}を単独で含むカルス誘導培地に，幼苗から摘出した茎頂分裂組織を置床したが，体細胞不定胚形成カルスを得るに至らなかった。しかし，同培地にさらに低濃度（0.01 mg L^{-1}）のベンジルアデニン（BA）を添加した培地を使用することでカルス形成が促進され，体細胞不定胚形成カルスを得ることができた。このことから，本系統の体細胞不定胚形成カルスの誘導には培地へのBA添加が有効であることが明らかとなった。得られた体細胞不定胚形成カルスを0.3 mg L^{-1}ジベレリン（GA）を添加した培地あるいは3 g L^{-1}活性炭を加えたホルモンフリー培地に置床することで植物体を再生させることができた。シュートには再生時に使用した培地間で形態的な差が認められ，GA添加培地上で形成されたシュートは葉の色が薄く，細長い形態を示し，軟弱であった。一方，活性炭を添加したホルモンフリー培地上では健全なシュートが多数形成された。このことから本系統の植物体再生には活性炭を添加したホルモンフリー培地が適していることが示唆された。

キーワード：活性炭，サトウキビ野生種，植物体再生，組織培養，ベンジルアデニン