

OPTIMIZING CULTURE MEDIUM FOR MERISTEM TISSUE CULTURE OF SEVERAL *SACCHARUM* SPECIES AND COMMERCIAL HYBRIDS

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ABSTRACT

The optimal range of medium nutrients and plant growth regulators (PGR) was investigated for *in vitro* culture of diverse *Saccharum* species and commercial cultivars. Macro-nutrients, nitrogen (N), phosphorous (P) and potassium (K), were essential for growth of meristems. Although the best concentration of N, P and K differed among species and cultivars, 41-66 mM of N, 0.6-1.25 mM of P and 10-30 mM of K were the optimal ranges for all tested sugarcane. An excess of N and P (1.5-2 fold of commonly used media) had an inhibitory effect on meristem growth. No obvious inhibitory effects of low (10 mM, half of normal media) or high (30 mM, 1.5 fold of normal media) concentrations of K were found. Cytokinins (BA and kinetin), GA₃ and auxin (NAA), were all critical for maintaining viability and growth of meristems. By adding PGR to the initiation medium, outgrowth and further shoot elongation were significantly increased. On average, over 60% of the shoots elongated on a medium containing 0.25 μM BA, kinetin, 0.1 μM GA₃, and 0.1 μM NAA. Cytokinins (0.5-1.0 μM) combined with low level of GA₃ and NAA were adequate for all species and cultivars.

INTRODUCTION

Sugarcane is an economically important crop that is propagated by clonal methods. In order to improve agricultural traits in currently used commercial cultivars, breeding programs must import diverse genetic resources such as foreign-grown cultivars or wild related species. However, the process of transferring clonally propagated sugarcane between countries includes the risk of introducing pathogens that may adversely affect a local commercial industry. All clonally propagated sugarcane entering the U.S. must be admitted to the USDA quarantine program and is released only when it tests free of detectable pathogens. Imported accessions that are positive for a prohibited pathogen must either be destroyed or pass through a therapeutic protocol to eliminate the pathogen.

Tissue culture techniques have been widely used in *Saccharum* spp. for various purposes. Meristem tip or shoot tip culture (using apical meristems) has been used as a tool to produce virus-free plants (Hendre et al., 1975; Fitch et al., 2001; Parmessur et al., 2002), which is especially important since vegetative propagation will maintain valuable traits in imported accessions. Early efforts in sugarcane *in vitro* culture used a medium developed by White in 1943 (Roth, 1969; Hendre et al., 1975). Later, a medium developed by Murashige and Skoog (1962) became more common as a basal salt medium with some modifications for either culture of meristems (Liu, 1981; Taylor and Dukic, 1993; Chatenet et al., 2001; Fitch et al., 2001; Wongkaew and Fletcher, 2004) or other tissues such as leaves (Irvine and Benda, 1985; Grisham and Bourg, 1989; Peros et al., 1990; Chengalrayan and Gallo-Meagher, 2001; Geijskes et al., 2003). Amino acids and PGR have been added as supplements to promote more efficient growth

(Thom et al., 1981; Lorenzo et al., 2001; Geijskes et al., 2003; Nieves et al., 2003; Wongkaew and Fletcher, 2004). These reports only focused on the effects of PGRs such as BA, kinetin and coconut water on the MS medium for *in vitro* culture of commercial hybrid cultivars and *S. officinarum*. The concentrations of the PGRs were also not optimized to accommodate a broad range of genotypes in most reports. In addition, there was no report to investigate optimal PGRs and their concentrations for meristem growth without intervening callus.

Plant quarantine programs, which receive diverse *Saccharum* germplasm, would greatly benefit from a universal medium that is capable of supporting growth of many species. The optimal medium and concentrations of nutrients and PGR to support the growth of a diverse range of *Saccharum* species were investigated in this study.

MATERIALS AND METHODS

Plant materials

Samples of six *Saccharum* species (*S. barberi* “Ganda Cheni”, *S. edule* “IJ 76-360”, *S. officinarum* “Barbados White Sport”, *S. robustum* “NG28-289”, *S. sinense* “Agual” and *S. spontaneum* “Guilin-1”), three hybrid commercial cultivars (CP78-1628, CP88-1762 and CP89-2143), and a hybrid of *Miscanthus sinensis* and *S. officinarum* (Fiji 57) were received as specie-representative samples either from the USDA-ARS Sugarcane Field Station (Canal Point, FL) or the Subtropical Horticultural Research Station (Miami, FL). The related genus, *Miscanthus sinensis*, was acquired from the USDA-ARS US National Arboretum in Washington, D.C. All sugarcane setts or stool pieces were germinated, planted in pots and maintained in a greenhouse. When plants were 3-4 months old, actively growing shoot tops were harvested and surface sterilized with a spray of 70% ethanol. Leaves surrounding meristem tissues were carefully removed until the apical meristem was revealed. Dissected meristem tissues, up to 1 mm in diameter at the base of the excision, were placed on medium and cultivated at 27-28°C with a 16-hr photoperiod. Cultures were transferred to fresh medium every 2 weeks. At the beginning of leaf growth, a white and pale green small dome is formed on the meristem. These explants were considered that gave meristem growth so they were counted as regenerated explants even if the growth ceased after the initial outgrowth. The numbers of explants achieving shoot growth (one or two extended leaves) were counted as elongated explants.

Effect of macro-nutrients on growth of meristem tissue

The concentration of each macro-nutrient, nitrogen (N), phosphorous (P) or potassium (K), in MS medium (Murashige and Skoog, 1962, 2 mg glycine, 100 mg myo-inositol, 0.5 mg nicotinic acid, 0.5 mg pyridoxine•HCl, 0.1 mg thiamine•HCl, 100 unit nystatin, and 30g sucrose were included per liter and pH was adjusted to 5.7 before autoclaving) was modified to determine their optimal concentrations for growth of meristem tissues. Various concentration combinations of N, P and K were tested and the growth evaluated on each medium. Compositions of media that were tested are described in Table 1.

Effect of supplemental PGR on shoot growth

Based on the results of the macro-nutrient tests, MS medium was chosen as basal medium. Four PGR, 6-benzyl aminopurine (BA), kinetin (KN), gibberellic acid (GA_3) and naphthaleneacetic acid (NAA), were added at a concentration of 1 μ M to the medium or 10% (v/v) coconut water (CW). All explants whether the growth ceased or continued were transferred

to fresh medium containing the same components every two weeks. After four weeks of culture, explants that gave shoot growth (extended leaves) were evaluated as elongated shoots.

Optimization of meristem tissue culture medium

Culture media with reduced concentrations of PGR (0.25 μM) were optimized for shoot growth from meristems to avoid growth of adventitious shoots. Combinations of BA and KN with or without GA_3 and NAA (0.1 μM each), and CW (10%) with or without GA_3 and NAA (0.1 μM each) were tested for shoot growth and elongation. Effects of the addition of PGR on meristem growth were observed by subculture on PGR-free medium or PGR media. All media were adjusted to pH 5.7 before adding a mixture of 0.1% gelrite and 0.3% agar. After autoclaving, the media were dispensed into 24-well cell culture plates. Elongated shoots were transferred to 20x150 mm glass test tubes containing 8 ml of medium.

Data analysis

Data were collected after four weeks of culture. Explants were considered 'regenerated' as long as there was an initial outgrowth following harvest of the meristems, whether or not the shoots continued to elongate after the initial growth phase. Shoots were considered 'elongated' if they continued to grow in length, with extended leaves past the initial outgrowth from the meristem. Four or five explants were cultured on each medium, with 3 replications of each experiment.

The SAS (SAS, 2003 Version 9.1th edition, SAS Institute, Inc., Cary, NC) Proc GLM was applied to determine the variation of trait among species, medium, species x medium and replication. The F test was based on the random effect model. Duncan's multiple range test was used to study the difference among species, medium and species x medium.

RESULTS

During the meristem excision process, the basal tissue surface was initially white, and then turned reddish brown when exposed to oxygen. While on media, some meristems remained white while others turned dark brown after a day of culture. At the beginning of leaf growth, a white and pale green small dome is formed on the meristem. When the first leaves developed from excised meristems, they were clear to white. As explants grew, they would begin to secrete exudates into the medium. If they remained on media with increased exudates, most explants would die. When transferred to fresh medium, the explants would either continue to grow or would suspend growth depending on the medium and the number of serial transfers to fresh media.

Effect of macro-nutrients on growth of meristem tissue

Nitrogen

For all *Saccharum spp.* and commercial cultivars tested, slightly more than 20% of cultivated meristems grew on media totally devoid of N, while the remaining meristems did not respond at all and died within a few of days of culture. However, those that initially grew eventually became necrotic and died, even after transfer to fresh media. The percentage of plants that grew, significantly increased when nitrogen was added to the medium, with the highest (78 and 83%) on media containing 41 and 66 mM of N, respectively. A high concentration of N (91 mM), which is 1.5 times that in the original MS medium, had an inhibitory effect on the growth

of meristems from most species. Statistical analysis showed that 41 and 66 mM of N were most effective for growth followed by 16 and 91 mM (Table 2).

There were significant differences in the average growth of species and cultivars. Six of the nine *Saccharum* species and cultivars tested, *S. barberi* (69%), *S. edule* (67%), *S. officinarum* (100%), *S. robustum* (89%), *S. sinense* (75%) and CP88-1762 (100%), grew best on medium containing 66 mM of N. Two of the hybrid cultivars (CP78-1628 and CP89-2143) preferred less N (41 mM) and *S. spontaneum* gave the best growth (100%) on 91 mM. Growth of hybrid cultivars (CP series) developed at the Sugarcane Field Station in Canal Point, FL was more vigorous than wild species, and *S. barberi* had the lowest average growth (47% on average). Considering the growth and habit for all tested species and cultivars, N concentrations between 41 mM and 66 mM, similar to 60 mM in the MS medium, were optimal for meristem tissue culture.

Phosphorus

With regard to relative concentration of P in growth medium, the growth habit of explants was similar to those on media tested for N. Compared to N, a more narrow range of P was found to affect the growth of meristems (Table 3). An average of 36% of total explants across species grew on medium lacking P. Growth of explants increased as the concentration of P increased up to 1.25 mM (68%), but was decreased at a concentration of 2.5 mM (50%). The highest growth was obtained on medium containing 1.25 mM of P which is the same concentration of P in MS medium. The optimal range of P was determined to be between 0.6 mM and 1.25 mM. As shown in the responses to N, *Miscanthus sinensis* (36%), *S. barberi* (47%) and *S. edule* (47%) had the lowest growth, and commercial cultivars (CP89-2143, 67% and CP78-1628, 58%) showed higher growth with the exception of one cultivar (CP88-1762, 27%) which was very sensitive to P.

Potassium

Meristems grew on the medium without K, although the growth was low. The percent of explants that grew significantly increased with supplemental treatments of K (from 30% in 0 mM to 60% in 20 mM); however, there was no significant difference among the concentrations, 10-30 mM (Table 4). The optimal range of K was broad, ranging from 10 to 30 mM which is half or 1.5 fold the level in the MS medium (20 mM). Growth of meristems varied among species and cultivars with *S. barberi* (31%), *S. robustum* (43%), *Miscanthus sinensis* (44%), and *S. edule* (45%) having the lowest percent growth of harvested meristems. All three hybrid cultivars demonstrated relatively better growth (50-60%) than wild species except few species a trend more closely aligned with responses to N than to P.

The optimum nutrient levels determined for each macro-nutrient (N, P and K) were 41-66 mM of N, 0.6-1.25 mM of P and 10-30 mM of K, respectively. The levels were suitable for initial growth of all tested sugarcane species and cultivars and fell within the range of MS medium. However, after initial growth of one or two leaves, meristems suspended growth and became necrotic. Only a few (less than 10%) of the meristems in all tested media achieved shoot elongation even after several subcultures to fresh medium suggesting that additional treatments such as PGR were needed for better growth from meristem tissues.

Effect of supplemental PGR on shoot growth

The explants growing on media supplemented with PGR secreted more exudates than those in PGR-free media. Leaf color was white to pale green, and the excised surface expanded. Growth of meristems was not influenced by the specific type of PGR. One μM of cytokinins, GA_3 , NAA or 10% coconut water proved effective in stimulating shoot growth and maintaining its viability. However, the addition of PGR to growth media induced the formation of adventitious shoots, a characteristic not desirable in meristem tissue culture. The addition of NAA to the medium did not induce root formation. Consistent with other experiments in this study, *S. barberi* showed the poorest growth (59%) when compared to the other species/commercial cultivars (68-90%); however, its growth was significantly higher when compared to a PGR-free medium (29%). The response to the various PGR differed among the species/commercial cultivars tested. *S. barberi*, responded best to BA (83%); *S. edule* had an equally superior response to the addition of BA or KN (100%); *S. officinarum* had the best response when grown on media supplemented with KN (90%); *S. robustum* had the greatest increase in initial growth in the presence of CW (83%); BA and NAA supplemented media produced a superior growth response in *S. sinense* (100%); the three commercial hybrid cultivars responded well to BA, KN, and CW (100%); Fiji 57, a *Miscanthus sinensis* x *S. officinarum* hybrid, had the greatest response to KN (100%); and *Miscanthus sinensis* responded best to the inclusion of BA to the basal medium (100%). Although there were no statistical differences among the PGR when averaged across all accessions tested, many species and cultivars grew well on medium supplemented with the cytokinins, BA, KN and CW. Most of all, shoot elongation was improved along with initial growth by adding PGR to the medium (Table 5). The most effective PGR on shoot elongation was BA although statistically KN, NAA and CW had similar effect on it. The average shoot elongation of tested species and cultivars ranged from 15% (*S. officinarum*) to 64% (*S. sinense*).

Optimization of meristem culture medium

On the basis of the results of above experiments, two cytokinins (BA and KN) were chosen to supplement the basal medium as they optimized growth for the majority of the genotypes tested. They were added at a low concentration (0.25 μM each) to avoid the induction of adventitious shoots. GA_3 and NAA were added to create a synergistic effect for shoot elongation at the concentration of 0.1 μM each. As a comparison, the addition of 10% CW to the medium or 10 % CW plus GA_3 and NAA (0.1 μM each) were used. In all media, leaf growth was achieved in 70-100% of all explants. The percent of shoot elongation was higher when GA_3 and NAA were supplemented in the media than when either cytokinins or CW alone were used (Table 6). There were also significant differences among species and cultivars; however, the average shoot elongation was above 50% in medium containing 0.25 μM of BA, KN, GA_3 and NAA. Initial growth and shoot elongation was influenced not only by the medium composition but also the explant's genotype and physiological status. Therefore, concentrations of PGR can be adjusted in the range of 0.1-1.0 μM depending on the genotype and explant's physiological status. Regardless of species or cultivars, PGR were critical for the initiation of meristem outgrowth and further shoot elongation (Fig.1). Meristems on a PGR-free medium for six weeks (Fig. 1, column A) died. When the explants were alternately transferred either from PGR-free to PGR medium or transferred from PGR medium to PGR-free medium, newly developed leaves became chlorotic and then died. The presence of PGR in the medium for the entire period of culture was necessary for significant growth of meristems. The presence of PGR was crucial in the initial growth stage of meristems and required at least four weeks of culture for shoot

elongation to occur. Explants grown on a PGR-free medium resumed growth by adding PGR in the second subculture but shoot growth was slow and viability was poor even in the medium containing PGR (Fig.1B). In contrast, explants grown on PGR medium in the initial culture lost their viability and became necrotic in the second and third subculture when PGR was removed. (Fig.1C). A continuous supply of PGR to the meristem was an important key for optimal growth (Fig.1D). The use of MS medium supplemented with a mixture of low levels ($\approx 1 \mu\text{M}$) of cytokinins and GA₃ and NAA will be most effective for the growth of the six species of sugarcane and several commercial hybrids tested for *in vitro* culture from meristems.

DISCUSSION

Meristems are usually the best material from which to produce virus free plants from an infected source as viruses generally are excluded from this region (Hendre et al., 1975; Fitch et al., 2001; Parmessur et al., 2002). In addition, outgrowth or direct differentiation from meristematic tissue, without intervening dedifferentiation, such as callus formation makes the genotype stable, which is important in crop propagation. However, small dissected meristems (usually less than 1mm in size) rarely survive *in vitro* culture because of their physiological status or the composition of the culture medium. Larger meristems might survive better, but the percentage of virus elimination decreases (Kantha and Gamborg, 1975).

Nutrients such as N, P and K play important roles in tissue differentiation. According to Ramage and Williams (2003), N, P and K uptake increased during meristem growth and differentiation when tobacco leaves were cultivated on the medium. N uptake was also high during the stages of pre-meristem formation and meristem initiation. In our study, very few meristems would grow on a N-free medium, and growth was significantly increased when low levels (8 mM) of N were introduced. Differences in leaf size and levels of chlorosis or necrosis on grown meristems were observed during extended culture periods correlating to changes in the concentration of N in the media. This agrees with the report by Kavanová et al. (2008), where leaf blade growth was inhibited when N was deficient. P deficiency was found to inhibit primary root apical meristem growth of *Arabidopsis* (Sánchez-Calderón et al., 2005). The effect of not only P deficiency, but also P excess, was determined in our study. Excess P caused necrosis in meristematic tissue and expanded leaves. The optimal range of P levels was narrow (0.6-1.25 mM). On the other hand, a broad range (10-30 mM) of K was acceptable for all species and cultivars, and obvious inhibitory symptoms were not observed at any concentration within that range.

It was observed that the presence of PGR was critical in promoting meristem growth when added to a medium containing the optimal nutrients. Shoot meristems are known to be sites that synthesize phytohormones that regulate meristem activity, organ production, and promote vegetative growth (Sakamoto et al., 2001 Clark, 2001). A delicate balance between cytokinins, GAs and auxins is necessary to maintain proper shoot apical meristem activity (Shani et al., 2006). Although these phytohormones are known to be synthesized in the shoot apex, meristems in this study needed exogenous PGR to give rise to leaf growth, possibly because the endogenous phytohormones were not present at proper concentrations. Most *Saccharum* spp. and hybrids in this study grew well on media supplemented with PGR, especially BA. Some species required a different type of PGR for growth. For instance, *S. barberi* and *Miscanthus* spp. grew well on a

BA containing medium whereas *S. robustum* responded better on a medium supplemented with coconut water. CW had been commonly used to promote shoot growth in sugarcane tissue culture alone or with additional PGR (Nickell, 1967; Heinz and Mee, 1969; Roth, 1969; Krishnamurthi and Tlaskal, 1974; Lee, 1987; Liu, 1981, 1993; Wongkaew and Fletcher, 2004). Recent analysis revealed CW is a complex of about 12 cytokinins with their concentrations below 0.00002 μM (isopentenyladenine (iP) and kinetin) except for the zeatin groups (0.04-0.07 μM) (Ge et al., 2006). The use of 10% CW was as effective as other PGR in stimulating leaf outgrowth from the meristem. The synergistic effect of cytokinins and other PGR was confirmed by observing shoot elongation. The combination of BA and KN plus GA₃ and NAA was more effective than CW plus GA₃ or NAA only. This is most likely because concentrations of cytokinins in coconut water are lower than the concentrations of two cytokinins, BA and KN. The presence of PGR was crucial in the initial growth stage of meristems and required at least four weeks of culture for shoot elongation to occur.

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Table 1. Composition of tested media, all other elements based on MS medium

	KNO ₃ (mg/L)	NH ₄ NO ₃ (mg/L)	KCl (mg/L)	Modification
N1 (0 mM)	0	0	1498	
N2 (8 mM)	815.5	0	802.8	
N3 (16 mM)	1630.9	0	198.8	
N4 (40 mM)	1630.9	1000	198.8	
N5 (66 mM)	1630.9	2000	198.8	
N6 (91 mM)	1630.9	3000	198.8	
P1 (0 mM)	1900	1650	93.3	
P2 (0.6 mM)	1900	1650	46.6	- 85 mg/L KH ₂ PO ₄
P3 (1.25 mM)	1900	1650	440	
P4 (2.5 mM)	1900	1650	440	+ 150 mg/L NaH ₂ PO ₄
K1 (0 mM)	0	1776	0	- CaCl ₂ •2H ₂ O + 544 mg/L Ca(NO ₃) ₂ •4H ₂ O*
K2 (10 mM)	1000	1500	0	
K3 (20 mM)	1000	1500	660	
K4 (30 mM)	1000	1500	1406	

* Ca(NO₃)₂•4H₂O was used for supplementing nitrogen and calcium.

Table 2. Growth (% , number of explants grow/total number of explants) on media containing various concentrations of nitrogen

Species Conc. of N	0 mM	8 mM	16 mM	41 mM	66 mM	91 mM	Mean	CV (%)
<i>S. barberi</i>	11	31	69	67	69	39	47 cd	60
<i>S. edule</i>	33	33	67	67	67	50	53 cd	47
<i>S. officinarum</i>	11	19	92	100	100	42	61 bc	44
<i>S. robustum</i>	17	56	69	69	89	50	58 bcd	43
<i>S. sinense</i>	19	50	67	75	75	75	60 bc	41
<i>S. spontaneum</i>	11	72	61	61	89	100	66 bc	44
CP78-1628	59	63	67	93	85	66	76 ab	44
CP88-1762	53	81	92	92	100	92	85 a	54
CP89-2143	11	53	81	100	89	89	70 ab	54
<i>Miscanthus</i> spp.	28	50	69	56	67	56	54 cd	44
Mean	25 d	51 c	73 b	78 a	83 a	66 bc		
CV (%)	96	71	44	46	42	51		

* Same letters are not statistically significant for $p < 0.05$.

Table 3. Growth (% , number of explants grow/total number of explants) on media containing various concentrations of phosphorus

Species Conc. of P	0 mM	0.6 mM	1.25 mM	2.5 mM	Mean	CV (%)
<i>S. barberi</i>	39	50	58	39	47 b	75
<i>S. edule</i>	50	47	47	44	47 b	40
<i>S. officinarum</i>	50	81	78	44	63 ab	53
<i>S. robustum</i>	50	78	81	61	67 a	51
<i>S. sinense</i>	31	75	63	63	58 ab	54
<i>S. spontaneum</i>	28	47	81	69	56 ab	52
CP78-1628	39	69	61	61	58 ab	34
CP88-1762	18	13	58	19	27 c	90
CP89-2143	43	77	92	55	67 a	42
<i>Miscanthus</i> spp.	11	28	58	47	36 b	85
Mean	36 c	56 ab	68 a	50 bc		
CV (%)	87	56	36	54		

* Same letters are not statistically significant for $p < 0.05$.

Table 4. Growth (% , number of explants grow/total number of explants) on media containing various concentrations of potassium

Species Conc. of K	0 mM	10 mM	20 mM	30 mM	Mean	CV (%)
<i>S. barberi</i>	11	42	49	20	31 cd	85
<i>S. edule</i>	25	49	53	52	45 b	77
<i>S. officinarum</i>	25	83	61	61	58 ab	64
<i>S. robustum</i>	33	53	43	42	43 bc	64
<i>S. sinense</i>	22	92	100	78	73 a	51
<i>S. spontaneum</i>	11	56	78	67	53 b	57
CP78-1628	44	44	44	67	50 b	45
CP88-1762	34	56	67	67	56 ab	54
CP89-2143	42	58	58	81	60 ab	60
<i>Miscanthus</i> spp.	29	49	48	51	44 bc	44
Mean	30 b	58 a	60 a	59 a		
CV (%)	96	50	56	51		

* Same letters are not statistically significant for $p < 0.05$.

Table 5. Growth (% , number of explants grow/total number of explants) and shoot elongation (% of shoots growing past initial growth) on the medium containing various PGR

Growth	PGR free	BA	GA ₃	KN	NAA	CW	Mean	CV (%)
<i>S. barberi</i>	29	83	82	53	71	36	59 bc	61
<i>S. edule</i>	78	100	94	100	72	94	90 a	45
<i>S. officinarum</i>	75	63	58	90	70	84	73 bc	49
<i>S. robustum</i>	58	58	67	75	67	83	68 bc	46
<i>S. sinense</i>	53	100	92	92	100	92	88 a	34
CP78-1628	43	67	85	85	75	93	75 abc	50
CP88-1762	50	100	83	58	83	83	76 abc	36
CP89-2143	70	67	77	83	43	83	71 bc	32
Fiji57	78	78	69	100	89	100	86 ab	28
<i>Miscanthus</i> spp.	50	100	67	83	75	67	74 bc	45
Mean	55 b	82 a	77 a	82 a	75 a	82 a		
CV (%)	60	34	32	28	42	34		
Shoot elongation	PGR free	BA	GA ₃	KN	NAA	CW	Mean	CV (%)
<i>S. barberi</i>	27	33	25	33	36	45	33 c	91
<i>S. edule</i>	29	64	50	36	50	46	46 b	42
<i>S. officinarum</i>	0	27	19	20	13	13	15 d	74
<i>S. robustum</i>	10	20	20	20	30	20	20 d	81
<i>S. sinense</i>	20	75	69	75	82	63	64 a	49
CP78-1628	20	33	33	33	33	17	28 d	96
CP88-1762	0	33	17	33	17	17	20 d	68
CP89-2143	13	38	25	50	13	50	32 c	82
Fiji57	10	40	50	50	56	44	42 bc	57
<i>Miscanthus</i> spp.	25	50	33	33	25	33	33 c	62
Mean	15 c	41 a	33 b	38 ab	35 ab	35 ab		
CV (%)	83	72	76	76	99	84		

* Same letters are not statistically significant for $p < 0.05$.

Table 6. Shoot elongation (% , shoots growing past initial growth) on the medium supplemented with combinations of PGR

	BA+KN	CW	BA+KN+ NAA+GA ₃	CW+ NAA+GA ₃	Mean	CV (%)
<i>S. barberi</i>	25	25	88	42	45 ab	76
<i>S. edule</i>	33	33	52	42	38 b	61
<i>S. officinarum</i>	27	63	70	53	53 a	48
<i>S. robustum</i>	50	33	42	33	40 ab	22
<i>S. sinense</i>	67	40	67	50	56 a	22
CP78-1628	27	27	50	19	25 b	69
CP89-2143	33	20	100	100	63 a	69
Fiji57	37	12	37	20	27 b	22
Mean	37 ab	32 b	63 a	45 ab		
CV (%)	50	72	42	63		

* Same letters are not statistically significant for $p < 0.05$.

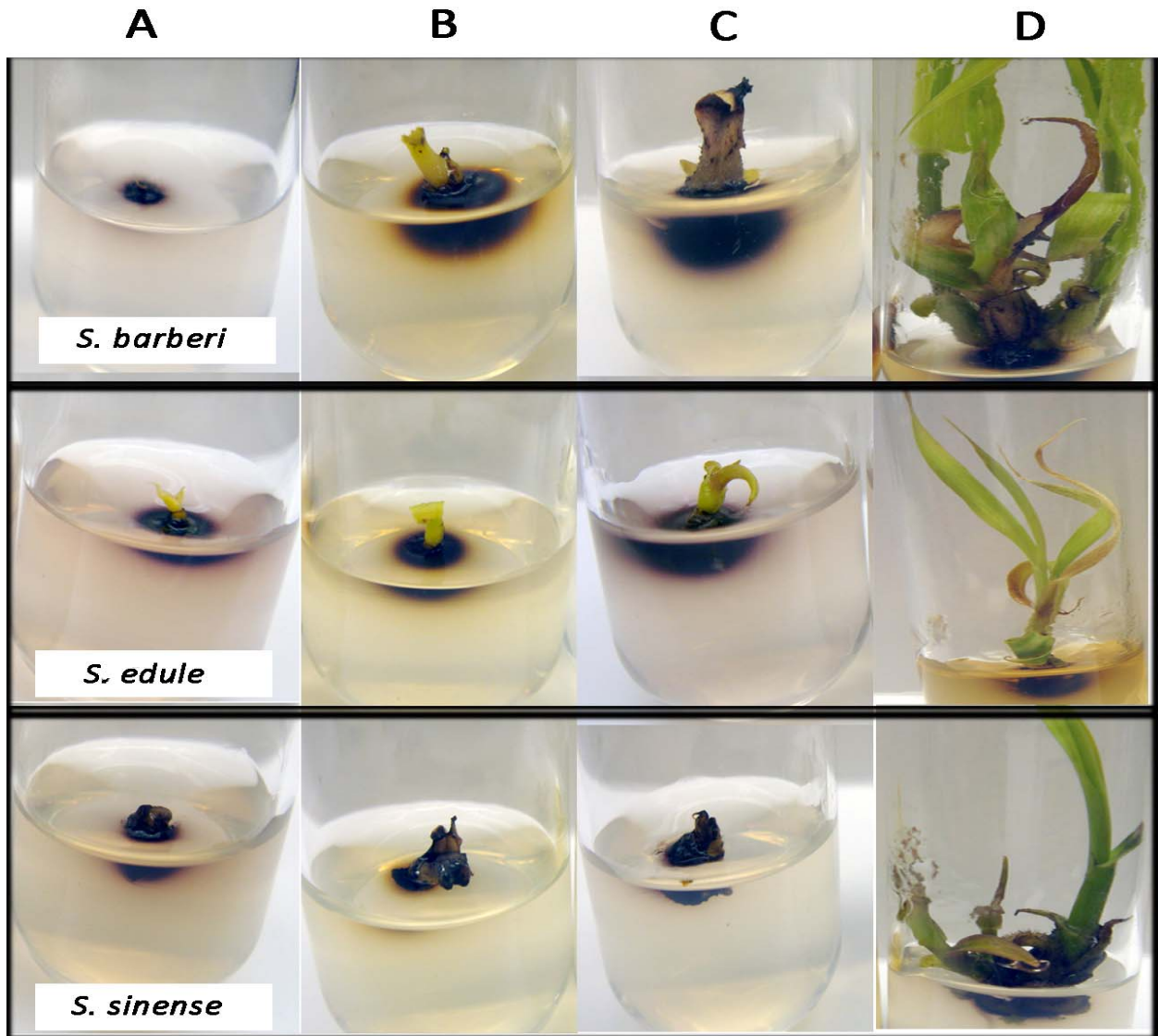


Fig.1. Shoot growth by alternate subculture on medium with or without supplemental PGR Column A, PGR-free→PGR-free→PGR-free; Column B, PGR-free→PGR→PGR; Column C, PGR→PGR-free→PGR free; Column D, PGR→PGR→PGR. Explants were subcultured on fresh medium every two weeks. Total culture period was six-weeks.