Bioscience Methods 2012, Vol.3, No.2, 7−20
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Research Article Open Access

Establishment of an *in vitro* Regeneration System as a Milestone for Genetic Transformation of Sugarcane (*Saccharum officinarum*. L) against *Ustilago scitaminea*

Naweed Anjum, Siddra Ijaz, Iqrar Ahmad Rana, Tariq Manzoor Khan, Iqrar Ahmad Khan, Muhammad Naeem Khan, Ghulam Mustafa, Faiz Ahmad Joyia, Ahsan Iqbal
Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad
Corresponding author email: iqrar_rana@uaf.edu.pk; Author
Bioscience Methods 2012, Vol.3, No.2 doi: 10.5376/bm.2012.03.0002
Received: 18 Feb., 2011
Accepted: 01 Mar., 2011
Published: 21 Mar., 2012

Preferred citation for this article as:
Anjum et al., 2012, Establishment of an *in vitro* Regeneration System as a Milestone for Genetic Transformation of Sugarcane (*Saccharum officinarum*. L) against *Ustilago scitaminea*, Bioscience Methods, Vol.3, No.2 7−20 (doi: 10.5376/bm.2012.03.0002)

Abstract Optimization of stable *in vitro* regeneration system is indispensible to apply molecular approaches in crops. Due to its profound impact on genetic transformation studies, we established a reproducible and effectual in *vitro* regeneration system, in two whip smut (Ustilago scitaminea) susceptible genotypes viz., S−2003−us−127 and S−2003−us−371. Twelve callus formation media (CFM) were investigated for callus formation, in which four levels of 2,4−D (1 mg/L, 2 mg/L, 3 mg/L and 4 mg/L), two levels of BAP (0 mg/L and 0.5 mg/L) and two levels of kinetin (0 and 0.1 mg/l) were used in different combinations with basal MS Salt. CFM3 (3 mg/L 2,4−D), CFM4 (4 mg/L 2,4−D), CFM11 (3 mg/L 2,4−D and 0.1 mg/L kinetin) and CFM12 (4 mg/L 2,4−D and 0.1 mg/L kinetin) were proved to be the best for callus formation response in genotype S−2003−us−127. But in case of genotype S−2003−us−371, CFM3, CFM11 and CFM12 showed good response for callus induction. Among good responsive CFM, we selected CFM with low dose of 2,4−D (CFM3 and CFM11) for our regeneration experiment. For regeneration study, four regeneration media (RM) with different plant growth regulators viz., 2,4−D (0.1 mg/L), BAP (0.5 mg/L and 1 mg/L), kinetin (0.25 mg/L) and proline (250 mmg/L) plus MS salt were used. Calli of three (3) different ages, viz., 21 days, 28 days and 35 days from CFM3 and CFM11 were shifted on four regeneration media (RM). Among these four regeneration media, RM2 (0.1 mg/L 2,4−D and 1 mg/L BAP) gave an excellent regeneration response for genotype S−2003−us−127, when 28 days old calli from CFM11 were transferred to this. This combination was selected as combination of choice in genotype S−2003−us−127 for genetic transformation studies. Genotype S−2003−us−371, showed maximum regeneration, when 35 days old calli from CFM11 were kept on RM4 (0.1 mg/L 2,4−D, 1 mg/L BAP, 0.25 mg/L kinetin and 250 mg/L proline). Genetic stability of regenerated plants of selected media combination was confirmed with RAPD (PCR) analysis by using 5 RAPD primers.
Keywords Biotechnology; Tissue culture; Plant Transformation; Disease resistance

Background

Sugarcane (*Saccharum officinarum* L.) belongs to genus *Saccharum*, family Poaceae and characterized by high levels of polyploidy (2n=80~270) and frequently aneupolidy (Heinz and Mee., 1969). It accounts for approximately 80% of world sugar production FAO, (2009). This grass is the most suitable promising crop which could be utilized mainly for sugar production and then for power generation, paper making, live stock feed, chipboard, cane wax, fertilizer, bioethanol, syrup and mulch (Chaudhry and Naseer, 2008). Sugarcane is the second major cash crop in Pakistan after cotton. Many factors are involved in low cane and sugar yields in which drought or low rainfall, salinity, insect pests and diseases are remarkable especially whip smut (*Ustilago scitaminea*) a fungal disease which causes 30%~100% economic damage (Rangashawami, 1996; Nasir et al., 2000; Gururaj, 2001; Khaliq et al., 2005; Ajit, 2006). Gene introduction by conventional breeding becomes more difficult due to limited flower production, environmental interactions, large complex genome, slow breeding advances, back crossing, low fertility, susceptibility to insect, pest and diseases especially whip smut (Gururaj, 2001).

Flowering is a major constraint for sugarcane improvement by adopting breeding tool Khan et al (2004). In Pakistan, flowering and viability are still a major problematic issue due to lack of favorable environmental conditions (Khan et al., 2007). Thus, unavailability of viable fuzz makes this crop unsuitable under the umbrella of conventional breeding in Pakistan. In conventional breeding and selection system, 10 to 15 years are tentative time span for commercial release of variety with improved characters James (2004).

Moreover, during vegetative propagation, the pathogens keep on accumulating generation after generation, which ultimately results in the decline of the variety. Preservation of germplasm collections is an integral part of all breeding programmes. Current methods for this purpose include conservation stands and greenhouse collections, requiring land and facilities, which are labor intensive and expensive to maintain. Furthermore, under such conditions, there is a high risk of germplasm loss through natural disasters, pest and disease infestations. Traditional plant breeding techniques have been widely used to enhance important economic traits in agronomic crops, but this approach is laborious and time-consuming, especially in vegetatively propagated species like sugarcane. Moreover, various important traits such as resistance to insects, viruses, and herbicides are often absent from the normal sugarcane germplasm. DNA-mediated plant transformation can serve an important function to introduce useful genes into sugarcane that otherwise would be difficult or impossible by standard procedures.

Tissue culture plays an important role in crop improvement. Sugarcane *in vitro* regenerants have higher yield potential in terms of excellent sugar recovery, high tiller ratio, more weight and excellent ratooning performance (Comstock and Miller, 2004). Through tissue culture successful attempts were made to eliminate diseases in sugarcane. Sugarcane yellow leaf virus (SCYLV) and sugarcane yellows phytoplasma (SCYP) were eliminated in nineteen cultivars and showed no disease attack for one year in green house (Parmessur et al., 2002; Ramgareeb et al., 2010). *In vitro* culture system is also used for screening of diseases viz., eye spot disease, fiji disease and downy mildew and whip smut in sugarcane (Singh et al., 2005). One advantage of the use of sugarcane rolled leaf and sheath tissue for embryogenic callus initiation is that it is relatively easy to arrange year-round availability of this explant type from field-grown plants, so that fresh callus batches can be regularly initiated to minimize time in culture for gene transfer. By comparison with more recalcitrant related species such as sweet sorghum (Raghuwanshi and Birch, 2010), the surface layers of sugarcane embryogenic callus evidently have a higher proportion of cells that are able to proliferate and regenerate under conditions that permit the selection of transformed plantlets.

The application of plant biotechnology approaches like genetic transformation of foreign genes into the plant genome, it is very crucial to be the optimization of efficient regeneration system in terms of homozygous plantlet formation through tissue culture with 100% purity to the mother plant. Therefore, assessment of the genetic stability of *in vitro* regenerated planets is an important step in the application of biotechnology. For the application of *in vitro* culture system and clonal propagation, it is important to determine genetic purity (Rani et al., 2000). Hence testing the genetic homogeneity of *in vitro* regenerated plants is very essential and important. The use of molecular markers is becoming widespread for the identification of somaclonal variants and the assessment of in vitro regeneration protocols (Taylor et al., 1995). Different molecular markers (ISSR, RAPD, Trap, RFLP, AFLP and microsatellites etc) are used to detect and characterize somaclonal variation at the genomic DNA level by Ford-Lloyd et al (1992) and Cloutier and Landry (1994).

Among the various molecular marker techniques, RAPD marker is found to be the most useful one to detect genetic changes at DNA level by Taylor et al (1995); Soliman et al (2003) and Anand (2003). RAPD analysis technique is the quick, simple, easy to perform, require small amount of DNA for analysis and the most important advantage of this marker is that the independence of prior information requirement

to detect genetic stability of *in vitro* regenerated plants by Williams et al (1990). These benefits justify the frequent application of the technique in genetic variability studies by Mondal and Chand (2002); Bennici et al (2003) and Feuser et al (2003).

Biotic stresses such as insect, pests and diseases are the alarming threats for sugarcane grass. Whip smut, Shoot borer, giant borer, Red rot, Leaf scald, Eye spot, Mosaic virus, Pineapple disease, Ratoon stunting disease are the major threats for sugarcane. Whip smut is a serious threat for sugarcane and occurs in almost all sugarcane growing countries Comstock (2000). Few attempts have been made to develop resistance against the most devastating disease. These are pre-plant heat therapy of planting sets of sugarcane; pre-plant fungicidal dips of planting sets and screening of sugarcane clones for identification of resistant varieties against the pathogen. Genetic mapping with SSR marker was done for sugarcane smut resistance by Raboin et al (2001). A recent report describes the use of cDNA-AFLP and suppression subtractive hybridization (SSH) to identify differentially expressed sugarcane genes upon inoculation with the sugarcane smut fungus *U. scitaminea*. Using a Restriction Fragment length Polymorphism (RFLP) approach, markers were developed and used on a population of 78 well characterized sugarcane genotypes that are used in a breeding program. In this study, 59 polymorphisms showing correlation with smut resistance were identified. PCR and microscopy were used for smut disease assessment in sugarcane (Singh et al., 2004). Amplified Fragment length Polymorphism (AFLP) analysis of cDNA was used to identify sugarcane genes differentially expressed in disease-resistant but not in susceptible sugarcane somaclones (Hidalgo et al., 2005).

Several strategies have been used to improve plant defense against insects and pathogens. The activation of stress-response transcription factors was found to enhance plant tolerance to fungal and bacterial pathogens in transgenic plants by Gu et al (2002). However, little is known about the function of other components of the plant transcription machinery during stress. The identification and characterization of agronomically interesting genes related to herbivores and pathogens are a major challenge for sugarcane functional genomics. One of the most promising areas is to improve insect control through the use of proteinase inhibitor genes Allsopp et al (1997) and Nutt et al (1999) or Bt genes Arencibia et al (1997) against the sugarcane borer (*Diatraea saccharalis*), responsible for considerable losses in the field. For over ten years now, the directed genetic modification of sugarcane has been a reality in laboratories and field trials has been conducted (Bower and Birch, 1992; Gallo-Meagher and Irvine, 1996; Leibbrandt and Snyman, 2003; Manickavasagam et al., 2004). Genes can be silenced or over expressed to study their function and to produce new phenotypes not possible through conventional breeding. But genetic manipulation through biotechnology such as marker assisted tool (Butterfield, 2005), DNA mapping (Grivet et al., 1996) and genetic transformation have emerged as novel approaches. Successful genetic transformation of cry1Ab gene for shoot borer (*Chilo infuscatellus*) (Arvinth et al., 2010), *Chitinase* and *Chitosanase* genes against *Colletotrichum falcatum* which cause Red rot disease in sugarcane crop (Ijaz, 2012).

1 Results

1.1 Sugarcane *in vitro* studies

Disease free planting material is a necessary component for propagation of next generation for getting higher yield. Tissue culture is an ideal technique for the production of problem free plants in a short time from small amount of planting material. *In vitro* culture of sugarcane provides the planting material throughout the year for the formation of stable transformant lines. Callus formation followed by regeneration varies among genotypes which depicts that genotype plays a significant role in callus formation and regeneration. Therefore, it is significant for every genotype to optimize media with different levels of plant growth regulators along with growing conditions so that optimum regeneration could be attained. Yield is a major constraint to make it a green profitable crop which mainly attributed to insect pest and diseases. Whip smut is a major hurdle for getting good yield. Therefore, under these circumstances this study was

conducted to develop stable *in vitro* regeneration system for genetic transformation experiment in selected genotypes.

1.2 Callus formation

Two whip smut susceptible genotypes S−2003−us−127 and S−2003−us−371 with good agronomic features and an excellent sugar recovery were selected for this study. Good callus mass is required for efficient regeneration. Embryogenic callus also have significant role in effective regeneration. In the present study, immature young leaves of selected sugarcane genotypes were surface sterilized and cultured on callus formation media (CFM). Combinations of different plant growth regulators (2,4−D, BAP and Kinetin) were used in CFM. For callus induction study, four levels of 2,4−D (1 mg/L, 2 mg/L, 3 mg/L and 4 mg/L); two levels of BAP (0 mg/L and 0.1 mg/L) and two levels of Kinetin (0 mg/L and 0.1 mg/L) were used for both genotypes. Data for callus formation response of both genotypes were scored on the basis of callus proliferation rate. Analysis of variance of data revealed significant variability between genotypes as well as among CFM. Interaction between genotypes and CFM was also highly significant (Table 1). Data over five weeks of culturing, revealed that callus formation response of both genotypes was good at all 2,4−D levels, alone or in combination with kinetin, but less response was observed when BAP was used in combination with different levels of 2,4−D. In genotype, S−2003−us−127, when BAP was used in combination with 2,4−D no callus mass formation was observed but swallowing of ex-plant was noted (Figure 1). But in genotype S−2003−us−371, BAP in combination with 2,4−D gave very little callus formation with mean value of 1.00.

Figure 1 Ex-plant swelling in genotype S−2003−us−127
Note: In this genotype, explant swelling was observed and no callus formation was observed when 2,4−D was used in combination with BAP

Genotype S−2003−us−127 showed an excellent callus mass proliferation by scoring the mean value of 5.00 on CFM3, CFM4, CFM11 and CFM12. Genotype S−2003−us−371 gave highest mass of calli on CFM3, CFM11 and CFM12 with mean value 4.00. Comparison among callus formation media (CFM) revealed that the response of CFM3, CFM11 and CFM12 was the best with mean of 4.50, followed by the response of CFM4 and CFM10 by scoring the mean value of 3.50. As for as, genotypes are concerned, the response of genotype S−2003−us−127 was overall good with mean score of 2.67 followed by the genotype S−2003−us−371 with mean value of 2.33 (Table 2; Figure 2).

These both genotypes gave embryogenic calli on callus induction media. Different stages of calli of both genotypes can be seen in Figure 3 and Figure 4.

1.3 Regeneration Response in sugarcane genotypes

In order to obtain an efficient regeneration, plant growth regulators (Auxin and cytokinin) as well as amino acid play a key role for *in vitro* regeneration. CFM3 and CFM11 were selected for *in vitro* regeneration study, because on these media, both genotypes

Table 1 Analysis of variance (ANOVA): Statistical analysis (ANOVA) for callus formation in different sugarcane genotypes on different CFM

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Genotype	1	2.040	2.040	15.06**
Callus formation media (CFM)	11	182.520	16.593	122.53**
Genotype x CFM	11	24.840	2.258	16.67**
Error	48	6.500	0.135	
Total	71	215.901		

Note: **: Highly significant (P<0.01)

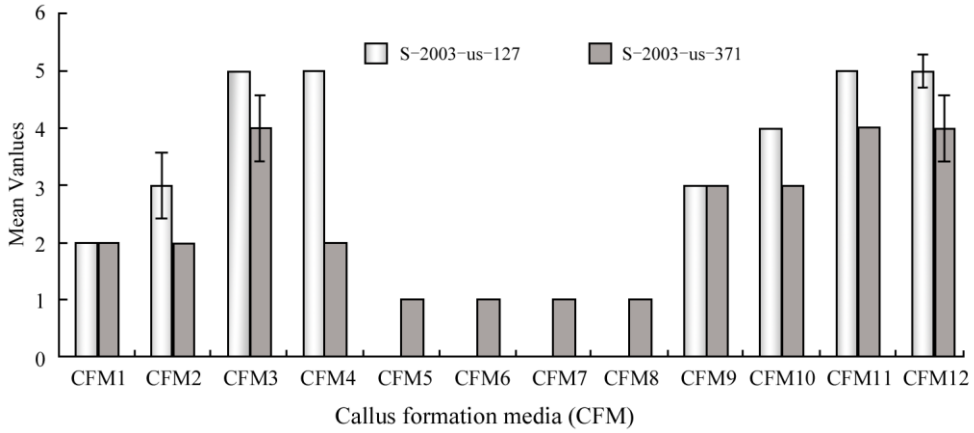

Figure 2 Callus formation response of sugarcane genotypes on different callus formation media (CFM)

Note: Genotype S−2003−us−127 gave highest callus mass on CFM3 (3 mg/L 2,4−D), CFM11 (3 mg/L 2,4−D, 0.1 mg/L Kinetin) and CFM12 (3 mg/L 2,4−D, 0.1 mg/L Kinetin), the most effective media for callus induction

Table 2 Mean interaction of Genotypes and CFM

Callus formation media CFM	Genotype		Overall mean of CFM
	S−2003−us−127	S−2003−us−371	
CFM1	2.00±0.00 d	2.00±0.00 d	2.00±0.00 E
CFM2	3.00±0.58 c	2.00±0.00 d	2.50±0.34 D
CFM3	5.00±0.00 a	4.00±0.58 b	4.50±0.34 A
CFM4	5.00±0.00 a	2.00±0.00 d	3.50±0.67 B
CFM5	0.01±0.00 f	1.00±0.00 e	0.51±0.22 F
CFM6	0.01±0.00 f	1.00±0.00 e	0.51±0.22 F
CFM7	0.01±0.00 f	1.00±0.00 e	0.51±0.22 F
CFM8	0.01±0.00 f	1.00±0.00 e	0.51±0.22 F
CFM9	3.00±0.00 c	3.00±0.00 c	3.00±0.00 C
CFM10	4.00±0.00 b	3.00±0.00 c	3.50±0.22 B
CFM11	5.00±0.00 a	4.00±0.00 b	4.50±0.22 A
CFM12	5.00±0.29 a	4.00±0.58 b	4.50±0.37 A
Overall means of genotypes	2.67±0.36 A	2.33±0.21 B	

Note: Table 2 means sharing similar letter in a row or in a column are statistically non-significant P>0.05; Small letters represent comparison among interaction means and capital letters are used for overall mean

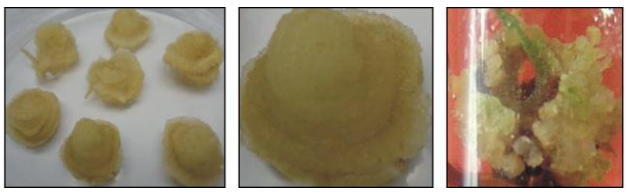

Figure 3 Stages of calli of genotype S−2003−us−127

Figure 4 Stages of calli of genotype S−2003−us−371

produced maximum callus. Despite of an excellent callus formation response of both genotypes also on CFM12, this medium was rejected because high level of 2,4−D might disrupt genetic stability of *in vitro* regenerants which is significantly not desired for genetic transformation approaches. For regeneration study, four regeneration media (RM) were used in which different plant growth regulators viz., 2,4−D

(0.1 mg/L), BAP (0.5 mg/L and 1.0 mg/L), kinetin (0.25 mg/L) and proline (250 mg/L) were used. Calli of 3 different ages, viz., 21 days, 28 days and 35 days were used for regeneration experiment. Calli of both genotypes, those were induced on CFM3 and CFM11, shifted on four regeneration media (RM). Data were collected in the form of total number of shoots per explants. Analysis of variance for regeneration showed that the interaction between days, genotype, CFM and RM is highly significant (Table 3).

Among genotypes, the genotype S−2003−us−127 produced more number of shoots per explant. Calli of 28 days from CFM11 produced 380 shoots per explant in genotype S−2003−us−127 on RM2 (Figure 5). This proved to be the best combination for regeneration; followed by 35 days old calli of this genotype on the same medium (RM2) gave 136.67 shoots per explant. Calli of age 35 days from CFM3 gave 73 shoots per explants on RM1, in genotype S−2003−us−127 followed by 28 days old calli of this genotype on the same regeneration media (RM1) produced 31.67 shoots per explants (Table 4; Figure 5). Contrary to this, the

Table 3 Analysis of variance table for regeneration: Statistical analysis (ANOVA) for Regeneration in different sugarcane genotypes on different RM

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Days	2	49 124.042	24 562.021	39.85**
Genotype	1	61 297.507	61 297.507	99.45**
Days ×Genotypes	2	36 548.931	18 274.465	29.64**
CFM	1	65 749.507	65 749.507	106.67**
Days ×CFM	2	50 779.347	25 389.674	41.19**
Genotype ×CFM	1	29 842.563	29 842.563	48.41**
Days ×Genotype ×CFM	2	41 821.292	20 910.646	33.92**
Regen. Media (RM)	3	31 122.021	10 374.007	16.83**
Days ×RM	6	17 378.292	2 896.382	4.69**
Genotype ×RM	3	38 237.188	12 745.729	20.67**
Days ×Genotype ×RM	6	16 916.958	2 819.493	4.57**
CFM ×RM	3	33 178.188	11 059.396	17.94**
Days ×CFM ×RM	6	23 106.208	3 851.035	6.24**
Genotype ×CFM ×RM	3	35 131.243	11 710.414	18.99**
Days ×Genotype ×CFM ×RM	6	19 850.486	3 308.414	5.36**
Error	96	59 170.667	616.361	
Total	143	609 254.438		

Note: **: Highly significant (P<0.01)

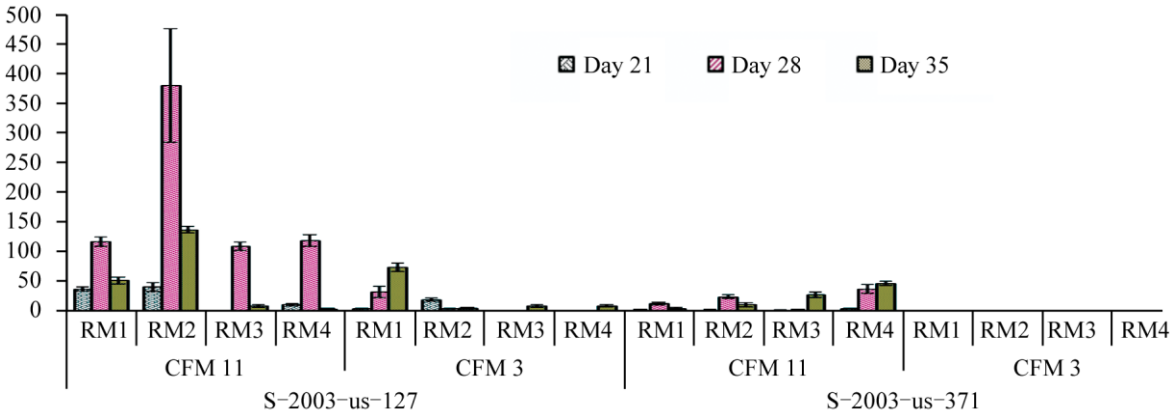

Figure 5 Regeneration response of sugarcane genotypes on different regeneration media (RM)
Note: 28 days old calli of genotype S−2003−us−127 gave maximum shoot formation on RM2 (0.1 mg/L 2,4−D and 1 mg/L BAP)

Table 4 Means for interaction among Days ×Genotype ×CFM ×RM

Genotype	CFM	RM	Day		
			21 Days	28 Days	35 Days
S−2003−us−127	CFM11	RM1	36.33±3.48 def	116.33±7.80 b	51.00±5.86 de
		RM2	40.00±7.51 def	380.00±96.1 a	136.67±5.49 b
		RM3	0.00±0.00 f	108.67±6.96 bc	8.00±2.52 ef
		RM4	10.00±2.08 ef	118.33±9.56 b	2.67±0.88 ef
	CFM3	RM1	3.00±0.58 ef	31.67±9.53 def	73.00±6.81 cd
		RM2	18.33±2.73 ef	3.33±0.88 ef	4.00±1.73 ef
		RM3	0.00±0.00 f	0.00±0.00 f	8.00±2.52 ef
		RM4	0.00±0.00 f	0.00±0.00 f	8.33±2.19 ef
S−2003−us−371	CFM11	RM1	2.00±0.58 ef	11.67±2.33 ef	3.33±2.03 ef
		RM2	1.67±0.33 ef	23.33±3.18 ef	10.00±3.51 ef
		RM3	1.33±0.33 ef	1.33±0.88 ef	26.67±4.67 def
		RM4	3.33±0.88 ef	36.67±7.31 def	46.00±3.79 def
	CFM3	RM1	0.00±0.00 f	0.00±0.00 f	0.00±0.00 f
		RM2	0.00±0.00 f	0.00±0.00 f	0.00±0.00 f
		RM3	0.00±0.00 f	0.00±0.00 f	0.00±0.00 f
		RM4	0.00±0.00 f	0.00±0.00 f	0.00±0.00 f

Note: Table 4 means sharing similar letter are statistically non-significant (P>0.05)

genotype S−2003−us−371, gave maximum number of shoots on RM4. Thirty five (35) days old calli from CFM11 produced 46 shoots per explant on RM4 followed by 28 days old calli from CFM11 gave 36.67 shoots per explant on the same regeneration medium (RM4).

Genotype S−2003−us−371showed no regeneration response, when calli from CFM3 were shifted to all regeneration media (RM), just calli proliferation was observed, and callus mass was increased with no regeneration response (Figure 6).

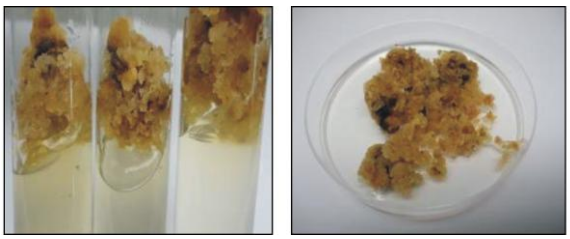

Figure 6 Calli of CFM3 was being continuously proliferated and mass was increased on all four regeneration media in genotype S−2003−us−371

Albino plants formation was observed when calli of genotype S−2003−us−371 from CFM11 were shifted to RM2 (Figure 7). Whitish pink shoots were formed

Figure 7 Formation of shoots with whitish pink colour in genotype S−2003−us−371, when calli induced on CFM11 were transferred to RM2

on this regeneration medium.

Different regeneration stages of both genotypes (S−2003−us−127 and S−2003−us−371) on regeneration media as well as root induction medium can be seen in Figure 8 and Figure 9.

In vitro regenerated plants of both genotypes were acclimatized in green house. Acclimatization of these *in vitro* regenerated under green house condition can be seen in Figure 10.

1.4 Genetic stability determination

Genetic stability determination is very crucial for *in vitro* regenerated plants of selected media combination. For genetic transformation, it is necessary that the

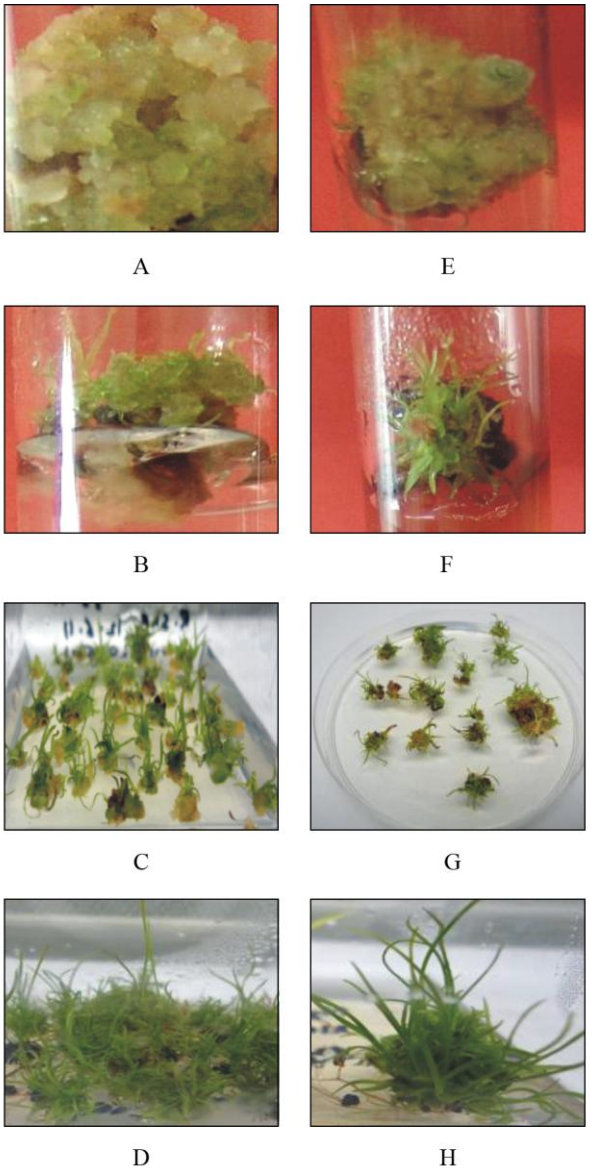

Figure 8 Different stages of regenaration of both genotypes
Note: A, B, C and D= Regeneration response of genotypes S−2003−us−127 from shoot emergence from callus to complete plantlets formation; E, F, G and H= Regeneration response of genotypes S−2003−us−371 from shoot emergence from callus to complete plantlets formation

variation could only induce through genetic transformation and not due to tissue culture regime. For this purpose, five random primers were used. All Random Amplified Polymorphic DNA primers showed genetic stability and same banding pattern was observed in regenerants and wild type (Figure 11).

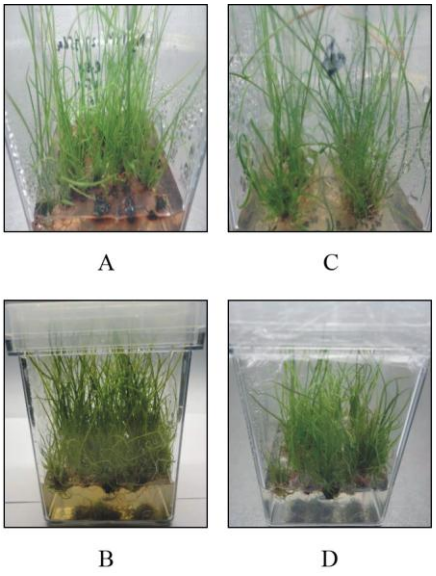

Figure 9 Plantlets of both genotypes on root induction medium (1/2MS)
Note: A and B: Genotypes S−2003−us−127, C and D: Genotypes S−2003−us−371

Figure 10 Acclimatization of *in vitro* regenerated plants under greenhouse conditions

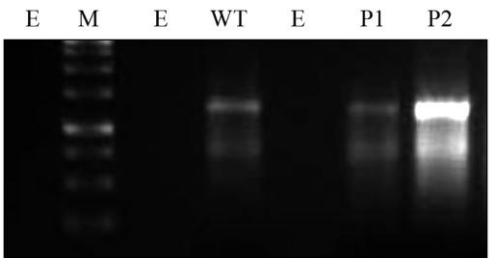

Figure 11 GL decamer K−07
Note: M: 1 kb ladder; E: Empty; WT: Wild type plant; P1, P2: *In vitro* regenerated plants of selected media combination

2 Discussion

An efficient and reproducible regeneration system is indispensible for genetic transformation to improve the yield potential and resistance for biotic and abiotic stresses. Callus is the most suitable target for the

introduction of desired gene into this grass Synman et al (1996). Immature young leaves was used as an explant because of high differentiation potential for regeneration system and reported as an ideal source for rapid callus formation Niaz and Quraishi, (2002). For callus induction, four levels of 2,4−D (1 mg/L, 2 mg/L, 3 mg/L and 4 mg/L), two levels of BAP (0 mg/L and 0.1 mg/L) and two levels of Kinetin (0 and 0.1 mg/L) were studied for both genotypes viz., S−2003−us−127 and S−2003−us−371. Both genotypes showed different response on all CFM which depicts genotype based response. Similar results were observed by Gandonou et al (2005). In their experiment callus formation differ in genotypes studied which depicts that callus formation ability is highly genotype dependent. Genotype response is more influential for callus induction in sugarcane (Badawy et al., 2008; Burner, 1992). Auxin and cytokinin play key role in callus induction (Bhansali and Singh, 1984). For callus induction both genotypes S−2003−us−127 and S−2003−us−371 responded well when 2,4−D was used alone at the level of 3 mg/L. These results are agreed with the results of Sadat et al (2011) and Goel et al (2010) in sugarcane in which he observed good callus induction at 3 mg/L 2,4−D. Ather et al (2009) also observed that 3 mg/L dose rate of 2,4−D gave 100 percent callus induction.

Genotype S−2003−us−127 also showed better callus mass proliferation on CFM4 when 2,4−D was used alone with dose rate of 4 mg/L. Similar results were observed by Ramanand et al (2006) when he used 2,4−D at 4 mg/L. While the genotype S−2003−us−371 showed less response at the same level of 2,4−D. This type of response indicates that genotype play key role in callus formation. Contrary to this, both genotypes showed less response at 1 mg/L and 2 mg/L 2,4−D levels. Our results are matched with Goel et al (2010) finding in which he observed low callus induction frequency at 1 mg/L and 2 mg/L 2,4−D. But our results deviate from the results of Behera and Sahoo, (2009) in which they observed maximum callus formation at 2.5 mg/L 2,4−D.

Kinetin also plays critical role for callus formation. In our study, the addition of kinetin in CFM at 0.1 mg/L gave an excellent response in both genotypes. Both genotypes showed an excellent response at CFM11 and CFM12 with mean score of 5 and 4 respectively. Similar results were produced by Khan et al (2006). Excellent shoots were developed when he used kinetin at 0.1 mg/L. While Ali et al (2010) used kinetin at 1 mg/L and observed excellent callus proliferation in all genotypes.

When 0.1 mg/L BAP was used in combination with 2,4−D (1 mg/L, 2 mg/L, 3 mg/L and 4 mg/L) in CFM, we obtained different results. Genotype S−200−us−371 gave very little callus proliferation with mean of 1.00, but genotype S−2003−us−127 showed no callus induction and just swallowing of explants was observed. Ali et al (2008) also observed less response when he used BAP for callus induction. But our results have no similarity with the results of Sadat et al., 2011 in which he used BAP at 0.1 mg/L and 0.3 mg/L and found an excellent callus growth at both levels. Naz and Jahangir, 2008 obtained callogenesis at 2 mg/L and 3 mg/L BAP level. Behera and Sahoo (2009) and Goel et al (2010) used NAA and IBA for callus induction.

Callus age is also an important factor in *in vitro* regeneration and has significant impact in regeneration. Effect of callus age on *in vitro* regeneration was first reported by Ijaz et al (2012). They used 15 days, 21 days, 28 days and 35 days old calli in regeneration. Here in this study, 21 days, 28 days and 35 days old calli were used for in vitro regeneration and 28 days old calli gave maximum number of shoots per explant in genotype S−2003−us−127 and 35 days old calli gave maximum number of shoots in case of genotypes S−2003−us−371. But this results deviate with the study of Ijaz et al (2012), in which they obtained maximum numbers of shoots from 21 days old calli. These results depicts that this factor is also genotype dependent.

Same response of genotype S−2003−us−127 at 3 mg/L and 4 mg/L 2,4−D alone and even in combination with kinetin was observed but 3 mg/L 2,4−D level alone or in combination with kinetin was selected for further studies instead of 4 mg/L 2,4−D alone or in

combination with kinetin because at high 2,4−D level might disrupt genetic stability of *in vitro* regenerants which is not desired for genetic transformation approaches. Same criteria of CFM selection were kept for genotype S−2003−us−371. High dose rate of this auxin also decrease regeneration potential (Ather et al., 2009). Due to an excellent response of both genotypes on CFM11 and CFM3 are selected as the best media among others for good callus formation. Low dose rate of auxin, cytokinin and amino acid are found to be good for getting better regeneration. BAP at 1 mg/L dose rate has significant impact for getting good regeneration. Genotype S−2003−us−127 showed excellent shoot formation on RM2 in which 1 mg/L dose rate of BAP was used for the 28 days old calli from CFM11. Sadat et al (2011) also obtained an excellent regeneration at 1 mg/L BAP level. Similarly Gopitha et al (2010) also observed excellent shoot formation at 1 mg/L BAP level. Similar types of results were also described found by Ather et al (2009). Our results are also matched with the findings of Khan et al (2009) and Khan et al (2006) in which they got significant shoot formation response with the inclusion of BAP at 1 mg/L dose rate.

Response of genotype S−2003−us−127 at 0.5 mg/L is less than the response of this genotype at 1 mg/L BAP but excellent regeneration response at 0.5 mg/L BAP was observed by Pathak (2009); Baksha et al (2002) and Goel et al (2011). BAP is a synthetic cytokinin which is used by many researchers for regeneration experiment. Dibax et al (2011) found 0.25 mg/L BAP dose rate to be good for regeneration. Khan et al (2009) used 1.5 BAP level while Behera and Sahoo (2009) studied 2 mg/L BAP level and found good shoot formation.

Contrary to this, calli CFM11 showed poor response on RM1 and RM2 for the genotype S−2003−us−371. 35 days old calli of this genotype from CFM11 showed good response at 1 mg/L BAP but in combination with 0.25 mg/L kinetin and 250 mg/L proline. Here our results were also similar with the study of Ali et al (2008) and Baksha et al (2002) in which he found excellent regeneration response at 0.25 mg/L kinetin level and observed that proline has significant impact on shoot formation.

Molecular approaches are more convincing and more reliable as compared with phenotypic observations for determining variations (Leroy et al., 2000). Among various molecular marker techniques, RAPD technique was found to be more powerful tool to determine variation *in vitro*-regenerated plants (Isabel et al., 1993; Rani et al., 1995; Shoyama et al., 1997; Goto et al., 1998). Jain et al., 2005 used the RAPD marker and Isozyme to find out the genetic purity of *in vitro* regenerants of sugarcane. 28 days calli from CFM 11 was selected in which 3 mg/L 2,4−D was used in combination with 0.1 mg/L kinetin and these calli were shifted to RM 2 (0.1 mg/L 2,4−D and 1 mg/L BAP) gave maximum number of plants and became the media combination of choice. The regenerants of this media combination of choice were characterized at molecular level by using RAPD analysis to verify their genetic stability. Because in this study our objective is to generate variability, due to our transgene and not due to tissue culture regime. These regenerants of selected media combination showed genetic stability by giving same banding pattern as found in wild type pant. This showed that for callus induction exposure of ex-pant to 3 mg/L 2,4−D brings no genetic change in plant genome. Jayanthi and Mandal (2001) also observed no genetic variation at 3 mg/L 2,4−D. Mohanty et al (2011) also confirmed genetic uniformity in in vitro regenerants in which they used Kinetin (1.0~3.0 mg/dm^3) along with different levels of NAA, IAA and adenine sulphate. Similar finding was in the study of Ijaz et al (2012) in which they proved that 3 mg/L 2,4−D level did not bring any genetic change in the regenerants of selected combination.

3 Material and Methods

3.1 Germplasm collection

Two sugarcane genotypes viz., S−2003−us−127 and S−2003−us−371 with good agronomic traits but susceptible to *U. scitaminea* were selected.

3.2 Callus mass formation

Young immature leaf was used as explants which was collected from six month old healthy plants. The field-collected material was washed two to three times

with running tap water and then sterilized with 70% ethanol. Leaf rolls were peeled off under sterile conditions inside horizontal laminar air flow hood and cut into cylindrical pieces approximately 2~3 mm in diameter. Five slices were cultured per plate on twelve callus formation media (CFM), which were supplemented with different plant growth regulators and basal MS salts in common. In callus formation media, 2,4−D, BAP and Kinetin were used in different concentrations (Table 5). The culture plates were placed in a controlled temperature room at (26±2)℃ under dark conditions. The cultures were transferred on to fresh callus formation media (CFM) after twenty days. Data of callus formation were recorded in the form of callus score.

3.3 Shoot induction

Proliferated calli of both genotypes with different ages viz., 21 days old, 28 days old and 35 days old, were shifted to four different regeneration media. Regeneration media were composed of basal MS salt in common with different levels of 2,4−D, BAP, Kinetin and Proline (Table 6).

3.4 Root induction

For the development of roots, 1/2 MS medium was used (Table 7). Plantlets were shifted to 1/2 MS medium, for root induction. After the formation of profuse root system, plants of all regeneration media were counted and shifted to peat and moss in poly ethylene bags.

Acclimatization After shifting to peat moss, these plants were acclimatized in green house (Figure 12).

3.5 Genome stability analysis

DNA was isolated by the method described by Pallotta et al (2000). Young leaf tissues (200 mg) were ground

Table 6 Regeneration media (RM) with different plant growth regulators (2,4−D, BAP, Kinetin and Proline

Ingredients	Regeneration media			
	RM1	RM2	RM3	RM4
Ms Salts (g/L)	4.33	4.33	4.33	4.33
Sucrose (g/L)	30	30	30	30
Myoinositol (mg/L)	100	100	100	100
Thiamine HCL (g/L)	2	2	2	2
Nicotinic acid (g/L)	1	1	1	1
Glycine (g/L)	4	4	4	4
Pyridoxine HCl (g/L)	1	1	1	1
2,4−D (mg/L)	0.1	0.1	0.1	0.1
BAP (mg/L)	0.5	1	0.5	1
Kinetin (mg/L)	0	0	0.30	0.30
Proline (mg/L)	0	0	250	250
Phytagel (g/L)	2.6	2.6	2.6	2.6

Table 7 Root induction medium for root development

½ MS medium	Content
Ms Salts (g/L)	2.165
Sucrose (g/L)	30
Thiamine HCL (g/L)	2
Nicotinic acid (g/L)	1
Glycine (g/L)	4
Pyridoxine HCL (g/L)	1
Phytagel (g/L)	2.6

Table 5 Callus Formation Media (CFM) with different plant growth regulators (2,4−D, BAP and Kinetin)

Ingredients	Callus formation media											
	CFM1	CFM2	CFM3	CFM4	CFM5	CFM6	CFM7	CFM8	CFM9	CFM10	CFM11	CFM12
Ms Salts (g/L)	4.33	4.33	4.33	4.33	4.33	4.33	4.33	4.33	4.33	4.33	4.33	4.33
Sucrose (g/L)	30	30	30	30	30	30	30	30	30	30	30	30
Myoinositol (mg/L)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Thiamine HCL (g/L)	2	2	2	2	2	2	2	2	2	2	2	2
Nicotinic acid (g/L)	1	1	1	1	1	1	1	1	1	1	1	1
Glycine (g/L)	4	4	4	4	4	4	4	4	4	4	4	4
Pyridoxine HCl (g/L)	1	1	1	1	1	1	1	1	1	1	1	1
2,4−D (mg/L)	1	2	3	4	1	2	3	4	1	2	3	4
BAP (mg/L)	0	0	0	0	0.1	0.1	0.1	0.1	0	0	0	0
Kinetin (mg/L)	0	0	0	0	0	0	0	0	0.1	0.1	0.1	0.1
Phytagel (g/L)	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6

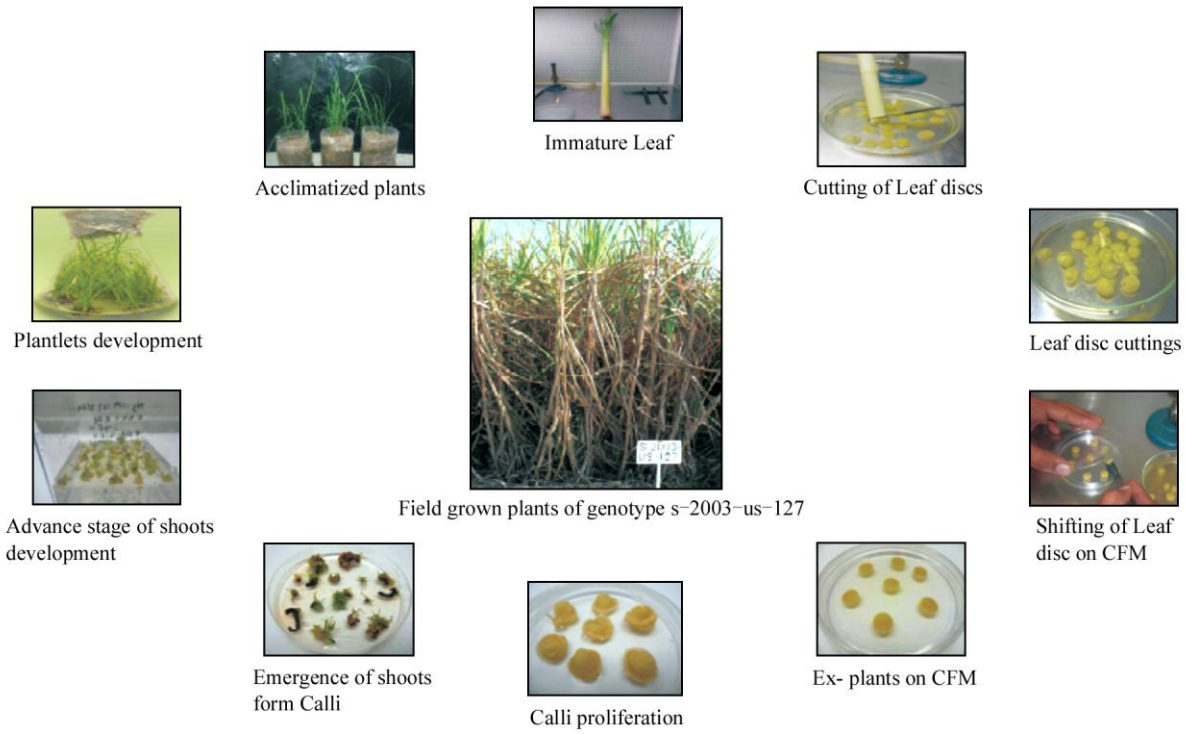

Figure 12 General Tissue culture strategy adopted

in liquid nitrogen through mixture mill. Extraction buffer (700 μL) were added to each eppendorf tube and inverted. Detail of extraction buffer is shown in table 8. Then 800 μL phenol chloroform isomyl alcohol, (25:24:1) was added into each eppendorf tube. Centrifugation was performed for 3 minute at 5 000 rpm at 4℃. Supernatant was taken in to each new eppendorf followed by addition of 1/10 of sodium acetate. Then equal volume of Iso-propanol was added into each tube. Centrifugation was done at 13 200 r/min for 15 minutes. Supernatant was removed. Washing of pellets with 80% ethanol was done, followed by air drying the pellets. The pellet was dissolved in R40 (RNase and TE). DNA quality was checked by running the isolated DNA samples on agarose gel electrophoresis. DNA quantity was determined by using nanophotometer.

Table 8 Genomic DNA Extraction buffer

DNA Extraction buffer	Content
Lauryl Sarcosyl	1%
Tris HCl	100 mmol/L
NaCl	100 mmol/L
EDTA	10 mmol/L

3.6 RAPD (PCR) analysis

For determining genetic stability of *in vitro* regenerated plants of selected combination compared with wild type, five random primers were used (Table 9). Random amplified polymorphic DNA (RAPD) analysis of genomic DNA was carried out in 25 μL reaction mixture containing 2.5 μL of template (Genomic DNA), 3 μL $MgCl_2$, 4 μL of dNTPs, 0.2 μL of *Taq* Polymerase and 2 μL of primer in 1× reaction buffer. The amplification reaction was performed in the Master cycler with an initial denaturation for 5 minutes at 94℃, followed by 40 cycles: 1 minute denaturation at 94℃; 1 minute annealing at 36℃; 2 minute extension

Table 9 RAPD primers with their sequence and annealing temperature

Primer name	Primer sequence	Annealing Temperature
GL Decamer K−07	AGCGAGCAAG	36℃
GL Decamer K−20	GTGTCGCGAG	36℃
GL Decamer B−02	TGATCCCTGG	36℃
GL Decamer B−03	CATCCCCCTG	36℃
GL Decamer D−11	AGCGCCATTG	36℃

at 72℃. Further 10 minute final extension was carried out at 72℃. Then gel electrophoresis of PCR product was done on agarose gel electrophoresis. By using gel documentation system photograph was taken.

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