THE EFFECT OF DICHLOROPHENOXYACETIC ACID (2,4-D) CONCENTRATIONS ON CALLUS INDUCTION IN SUGARCANE (*SACCHARUM OFFICINARUM*)

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(Received 27th Apr 2024; accepted 16th Jul 2024)

Abstract. Tissue culture serves as a valuable method for enhancing genetic diversity in sugarcane, which is essential for any successful breeding initiative. This research aimed to identify the optimal concentration of Dichlorophenoxyacetic acid (2,4-D) for inducing callus formation in sugarcane. Explants from the apical meristematic region of eight-month-old sugarcane plants underwent sterilization with a 70% bleach solution for 30 min, followed by cutting into small segments. These segments were then subjected to callus induction using MS media enriched with hormones, maintained at a temperature of 28 ± 2°C and a photoperiod of 16 h. Various concentrations of 2,4-D had a significant impact on the observed parameters. The application of 2 mg/L 2,4-D resulted in the highest callus weight (2.04 g), somaclones (63.60/callus), shoot length (9.04 cm), and chlorophyll mutants (9.60). Similarly, the outcomes obtained with 3 mg/L 2,4-D treatment were comparable to those achieved with 2 mg/L 2,4-D. The induction of callus required a minimum of 15.90 days at 4 mg/L and a maximum of 23.80 days at 5 mg/L 2,4-D concentration. Among the sugarcane varieties studied, Th-2019 exhibited superior performance in terms of callus induction and callus weight, while Th-326 showed the highest number of somaclones/callus. The number of roots per jar was statistically similar for both Th-2109 and Th-326 varieties. However, the interaction between treatment, variety, and all parameters varied. Following in vitro cultivation, the plantlets were transferred to the net house for further hardening. The establishment of callus culture methodologies holds promise for developing elite sugarcane genotypes, thereby enriching the genetic diversity pool. Consequently, this technique presents a valuable tool for sugarcane breeding endeavors in Pakistan.

Keywords: *hormone, chlorophyll mutant, genetic diversity, regeneration, somaclonal variation*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_49514960

Introduction

Sugarcane *(Saccharum officinarum* L.*)*, a member of the Poaceae family, serves as the primary raw material for approximately 70-75% of global sugar production (Yadav and Ahmad, 2013). Typically cultivated in tropical and subtropical regions, sugarcane varieties exhibit diverse characteristics such as cane girth, height, weight, color, hardness, and sugar content (Naidu, 1987). Given its high polyploidy (2n = 80-270), developing new sugarcane genotypes with desired traits poses significant challenges due to slow conventional breeding practices reliant on recombination (Sobahkumari, 2012). Negative correlations among attributes like yield, sugar content, and disease resistance complicate matters further. Additionally, the low viability of fuzz in Pakistan hampers traditional breeding efforts, necessitating the exploration of alternative approaches such as in-vitro culture and induced transformation to generate genetic diversity in sugarcane.

In light of these breeding challenges, biotechnological techniques offer promising possibilities for enhancing both quantitative and qualitative traits in sugarcane. Callus culture, a key strategy in this regard, has been successfully established using shoot pieces, young leaves, and shoot tips as explants. The resulting variation in regenerated plants, known as somaclonal variation, represents a valuable reservoir of potentially beneficial germplasm for plant improvement (Larkin and Scowcroft, 1981). Somaclonal variations, which occur at high frequencies, can introduce novel changes in adapted genotypes, offering means to develop desired traits in sugarcane.

For years, callus-cultured plantlets have been screened for traits such as high yield, sucrose content, and resistance to biotic and abiotic stresses in field trials. Consequently, tissue culture-derived variations hold promise for enhancing sugarcane crop improvement programs by incorporating new and desirable traits into promising genotypes. In Pakistan, limited studies have been conducted on callus induction from shoot apical meristems, which serve as a basis for genetic studies aimed at developing high-yielding sugarcane varieties resistant to diseases and pests. This study aimed to optimize conditions for callus induction in sugarcane, particularly focusing on determining the most suitable concentration of 2,4-D for in-vitro callus production. The optimized protocol outlined in this article lays the groundwork for establishing an efficient system for genetic transformation in callus cultures of promising sugarcane varieties.

Materials and methods

The study was conducted at the Tissue Culture Laboratory, PARC-National Sugar and Tropical Horticulture Research Institute, Thatta, during 2017-18. Young sugarcane shoot tips with actively dividing cells were selected as explants for callus induction. Shoot apical meristems from eight-month-old sugarcane plants of Thatta-10, Thatta-2109, and Thatta-326 varieties were utilized. The process of selecting, preparing, sterilizing, and culturing the explants involved several steps. Twelve replicates of each genotype were chosen, and the outer leaves of the cane top were removed to obtain 10 mm-sized explants. These explants were then sterilized using a 70% bleach solution for 30 min, followed by rinsing with double distilled water three times. After sterilization, the explants were cut into round shapes with sizes ranging from 0.5 to 0.8 mm, and three explant samples were placed in each jar and weighed individually. The culture media for callus/somatic embryogenesis consisted of MS medium enriched

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_49514960

with different concentrations of dichlorophenoxyacetic acid (2,4-D), labeled as CT-1 (1 mg/L), CT-2 (2 mg/L), CT-3 (3 mg/L), CT-4 (4 mg/L), and CT-5 (5 mg/L). Sucrose (30 g) served as the carbon source, and the media was solidified with 3 g/L gallon gum. After 28 days of explantation, the callus was weighed, and one gram of callus was cultured on a regeneration medium containing 2.5 mg/L each of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and Kinetin (Kin). The green shoots developed from the callus were counted for shoot organogenesis, and chlorophyll mutants were enumerated from the regenerated shoots. When the plantlets reached a height of 6-8 cm, they were transferred to rooting media containing different concentrations of IBA, labeled as CI-1 (1 mg/L), CI-2 (2 mg/L), CI-3 (3 mg/L), CI-4 (4 mg/L), and CI-5 (5 mg/L). All sterilization and culturing procedures were conducted under aseptic conditions in a laminar flow cabinet, and the cultures were incubated at 28 ± 2°C with a 16-h photoperiod for 28 days. Finally, the in-vitro grown plantlets were transferred to the net house for hardening using plastic bags filled with peat moss.

Statistical analysis

The statistical analysis of the recorded data was performed using Statistix 10. For this purpose, analysis of variance (ANOVA) and Tukey's HSD test were employed.

Results and discussion

Three approved sugarcane varieties of the Pakistan Agricultural Research Council (PARC) were used in this experiment. The varieties showed significant differences in callus formation, regeneration, and the number of chlorophyll mutants at various concentrations of 2, 4-D. The parameters of interest in this study included size/weight of explants, the weight of callus, number of days required for callus induction, number of plantlets/jar, shoot length, number of chlorophyll mutants/jar, and number of roots/plantlet.

Weight of explants/jar

The mean explant weight per jar was 0.47 g for Th-10, 0.53 g for Th-2109, and 0.66 g for Th-326 (*Table 1*). In terms of mean weights with regard to different 2, 4-D concentrations, maximum weights of 0.66 and 0.61 g/jar were observed at CT-2 and CT-3, respectively, but the difference was statistically insignificant. Similarly, the weights noted at CT-1 (0.51 g), CT-4 (0.51 g), and CT-5 (0.48 g) were also statistically non-significant.

Table 1. *Weight of explants (g) under different 2,4-D concentrations*

Treatment (concentration of 2,4-D)	Variety			Mean
	Th-10	Th-2109	Th-326	
CT-1 (1 mg/L)	0.45 c	0.56 bc	0.52 bc	**0.51 B**
CT-2 (2 mg/L)	0.48 c	0.65 b	0.85 a	**0.66 A**
CT-3 (3 mg/L)	0.52 bc	0.43 c	0.86 a	**0.61 A**
CT-4 (4 mg/L)	0.46 c	0.50 bc	0.57 bc	**0.51 B**
CT-5 (5 mg/L)	0.44 c	0.50 bc	0.50 bc	**0.48 B**
Mean	**0.47 C**	**0.53 B**	**0.66 A**	

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_49514960

ANOVA Test					
Source	**DF**	**SS**	**MS**	**F**	**P**
Replication	4	0.02619	0.00655		
Treatment	4	0.34544	0.08636	14.43	0.0000
Varieties	2	0.46763	0.23382	39.06	0.0000
Treatment × varieties	8	0.47832	0.05979	9.99	0.0000
Error	56	0.33519	0.00599		
Total	74	1.65278			
CV %	13.91				

Tukey's HSD test			
Parameters	**Standard error**	**Q value**	**Tukey's HSD $_{(0.05)}$**
Treatment	0.0283	3.987	0.0796
Variety	0.0219	3.405	0.0527
Treatment × variety	0.0489	5.012	0.1734

Weight of callus

The statistical analysis indicated that the effect of 2, 4-D treatment was highly significant ($P \leq 0.05$) for variety and interaction of both (treatment × variety). Treatment CT-2 and CT-3 gave statistically at par highest callus induction with a mean weight of 2.04 and 2.00 g, respectively. As for variety, the maximum weight of callus was observed in Th-2109 with the mean weight of 1.81 g, followed by Th-10 (1.69 g) and Th-326 (1.62 g). The interaction of treatment and variety produced the maximum weight of 2.12 g/callus at CT-3 × Th-2109 (*Table 2*). The results are in agreement with Misbah et al. (2017), who reported the best callus weight at 1 and 2 mg/L 2, 4-D. The findings are also consistent with the report of Dibax et al. (2011), who reported optimal proliferation under 2 mg/L concentration of 2, 4-D.

Table 2. *Effect of 2, 4-D concentrations on weight of callus (g)*

Treatment (concentration of 2,4-D)	**Variety**			**Mean**
	Th-10	**Th-2109**	**Th-326**	
CT-1 (1 mg/L)	1.51 bf	1.55 af	1.32 ef	**1.46 CD**
CT-2 (2 mg/L)	1.67 af	2.08 ac	1.45 df	**1.73 BC**
CT-3 (3 mg/L)	1.98 ad	2.12 a	2.02 ad	**2.04 A**
CT-4 (4 mg/L)	2.06 ad	2.11 ab	1.83 ae	**2.00 AB**
CT-5 (5 mg/L)	1.23 ef	1.21 f	1.47 cf	**1.30 D**
Mean	**1.69 AB**	**1.81 A**	**1.62 B**	

ANOVA test					
Source	**DF**	**SS**	**MS**	**F**	**P**
Replication	4	0.2128	0.05319		
Treatment	4	6.3241	1.58102	21.13	0.0000
Varieties	2	0.4908	0.24539	3.28	0.0450
Treatment × varieties	8	1.1502	0.14378	1.92	0.0747
Error	56	4.1909	0.07484		
Total	74	12.3688			
CV %	15.99				

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_49514960

Tukey's HSD test			
Parameters	**Standard error**	**Q value**	**Tukey's HSD $_{(0.05)}$**
Treatment	0.0999	3.987	0.2816
Variety	0.0774	3.405	0.1863
Treatment × varieties	0.1730	5.012	0.6131

Number of days required for callus induction

The effect of 2, 4-D concentrations, variety, and interaction of the both was highly significant ($P \leq 0.05$) for callus induction (*Table 3*). The maximum number of days (21.7) was required for callus induction under CT-5 concentration, followed by CT-1 (20.30 days). Variety Th-2109 needed the highest number of days. As for interaction of treatment and variety, the minimum number of 15.20 days was required for CT-4 × Th-2109, while the maximum number of 23.8 days were required at CT-5× Th-2109. The results are in accordance with various researchers who reported the best results of callus induction under 2 to 5 mg/L of 2, 4-D concentration. Tahir et al. (2011) and Karim et al. (2002) observed maximum callus induction at 3.0 mg/L of 2, 4-D. Moreover, Shahid et al. (2001) investigated callus formation in 7 sugarcane genotypes and reported 2 mg/L 2, 4-D as the best concentration. Fitch and Moore (1990) and Oropeza and Garcia (1996) also proposed 2-4 mg/L 2, 4-D as the best range of concentrations for producing callus in sugarcane.

Table 3. *Effect of 2, 4-D concentrations on number of days to callus induction*

Treatment (concentration of 2,4-D)	**Variety**			**Mean**
	Th-10	**Th-2109**	**Th-326**	
CT-1 (1 mg/L)	20.20 bc	21.60 b	19.00 cd	**20.30 B**
CT-2 (2 mg/L)	18.40 ce	19.60 bc	17.40 df	**18.30 C**
CT-3 (3 mg/L)	17.00 dg	17.00 dg	16.40 ef	**16.80 D**
CT-4 (4 mg/L)	16.40 eg	15.20 g	16.20 fg	**15.90 D**
CT-5 (5 mg/L)	21.20 b	23.80 a	20.20 bc	**21.70 A**
Mean	**18.6 B**	**19.40 A**	**17.80 C**	

ANOVA test					
Source	**DF**	**SS**	**MS**	**F**	**P**
Replication	4	5.680	1.4200		
Treatment	4	344.347	86.0867	99.77	0.0000
Varieties	2	32.000	16.0000	18.54	0.0000
Treatment × varieties	8	36.933	4.6167	5.35	0.0001
Error	56	48.320	0.8629		
Total	74	467.280			
CV %	4.98				

Tukey's HSD test			
Parameters	**Standard error**	**Q value**	**Tukey's HSD $_{(0.05)}$**
Treatment	0.3392	3.987	0.9562
Variety	0.2627	3.405	0.6327
Treatment × varieties	0.5875	5.012	2.0819

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Number of somaclones/callus

The regeneration of the somaclones was significantly ($P \leq 0.05$) affected by the treatment, variety, and interaction of both (*Table 4*). Application of CT-2 and CT-3 concentrations gave the maximum number of mean 63.60 and 58.30 somaclones/callus, respectively. However, the difference was statistically non-significant. Th-326 produced the highest number of mean 53.80 somaclones/callus, followed by Th-10 (47.20 somaclones/callus) and Th-2109 (45.30 somaclone/callus). In the case of interaction of treatment and variety, 71.00 somaclones were obtained in CT-2 × Th-10 (*Table 5*). Misbah et al. (2017) noted good quality callus in sugarcane at 2 mg/L 2, 4-D concentration. Similar results were reported by Schween and Schwenkel (2003) in Primula species and Hoque and Mansfield (2004) in rice.

Table 4. *Effect of 2, 4-D concentrations on regeneration of somaclones/callus*

Treatment (concentration of 2,4-D)	**Variety**			**Mean**
	Th-10	**Th-2109**	**Th-326**	
CT-1 (1 mg/L)	38.40 eg	42.60 ef	51.20 be	**44.0 B**
CT-2 (2 mg/L)	71.00 a	44.60 df	59.40 ad	**58.30 A**
CT-3 (3 mg/L)	64.20 ab	61.20 ac	65.40 ab	**63.60 A**
CT-4 (4 mg/L)	35.80 fg	41.80 eg	45.60 df	**41.00 BC**
CT-5 (5 mg/L)	26.80 g	36.40 eg	47.40 cf	**36.80 C**
Mean	**47.20 B**	**45.30 B**	**53.80 A**	

ANOVA test

Source	**DF**	**SS**	**MS**	**F**	**P**
Replication	4	333.3	83.31		
Treatment	4	8018.1	2004.51	43.12	0.0000
Varieties	2	988.6	494.29	10.63	0.0001
Treatment × varieties	8	2541.5	317.69	6.83	0.0000
Error	56	2603.1	46.48		
Total	74	14484.6			
CV %	13.98				

Tukey's HSD Test

Parameters	**Standard error**	**Q value**	**Tukey's HSD (0.05)**
Treatment	2.4896	3.987	7.0183
Variety	1.9284	3.405	4.6436
Treatment × varieties	4.3121	5.012	15.281

Shoot length

The influence of treatment, variety, and interaction of the both was highly significant ($P \leq 0.05$) with regard to 2, 4-D treatment for shoot length (*Table 5*). Treatment CT-2 and CT-3 gave statistically at par shoot lengths of 9.04 and 8.81 cm, respectively. Regarding variety, maximum shoot length was observed in Th-326 with a mean shoot length of 8.58 cm, followed by Th-10 (7.98 cm) and Th-2109 (5.69 cm). The interaction of treatment and variety gave the longest shoot of 11.20 cm at CT-3 × Th-326. The results are in agreement with Misbah et al. (2017), who obtained the best shoot length at

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_49514960

1 and 2 mg/L concentration of 2, 4-D. Similar outcomes were reported by Gunderson et al. (2005) and Raza et al. (2010).

Table 5. *Effect of 2, 4-D concentrations on shoot length (cm)*

Treatment (concentration of 2,4-D)	Variety			Mean
	Th-10	Th-2109	Th-326	
CT-1 (1 mg/L)	7.60 df	5.90 g	6.02 g	**6.52 C**
CT-2 (2 mg/L)	10.30 ab	7.96 de	8.82 cd	**9.04 A**
CT-3 (3 mg/L)	8.10 de	7.20 efg	11.12 a	**8.81 A**
CT-4 (4 mg/L)	7.50 df	4.40 h	9.78 bc	**7.25 B**
CT-5 (5 mg/L)	6.40 fg	3.02 i	7.19 efg	**5.55 D**
Mean	**7.98 B**	**5.69 C**	**8.58 A**	

ANOVA test

Source	DF	SS	MS	F	P
Replication	4	7.826	1.9566		
Treatment	4	133.338	33.3346	97.16	0.0000
Varieties	2	117.488	58.7441	171.23	0.0000
Treatment × varieties	8	71.207	8.9009	25.	0.0000
Error	56	19.213	0.3431		
Total	74	349.072			
CV %	7.88				

Tukey's HSD test

Parameters	Standard error	Q value	Tukey's HSD (0.05)
Treatment	0.2139	3.987	0.6029
Variety	0.1657	3.405	0.3989
Treatment × varieties	0.3704	5.012	1.3128

Number of chlorophyll mutants

The chlorophyll mutant plants with low green pigment, known as albino and viridis plantlets, indicate the presence of chlorophyll changes in plantlets. A chlorophyll deficient phenotype can also result from passive transformation (Han and Ullrich, 1993). The effect of treatment, variety, and interaction of both was significant ($P \leq 0.05$) with regard to chlorophyll mutants (*Table 6*). The concentrations of CT-1, CT-2, and CT-3 were statistically at par, having mean 8.30, 9.60, and 8.00 chlorophyll mutants, respectively. Similarly, CT-4 and CT-5 concentrations were also statistically non-significantly different (*Table 7*). As for varieties, the highest number of chlorophyll mutants were observed in Th-2109 with the mean number of 8.00 chlorophyll mutants, followed by Th-326 (6.60 mutant plants) and Th-10 (6.10 mutant plants). The maximum number of 11.80 chlorophyll mutant plants was observed in the interaction of CT-1× Th-2109. Moreover, the interaction CT-1 × Th-2109 was statistically at par with CT-1 × Th-10, CT-2 × Th-10, CT-2 × Th-2109, CT-3 × Th-2109, CT-2 × Th-326, and CT-3 × Th-326. The lowest number of 3.00 chlorophyll mutants was observed in Th-10 clones at the interaction of CT-4 and CT-5 concentrations (*Table 6*). The results achieved by Misbah et al. (2017) are in accordance with our results. Likewise, our results were also in line with those reported by Nair et al. (2006).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2205_49514960

Table 6. *Effect of 2, 4-D concentrations on chlorophyll mutants*

Treatment (concentration of 2,4-D)	Variety			Mean
	Th-10	Th-2109	Th-326	
CT-1 (1 mg/L)	9.00 ad	11.80 a	4.20 ef	**8.30 A**
CT-2 (2 mg/L)	9.80 ab	10.60 a	8.40 ae	**9.60 A**
CT-3 (3 mg/L)	6.00 bf	8.80 ad	9.40 ac	**8.00 A**
CT-4 (4 mg/L)	3.00 f	5.40 cf	5.00 df	**4.40 B**
CT-5 (5 mg/L)	3.00 f	3.60 f	6.20 bf	**4.20 B**
Mean	**6.10 B**	**8.00 A**	**6.60 B**	

ANOVA test

Source	DF	SS	MS	F	P
Replication	4	9.120	2.2800		
Treatment	4	353.253	88.3133	23.23	0.0000
Varieties	2	47.707	23.8533	6.27	0.0035
Treatment × varieties	8	190.827	23.8533	6.27	0.0000
Error	56	212.880	3.8014		
Total	74	813.787			
CV %	28.07				

Tukey's HSD test

Parameters	Standard error	Q value	Tukey's HSD $_{(0.05)}$
Treatment	0.7119	3.987	2.0070
Variety	0.5515	3.405	1.3279
Treatment × varieties	1.2331	5.012	4.3698

Number of roots

The effect of different concentrations of IBA concerning the number of roots was significant for treatment and variety but was non-significant for the interaction (treatment × variety) as presented in *Table 7*. The IBA concentration of CI-2 and CI-3 yielded the highest mean 12.40 and 14.40 roots per jar, respectively; however, the difference between the two concentrations was statistically non-significant. In the case of varieties, Th-2109 and Th-326 gave statistically at par roots with the mean number of 10.5 and 10.10, respectively. The interaction of CI-3 × Th-326 produced the maximum number of 15.40 roots/jar, but statistically, it was non-significant over CI-2 × Th-10, CI-3 × Th-10, CI-2 × Th-2109, CI-3 × Th-2109, and CI-2 × Th-326 (*Table 7*). The results were supported by Misbah et al. (2017), Nair et al. (2006), and Khatri et al. (2002), who reported that IBA application improves root initiation of tissue culture plants.

Table 7. *Effect of IBA concentrations on number of roots*

Treatment (concentration of IBA)	Variety			Mean
	Th-10	Th-2109	Th-326	
CT-1 (1 mg/L)	5.80 e	7.40 de	6.00 e	**6.40 B**
CT-2 (2 mg/L)	11.20 ad	14.20 ab	11.80 ad	**12.40 A**
CT-3 (3 mg/L)	14.80 a	13.20 ac	15.40 a	**14.40 A**
CT-4 (4 mg/L)	5.80 e	9.40 be	9.00 ce	**8.00 B**
CT-5 (5 mg/L)	5.80 e	8.40 ce	8.60 ce	**7.60 B**
Mean	**8.60 B**	**10.50 A**	**10.10 AB**	

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DOI: http://dx.doi.org/10.15666/aeer/2205_49514960

ANOVA test					
Source	**DF**	**SS**	**MS**	**F**	**P**
Replication	4	14.99	3.747		
Treatment	4	719.12	179.780	34.31	0.0000
Varieties	2	47.55	23.773	4.54	0.0149
Treatment × varieties	8	61.52	7.690	1.47	0.1899
Error	56	293.41	5.240		
Total	74	1136.59			
CV %	23.39				

Tukey's HSD Test			
Parameters	**Standard error**	**Q value**	**Tukey's HSD $_{(0.05)}$**
Treatment	0.8358	3.987	2.3563
Variety	0.6474	3.405	1.5590
Treatment × varieties	1.4477	5.012	5.1302

Conclusion

The varieties Th-10, Th-2109, and Th-326 produced genetically variable somaclones. The 2, 4-D concentration of 2 mg/L in tissue culture media produced the maximum mean weight of callus (2.04 g), the number of somaclones (63.60/callus), and shoot length (9.04 cm). The application of 4 mg/L required a minimum of 15.90 days for callus induction. As for varieties, Th-2019 showed better performance with respect to callus induction and callus weight, while Th-326 was better in the number of somaclones/callus, shoot length, and produced less chlorophyll mutant plants.

Acknowledgement. The authors extend their appreciation to the Researchers Supporting Project (project number: RSP2024R293) King Saud University, Riyadh, Saud Arabia.

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