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Asian Journal of Plant Science and Research, 2014, 4(3): 14-21

ISSN : 2249-7412
CODEN (USA): AJPSKY

Efficient callus induction protocol for *Sorghum bicolor*

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ABSTRACT

Sorghum, a hardy cereal crop, is a basic staple food foe many rural communities. Its adaptability to more drought prone areas gains attention for cultivation of this crop for food security. Six genotypes of Sorghum were examined for efficient protocol design in tissue culture studies, Tissue cultures were initiated from immature inflorescence on a modified MS-medium containing combination of auxins and cytokinins. Studies were carried out on callus induction, regenerable callus formation, callus fresh, dry and total weights, volume for the development of an effective regeneration. Of all the six Sorghum genotypes IS 462, IS 2898, IS 3477, IS 3541, IS 5291, IS 27703 studied, genotype IS 3477 showed highest callus induction. Among all the genotypes the MS-medium containing 2, 4-D 1.5mg /L+ 0.5KN mg/L showed good results. The growth rates of total callus, callus fresh and dry weights were also highest for the genotype IS 3477. The total callus volume was highest (80mm) on 12th week for the genotype IS 3541.

Key words: *Sorghum*; Immature inflorescence; Callus induction; Explant.

INTRODUCTION

Sorghum is mainly cultivated in drier areas and can be regarded as a staple food for many rural communities all over the world. The nature of tolerance to more drought conditions made a better implication on this crop and rivet plant biotechnologists' interest. It is mainly grown on low potential, shallow soils with high clay content. It has the capacity to tolerate more alkaline salts and short periods of water logging. High frequency plant regeneration in *Sorghum* still requires standardized protocols using various explants because, *Sorghum* has been considered as one of the most difficult plant species to manipulate through tissue culture and transformation [4]. Advanced transgenic studies, genomic research, agronomy and breeding methodologies for *Sorghum* crop are mutually complementary along with tissue culture studies. The main objective focused on regeneration response and potential of immature inflorescence explant of *Sorghum* and effect of different concentrations of plant growth hormones on callus induction *invitro*. A reliable, efficient and reproducible tissue culture system is vital for the development of sorghum transformation. A number of researchers have concentrated on the development of successful regeneration methods. Most of them used immature embryos and immature inflorescences or shoot meristems as source material for efficient callus induction and subsequent plant regeneration. However, the rate of plant regeneration per explant is not satisfactory. This low level of regeneration response from callus cultures is associated with genotype specificity and gradual loss of regeneration ability. Further, Regeneration could be through either organogenesis or somatic embryogenesis. [16] reported that, tissue culture protocols are genotype specific and suitable protocols need to be developed when a new variety is to be used. They made a detailed study on callus induction and regeneration using different explants of *Sorghum*. The efficiency of callus induction depends on percentage of embryogenic callus, callus weight (fresh and dry), and callus volume. Later on it also plays a crucial role in regeneration of shoots during

sub-culturing. Therefore, standardization of callus induction protocol is a prerequisite for *Sorghum* tissue culture system.

MATERIALS AND METHODS

2.1 Genotypes and explants

Six *Sorghum* genotypes IS 462, IS 2898, IS 3477, IS 3541, IS 5291, IS 27703 were provided by International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. The explants studied for callus induction for the six genotypes is immature inflorescence.

2.2 Medium preparation

The media chosen for callus induction was prepared according to [8] with some modifications.

2.3 Surface sterilization of the explant

Immature inflorescences enclosed in the boot leaf were collected from field grown plants, swabbed in 70% ethanol after removing outer whorl of leaves. Small panicles (1-4cm) were aseptically cut open and were surface sterilized in 70% ethanol for a minute and 3% sodium hypochlorite for 10 minutes and then rinsed 10 times with sterile distilled water. This explant was cut into small pieces (0.5cm or less) and inoculated aseptically onto the callus induction medium, containing different concentrations and combinations of auxins and cytokinins. Cultures were maintained in the dark at 25 ± 2^0C and subcultured at an interval of 21 days. At the end of first subculture, those cultures containing smooth, shiny globular structures were defined as embryogenic callus and those having unorganized, creamy or yellow colored structures as non-embryogenic callus. For shoot regeneration, embryogenic calli were detached from the explants and cut into small pieces which were then transferred onto regeneration medium containing 0.5-3.0 mg/L of BAP/ZN/KN/TDZ.

2.4 Statistical Analysis

The experimental data were analyzed statistically by SPSS software. Data on the effects of variety of hormone treatments at various concentrations and combinations were tested by using ANOVA.

RESULTS AND DISCUSSION

3.1 Callus initiation

The explants started showing the callus development from the 3rd day after inoculation. Callus initiation occurred on the surface or cut ends of the explants 6-14 days after inoculation. A week after culturing, a dark brown and purple colour pigment began exuding from the cut ends of the explants. The pigment reached the medium, but it did not affect the callus growth and plant regeneration as observed visually. To control this phenolic secretion, the explants present in liquid MS medium were kept in a shaker at 200rpm for 24 hours. Then phenolic secretion was released into the liquid MS medium. Also activated charcoal (1%) or citric acid was used in culture medium to inhibit pigment formation. Frequent sub-culturing was also done to overcome this problem of pigmentation.

3.2 Callus derived from immature inflorescence

Immature inflorescence explants were cultured on MS medium supplemented with 2,4-D and kinetin (Kn) at different concentrations to study their response for callusing and further differentiation. Callus initiated by 12-14 days after inoculation from the inflorescence axis and spikelets. The primary callus was non-embryogenic, loose, white and yellowish but differentiated rapidly into a pale yellow, nodular and friable embryogenic callus within 2 weeks. Most of the callus turned embryogenic and only a very minor portion remained watery, yellow and soft, which was non-embryogenic, separated out during subculture. Embryogenic callus was separated from the primary culture and subcultured onto fresh medium at regular intervals for prolonged periods for further proliferation and development, which formed globular structures. These globular structures developed into somatic embryos on the surface of the callus when maintained on the same MS medium for 2-3 weeks. The frequency of embryogenesis increased with consecutive subcultures. The embryogenic calli on transfer to regeneration medium produced whitish embryoids, which later differentiated into plantlets on the same medium. The plantlets rooted 1-2 weeks later on MS basal medium and were then transferred to jiffy cups for hardening for a week. The plants were then transferred to glasshouse and were grown to maturity.

3.3 Effect of PGR concentration on callusing

Concentrations of 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0mg/L of 2, 4-D, IAA, IBA, BAP, NAA, KN and ZN were used to check the effect of them on callus formation. Callus initiation has appeared for all the varied concentrations of 2, 4-D, IBA, KN. But, there was no callus initiation at lower concentration of IAA, NAA and ZN. Callus induction frequency varied among the different concentrations of Plant growth regulators. The embryogenic callus frequency was maximum (84%) at 1.5 mg/L of 2, 4-D, followed by 2mg/L of IBA, IAA, and NAA each with 72% induction frequency (Table 1). Our observations also found that a higher concentration of auxins has hindered callus induction and growth. The callus induction response was also analyzed with a combination of auxin and cytokinin that ultimately gave a redundant outcome. Callus induction frequency was enhanced by 8-16% with a combination of 1.5mg/L 2, 4-D and 0.5mg/L of KN in all the genotypes. The genotype IS 3477 resulted maximum callus induction frequency (100%), followed by IS 2898 (80%), CS 3541 and IS 5291(each 72%), IS 462 (60%), and IS 27703 (52%) (Table 2).

3.4 Growth rate of total callus, embryogenic callus and dry callus weight

The callus growth rate of immature inflorescence was given in the (Table 3). The variety IS 3477 gave the highest response in terms of both total and embryogenic callus, callus fresh and dry weights (Figure 2, 3 and 4 respectively), followed by CS 3541, IS 2898, IS 5291, and IS 462. The least response was observed for the genotype IS 2773. These calli dry weights were tested by analysis of variance and genotypic differences were found to be significant ($p < 0.05$) (Table 4, 5, 6).

3.5 Total callus volume and embryogenic callus volume

The total volume of the callus was highest in the CS 3541, followed by IS 3477, IS 462, IS 2898, IS 27703 and lowest in IS 5291. The average quantity of total callus volume per explant ranged from a minimum of 26mm to a maximum 80mm. The results of these initial studies indicated that the E and NE could be distinguished by the end of three weeks after their culture inoculation. The average total callus volume in different genotypes at 3, 6, 9, and 12 week intervals is summarized in the (Figure 5). The embryogenic callus volume growth rate was highest in the variety IS 3477 followed by IS 2898, IS 462, IS 5291, IS 27703, CS 3541. The average quantity of embryogenic callus volume per explant ranged from a minimum of 24mm to a maximum 38mm. The average volume of E callus volume in different genotypes at 3, 6, 9, and 12 week intervals is summarized.

Broad range of varietal differences in callus formation and plant regeneration was observed by [5]. He reported that, sustained varietal differences displayed in callus formation and plant regeneration on *Sorghum* as well as in other major cereals. Similarly, a study on genotypic differences in callus induction in Barley by using mature embryo explant was done by [16]. [14] reported that, tissue culture protocols are genotype specific and suitable protocols need to be developed when a new variety is to be used. They made a detailed study on callus induction and regeneration using different explants of Sorghum. They reported multiple shoot induction and regeneration using 1-6 mg/L concentration of BAP. The effect of developmental stages on embryogenic callus induction was reported by [10]. They found that, the inflorescence of 4-5 cm in length with pinhead size spikelet primordial were the optimal stage for the production of efficient embryogenic callus production. *In vitro* response of different explants viz., shoot tip; immature inflorescence and immature embryo were studied by [9]. They found that shoot tip and immature inflorescence were more totipotent than the immature embryo. Moreover, immature embryos and immature inflorescences are considered to be excellent explants for establishing embryogenic cultures of several genotypes of sorghum. Researchers like [2], [7], [1], [3], [15], [12] and [6] worked on developing a standardized protocol design by using immature inflorescence as an explant.

This report describes parameters for induction of E. callus maintaining and subsequent regeneration of complete plants. [17] optimized callus induction and developed an efficient regeneration system for sweet sorghum genotypes by using germinating seeds. A rapid regeneration system from shoot tip explant of *Sorghum* was done by [13]. The effect of plant growth hormones were studied widely by various researchers. Many reports revealed that the combination of auxin and cytokinin in proper proportion showed synergistic effect on callus induction as well as its size. [11] *reported* that, the addition of casein hydrolysate, mixture of amino acids and vitamins to the callus induction medium promoted the production of embryogenic callus in Sorghum. He also reported that, the most important factors for embryogenic callus induction and regeneration were the physiological state of the immature embryo.

Table 1. Effect of Plant growth hormones on callus induction

Concentration of PGR							No. Of Explants Responded (25)	
2,4-D	IBA	IAA	NAA	BAP	KN	ZN	With E.C	E.C. Frequency
1.0							12	48
1.5							21	84
2.0							16	64
2.5							15	60
3.0							16	64
	1.0						12	48
	1.5						14	56
	2.0						18	72
	2.5						12	48
	3.0						14	56
		1.0					-	-
		1.5					-	-
		2.0					10	72
		2.5					14	56
		3.0					13	52
			1.0				14	56
			1.5				-	-
			2.0				18	72
			2.5				14	56
			3.0				16	64
				1.0			12	48
				1.5			12	48
				2.0			14	56
				2.5			15	60
				3.0			12	48
					0.2		8	32
					0.5		13	52
					1.0		10	40
					1.5		10	40
					2.0		12	48
						0.2	0	0
						0.5	0	0
						1.0	14	52
						1.5	10	40
						2.0	12	48

Table 2. Callus induction from immature inflorescence in combination with 2,4-D and KN

Variety	PGR concentration mg/L	Total explants inoculated	Explants with E. Calli	E. Calli Frequency
IS 462	2,4-D 1.5mg/L	25	13	52
	0.5KN mg/L	25	12	48
	2,4-D 1.5mg /L+ 0.5KN mg/L	25	15	60
IS 2898	2,4-D 1.5mg/L	25	18	72
	0.5KN mg/L	25	11	44
	2,4-D 1.5mg /L+ 0.5KN mg/L	25	20	80
IS 3477	2,4-D 1.5mg/L	25	21	84
	0.5KN mg/L	25	13	56
	2,4-D 1.5mg /L+ 0.5KN mg/L	25	25	100
CS 3541	2,4-D 1.5mg/L	25	15	60
	0.5KN mg/L	25	12	48
	2,4-D 1.5mg /L+ 0.5KN mg/L	25	18	72
IS 5291	2,4-D 1.5mg/L	25	16	64
	0.5KN mg/L	25	10	40
	2,4-D 1.5mg /L+ 0.5KN mg/L	25	18	72
IS 27703	2,4-D 1.5mg/L	25	11	44
	0.5KN mg/L	25	7	28
	2,4-D 1.5mg /L+0.5 KN mg/L	25	13	52

Table 3. Growth rates of total callus weight, embryogenic callus weight and dry weight of immature inflorescence (Mean ± SE)

Source of variation	N	Total Callus weight Mean ± SE	Embryogenic Callus weight Mean ± SE	Dry weight Mean ± SE
3rd week	300	238.23 ± 3.86	212.13 ± 3.20	169.37 ± 3.08
6th week	300	356.53 ± 3.92	313.13 ± 4.83	246.47 ± 3.95
9th week	300	481.43 ± 6.57	442.50 ± 6.19	369.83 ± 6.52
12th week	300	593.43 ± 7.89	548.73 ± 7.96	469.30 ± 8.01
Total	1200	417.41 ± 4.95	379.13 ± 4.69	313.74 ± 4.38

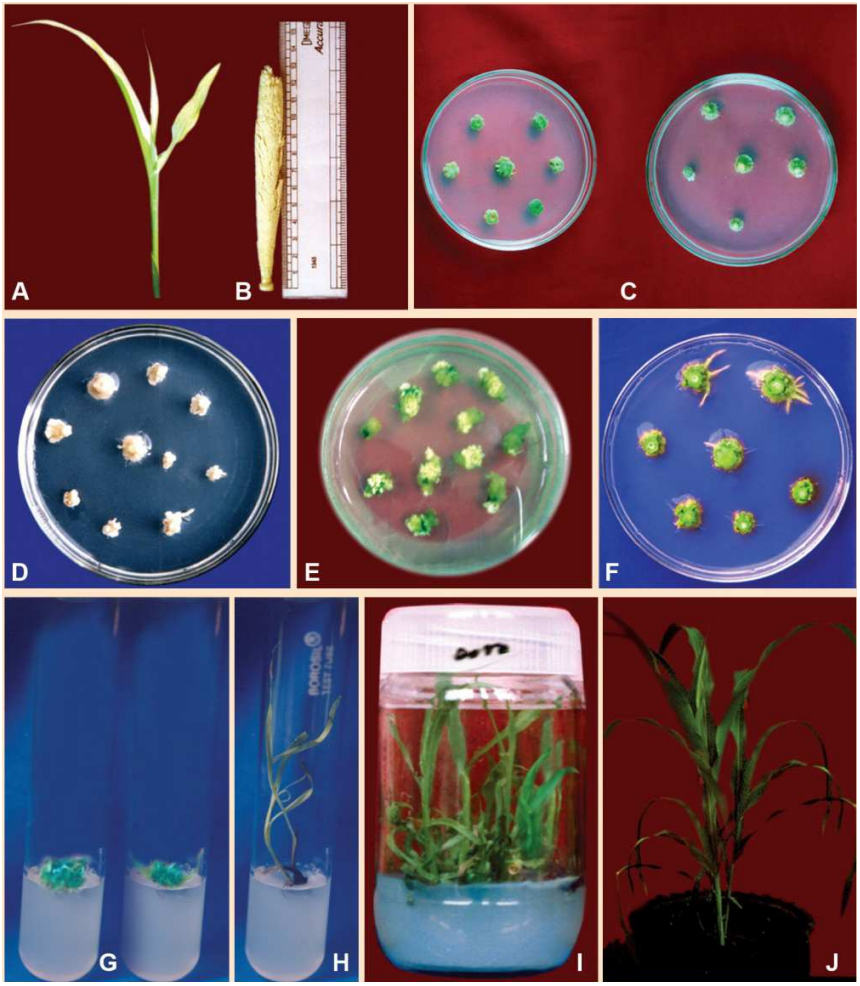

Figure 1. Callus induction and regeneration from immature inflorescence explant

a, b. Immature inflorescence explant
c. Inoculation of the explant
d. Callus induction
e, f. Regeneration
g, h. Shoot initiation
i. Plantlet

Table 4. Analysis of variance test for total callus weight (ANOVA)

Source of variation	Sum of Squares	Degree of freedom	Mean square	F Value
Between Groups	21268034	3	7089344.75	607.96*
Within groups (Error)	13946306	1196	11660.79	
Total	35214340	1199		

**Significant at p < 0.05*

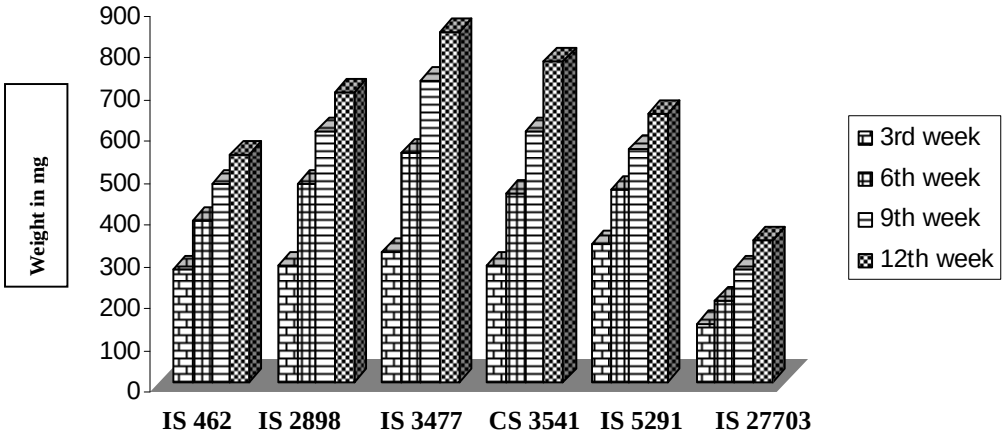

Figure 2. Growth rate of total callus weight in different genotypes

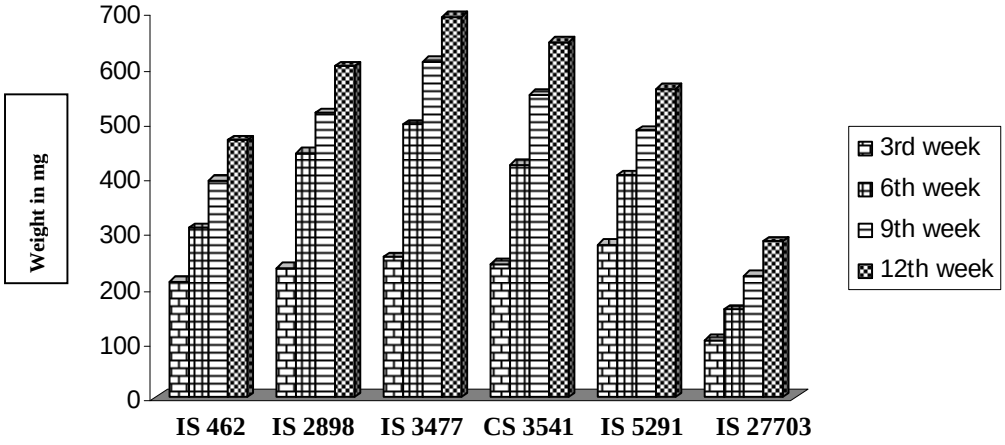

Figure 3. Growth rate of embryogenic callus weight in different genotypes

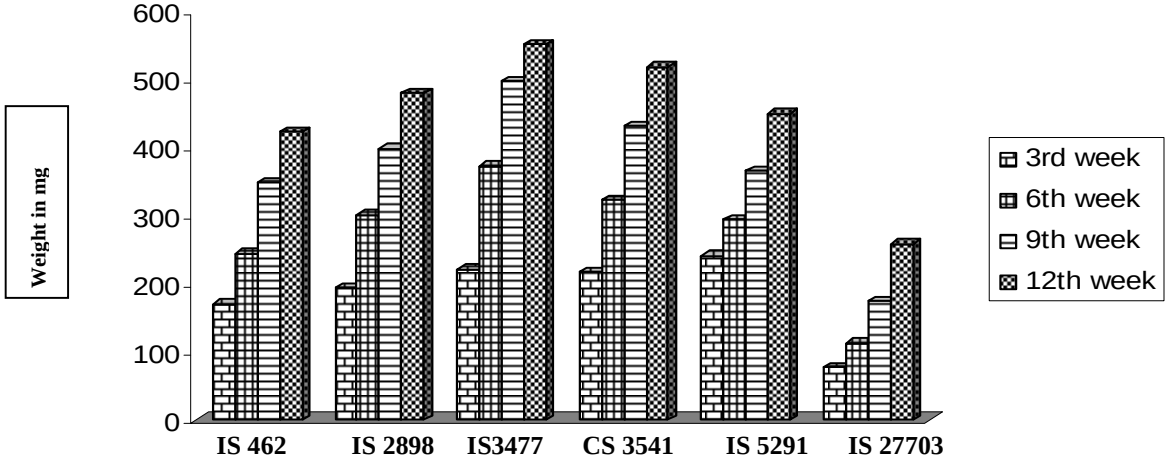

Figure 4. Callus dry weights in different genotypes

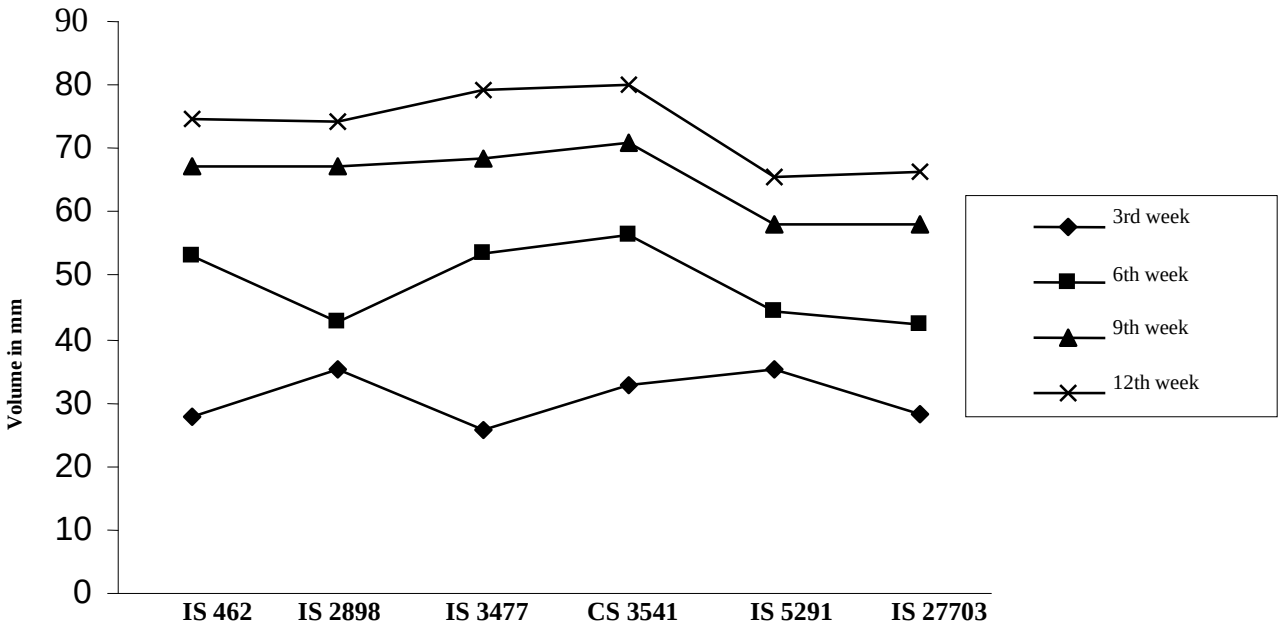

Table 5. Analysis of variance test for embryogenic callus weight (ANOVA)

Source of variation	Sum of Squares	Degree of freedom	Mean square	F Value
Between Groups	19507348	3	6502449.42	640.98*
Within groups (Error)	12132833	1196	10144.51	
Total	31640181	1199		

**Significant at p < 0.05*

Table 6. Analysis of variance test for dry weight (ANOVA)

Source of variation	Sum of Squares	Degree of freedom	Mean square	F Value
Between Groups	15814421	3	5271473.64	533.61*
Within groups (Error)	11815079	1196	9878.83	
Total	27629500	1199		

**Significant at p < 0.05*

Figure 5. Callus volume of six genotypes

Acknowledgements

Authors acknowledge University Grands Commission (UGC), New Delhi for the financial support provided to carry out our work.

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