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In Vitro Callus Induction Potentials of Wheat Genotypes using Mature Embryo as Ex-Plant Source under different Levels of Polyethylene Glycol (PEG)

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ABSTRACT: This work was carried out at Jigawa Research Institute Biotechnology Laboratory Kazaure to determine callus induction potentials of 16 wheat genotypes using matured embryo as ex-plant source under different (PEG) levels. The experiment had six treatments (0%, 5%, 10%, 15%, 20% and 25%) including control with each replicated three times and the necrotic percentage taken showed varieties 8(53.3%), 7(60%) and 28(61.1%) had minimal necrotic callus, while ANOVA shows that there is significance difference between the treatments percentage values ($P<0.05$), while there was positive correlation among the genotypes. Also the total number of callus recorded for each variety across treatment showed an encouraging callus production in varieties 8(38), 7(34), 2(32) and 6(31), however (ANOVA) shows significant difference between the treatment mean values ($p< 0.05$) while Pearson correlation coefficient among the genotypes indicated that there was positive correlation.
Keywords: Callogenesis, drought stress, embryo culture, tissue culture, *Triticum aestivum* L.

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is an important crop worldwide and is grown on about 200 million ha in a range of environments, with an annual production of more than 600 million metric tons (Singh *et al.,* 2007). Global wheat production must continue to increase 2 % annually until 2020 to meet future demands of imposed population and prosperity growth (Abdel-Ghany *et al.,* 2004). Approximately 32 % of the wheat-growing regions in developing countries experience some type of drought stress during the growing season (Rajaram, 2001). The frequency and severity of soil water deficit is generally greater for rainfed wheat crops. However, changing weather patterns and worldwide water shortages will likely result in irrigated wheat being grown with the loss of applied water, increasing the likelihood of a soil water deficit (Rebetzke *et al.,* 2006).

Global warming and concomitant increase in drought effected areas limit plant production is also restricted by drought exposed areas and this loss lead to considerable economic and social problems because of its great importance on human nutrition (Ilker *et al.,* 2011). One possible way to ensure future food needs of an increasing world population involves the better use of water through the development of crop varieties which need less water and are more tolerant to drought (El-Shafey *et al.,* 2009; Mafakheri *et al.,* 2010). Development of cultivars with high yield is the main goal in water limited environments but success has been modest due to the varying nature of drought and the complexity of genetic control of plant responses (Mirbahar *et al.,* 2009). Since yield is a complex trait and is strongly influenced by the environment, severe losses can be caused by drought, a stress common in most arid and semi-arid areas. Accordingly, drought tolerance is one of the main components of yield stability and its improvement is a major challenge to geneticists and breeders (Eid, 2009). Breeding for drought tolerance by selecting solely for grain yield is

difficult because the heritability of yield under drought conditions is low, due to small genotypic variance or due to the large variances in the genotype-environment interaction (Koszegi *et al.,* 1996). Recent progress in the genetic manipulation of plant cells through tissue culture has opened new possibilities in crop improvement in which crop species can be improved without interfering with a large portion of the genes and without introducing alien or exogenous genetic material. *In vitro* culture of plant cells and tissues such as mature embryos and immature embryos has attracted considerable interest over recent years because it provides a means to study plant physiological and genetic processes and offers a potential to improve cultivars by increasing genetic variability and are considered to be an important complement to classical plant breeding methods (Sorkheh *et al.,* 2011). *In vitro* selection technique has been used to improve abiotic environmental stresses such as cold hardiness, salt tolerance and drought tolerance (Zair *et al.,* 2003; Bajji et al., 2004; Gawande et al., 2005).

One of the screening techniques based on physiological traits is the use of various osmotica to induce stress in plant tissues. Germination in mannitol and polyethylene glycol (PEG) have been suggested for drought screening (Shabir, 2010; Geravandi *et al.,* 2011). General plant growth inhibition in response to mannitol has led numerous researchers to develop screening techniques using mannitol as an osmotic agent (Hsissou and Bouharmont, 1994). Because of its high molecular weight, PEG cannot cross membranes and cannot get into the cell to change its osmotic potential (Dragiiska *et al.,* 1996). It stimulates water deficit conditions in cultured cells in a manner similar to that observed in the cells of intact plants subjected to true drought conditions (Gawande *et al.,* 2005; Yao *et al.,* 2007). Osmotic solutions of NaCl, mannitol/sorbitol, and PEG have been used as *in vitro* stress factors for selecting salt- and droughttolerant genotypes in screening procedures for seed germination of durum wheat (Almansouri *et al.,* 2001), sunflower (*Helianthus annuus* L.) (Punia and Jain, 2002) and potato (Gopal and Iwama, 2007). In wheat genotypes, drought-tolerant genotypes were found to have better developed root systems under in vitro water stress mediated through PEG (Ye *et al.,* 2002). The objectives of the present investigations were: (i) screening bread wheat genotypes for drought tolerance under *in vitro* condition; (ii) evaluation of the ability of genotypes to induce callus using mature embryo culture; and (iii) screening *in vitro* indicators of drought tolerance.

METHODOLOGY

Place of Research

The Research was conducted at the Biotechnology Laboratory, Jigawa Research Institute, Kazaure, Jigawa State.

Seed source

Thirty wheat accessions (genotypes) of germplasm was obtained from Lake Chad Research Institute (a National Agricultural based Research Institute), Maiduguri, Nigeria.

Table: 1 Showing 16 Wheat Genotypes used for the Research

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S/N	Genotype NO	Name /Wheat Pedigree
1	2	INQALAB91*2/TUKURU//WHEAR
2	3	SERI.1B*2/3/KAUZ*2/BOW//KAUZ/4/KAUZ/FLORKWA-1
3	4	SAUA-/3/C80.1/3*BATA VIA//2*WBLLI/4/SAUAL#*1
4	5	ZAKIA-5
5	6	WBLLI/4/BOW/NKT/CBRD/3/CBRD/5/WBLLI*2……..
6	7	HUBARA-2/QAFZAH-21//DONIN-2
7	8	ATTILA 50Y//ATTILA/BCN/3/STAR*3/MUSK-3
8	9	ATTILA*/PBW 65*2/4/BOW/NTK/CBRD/3/CBRD
9	21	ATTILA GAN ATTILA
10	22	WBLLI/4/BOW/NKT/CBRD /3/CBRD/5/WBLLI*2/…
11	23	HOOSAM-8//CHAM-6/FLORKWA-2
12	26	KAUZ'S'/FLORKWA-1//GOUMAARIA-3
13	27	FRET2/TUKURU/FRET2/3/MONIA/CHTO/AMEL/4/…
14	28	WEAVER/WL3928//SW89.3064/3/KAUZ//MON/CROW'S'
15	29	ATTILA 50Y//ATILLA/BCN/3/STAR*/MUSK-3 (AISBW05-0043-9AP-OAP-OAP-2AP-OAP)
16	30	ATTILA 50//ATTILA/BCN/3/STAR'S'/KAUZ'S' (AISBW05-0006-2AP-OAP-OAP-2AP-OSD)

Seed Sterilization Procedure

Matured explants from a totipotent plants (mature seeds) of 30 wheat accessions (genotypes) (*Triticumaestivum*L) were surface sterilized with liquid detergent and two drops of tween 20 using tap water severally then rinsed with distilled water 5 times, thereafter the seeds were transferred to sterile bottles containing 20% benlete solution made from stock of hexaconozole 5% marketed control systematic fungicide for 30 minutes, and were decanted and washed with distilled water 5 times, subsequently the seeds were dipped in 70% ethanol for between 3- 5 minutes after which were decanted and wash with distilled water 5 times, the seeds were later dipped in 20% chlorox (commercially called bleech) which contained 3% sodium hypochlorite and finally rinsed with double distilled water (ddw). The sterile seeds were further soaked in double distilled water overnight. Matured embryos were aseptically excised from caryopsis using surgical knife and forcepts (Gupta *et al.,* 2014)

Embryo Excision

The sterile seeds were removed from bottles using sterile forceps and placed on a sterile laboratory mat, with the aid of sterile surgical knives the embryo were quizzed out under laminar air flow Rahman (*et al.,* 2008).

Inoculation of Embryo

The excised embryos were picked using sterile flamed forceps and inoculated the sterile media in 500ml sized bottles with each bottle containing five embryos of each genotype and three bottles per treatment (Rahman *et al.,* 2008).

Callus Production

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The inoculated sterile media contained in a 500ml bottles with 50ml media were removed from laminar air flow hood and kept in the dark room for three weeks at a temperature of 25±1°C to callus. The callus induction medium (CIM) consisted of mineral salts of Murashige and Skoog (MS) (Murashige and Skoog, 1962), 30g/L sucrose, (0, 1, 2, 3,4 and 5mg/L 2,4-D with each as treatment). Percentage of callus induction frequency at different levels of 2, 4-D were recorded based on the fifteen replicates (contained in three bottles with 5 embryo each) in each treatment of the genotype (Guptaet *al.,* 2014)

Proliferation of callus

The responsive varieties from callogenesis above were sub-cultured twice at two week intervals on fresh MS medium for their proliferation.

In vitro drought tolerance selection:

50mg clumps of the above mentioned proliferated calli (about 7weeks old) of wheat genotypes were transferred to drought selection medium under sterile condition, which contained MS salts, the promising 2,4-D (5mg/L 2, 4-D) in mg/L from callogenesis above, (similar to CIM) and polyethylene glycol (PEG) 6,000 of six levels of concentrations (0%,5%, 10%, 15%, 20% and 25%) were used including a control (0%) as treatment for osmoticum. The calli were incubated at 25±1°C in continuous darkness for a stress period of 4 weeks. However to avoid deficiency of mineral components in selection media during stress period, the stressed calli were shifted to fresh selection media after 15 days interval. Fifteen replicates (in three bottles of 5 embryo each) each of the sixteen ie (genotype numbers 2,3,4,5,6,7,8,9,21,22,23,26,27,28,29, and 30) genotypes that were selected from callus induction with 2, 4-D above (in three 500ml bottles) were made for each of the five PEG treatments [Gupta *et al.,* 2014].

The following treatments were studied during the course of this research work:

- P_0 polyethylene glycol 0% (PEG 6000) Control
- P_1 Polyethylene glycol 5% (`PEG 6000)
- P_2 polyethylene glycol 10% (PEG 6000)
- P_3 polyethylene glycol 15% (PEG 6000)
- P_4 polyethylene glycol 20% (PEG 6000)
- P_5 polyethylene glycol 25% (PEG 6000)

The callus induction were calculated by taken the numbers of surviving callus per treatment for all the treatments and adding them up to give the total sum of callus frequency of necrotic calli were calculated on percentage of dead callus per treatment for each variety were taken across treatments and the mean calculated. [Gupta *et al.,* 2014].

Statistics

Simple percentages, One-way Analysis of variance (ANOVA) and Pearson correlation coefficient were used. In all statistical analyses, confidence level was held at 95% and $P < 0.05$ (at 5% level of significance) was considered as significant.

RESULT AND DISCUSSION

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Table 2A shows that varieties 2(plates 3 and 4), 7 and 8 produce more total number of callus for the six polyethylene glycol levels including control i.e.0%, 5%, 10%, 15%, 20% and 25% PEG levels, though there were decrease in callus sizes across the treatments . Analysis of Variance (ANOVA) shows there were significant difference between the treatment mean values ($p < 0.05$) (Table 2B) while Pearson correlation coefficient among the genotypes indicated that there was positive correlation (Table 2C). Similar results were found by Zouzou *et al.* (2008) who reported that, response in tissue culture such as callus initiation is highly genotype dependent. Sakthivelu *et al.* (2008) reported that a meaningful decrease in the relative growth rate of callus cultures were observed for soybean cultivars, with increasing PEG concentrations to the MS medium. Maryam *et al.,* 2015 revealed that culture response was greatly influenced by the wheat genotypes and also emphasized a marked effect of genotypes on callus induction capacity. Table 2A, presented data on necrotic calli produced with respect to drought stress. Drought stress increased necrotic percentage in calli of wheat genotypes. As the level of polyethylene glycol (PEG) increased, necrotic percentage also increased (Table 3A). While it was lower at genotypes 8 (53.3%) and 7(60,0%) (Table 3A). The minimum value of (27.9%) was observed where no PEG was applied (Table 3A). Interaction between PEG and varieties indicated that all the genotypes produced necrotic masses at higher PEG levels, while it was lower in the case of control (0%) (Table 3A). ANOVA shows that there is significance difference between the treatments percentage values ($p<0.05$) (Table 3B), while there was positive correlation among the genotypes (Table 3C). Imran et al., 2012 who's work corroborate the present result state that Calli cultured on stress free medium (without PEG 6000) were found to be healthier with unrestricted growth (0-20% necrosis) than those on the stressed media (80-100% necrosis). Calli subjected to drought stress (-1.2 MPa) for four weeks exhibited very poor health. Callus culture for two weeks on media containing 5% PEG 6000 did not affect callus health and it looked light yellow to whitish in color with no browning. Whole callus tissues were turned brown when calli were cultured on the same media with 20% PEG 6000 for two, three or four weeks. Callus browning rate was found to be good indicator of callus sensitivity to PEG 6000 induced osmotic stress. Necrosis was more on the surface of the callus facing medium. These observations are in line with those of Hassan *et al.* (2004). They reported that *in vitro* osmotic stress of -1.0 MPa for eight months of culture onto PEG 6000 containing media is lethal for sunflower calli with evident necrotic tissues on callus surface.

Table 2A: Effect of varying levels of drought Stress (Polyethylene glycol (PEG) on callus of 16 Wheat Genotype (number of Callus)

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Genotype	0%	5%	10%	15%	20%	25%	Total
2	14	8	4	2	2	2	32
3	9	4	2	1	1	0	14
4	8	1	1	1	0	0	11
5	9	1	1	1	0	0	12
6	12	7	5	3	2	2	31
7	14	8	5	4	2	1	34
8	15	10	6	3	2	2	38
9	11	5	4	2	1	1	24
21	12	4	3	2	1	1	23
22	10	3	2	1	1	1	18
23	9	7	5	2	1	1	25
26	8	2	1	1	0	0	12
27	10	2	3	4	1	1	21
28	12	6	4	2	1	1	26
29	10	2	1	1	0	0	14
30	10	3	2	1	0	0	14
Total	173	72	47	29	15	13	

F= 24.97 d.f.= 15 (p< 0.05)

Table 2B: ANOVA of Varying levels of drought Stress (Polyethylene glycol (PEG) on callus of 16 Wheat Genotypes

Model		Sum of Squares	Df	Mean Square	F	Sig.
1	Regression	184.937	5	36.987	24.970	.000[b]
	Residual	14.813	10	1.481		
	Total	199.750	15			

a. Dependent Variable: PEG (0%)

b. Predictors: (Constant), PEG 5%, PEG10%, PEG 15%, PEG 20%, PEG 25%

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Table 2C: Correlation matrix of Varying levels of drought Stress (Polyethylene glycol (PEG) on callus of 16 Wheat Genotypes

		PEG 0%	PEG 5%	PEG 10%	PEG 15%
PEG 0%	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	16			
PEG 5%	Pearson Correlation	.932**	1		
	Sig. (2-tailed)	.000			
	N	16	16		
PEG 10%	Pearson Correlation	.833**	.934**	1	
	Sig. (2-tailed)	.000	.000		
	N	16	16	16	
PEG 15%	Pearson Correlation	.496	.572*	.744**	1
	Sig. (2-tailed)	.050	.021	.001	
	N	16	16	16	16
PEG 20%	Pearson Correlation	.873**	.880**	.871**	.726**
	Sig. (2-tailed)	.000	.000	.000	.001
	N	16	16	16	16
PEG 25%	Pearson Correlation	.843**	.814**	.850**	.654**
	Sig. (2-tailed)	.000	.000	.000	.006
	N	16	16	16	16

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

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Table 3A: Necrotic Percentage of Callus of Varying Drought Stress (PEG) on16 Wheat Genotypes

Genotype	PEG 0%	5%	10%	15%	20%	25%	Mean
2	6.7	46.7	73.3	93.3	86.7	93.3	66.7
3	40.0	80.0	93.3	86.7	93.3	93.3	81.1
4	46.7	93.3	93.3	93.3	100	100	87.8
5	40.0	93.3	93.3	93.3	100	100	86.7
6	20.0	73.3	80.0	80.0	86.7	93.3	72.2
7	6.7	53.3	66.7	66.7	80.0	86.7	60.0
8	00.0	33.3	60.0	60.0	80.0	86.7	53.3
9	26.7	53.3	66.7	73.3	86.7	93.3	66.7
21	20.0	86.7	80.0	100	73.3	93.3	75.6
22	33.3	73.3	86.7	86.7	93.3	100	78.9
23	40.0	66.7	73.3	73.3	86.7	93.3	72.2
26	46.7	86.7	93.3	93.3	100	100	86.7
27	33.3	60.0	73.3	86.7	93.3	93.3	73.3
28	20.0	46.7	66.7	73.3	73.3	86.7	61.1
29	33.3	93.3	93.3	93.3	100	86.7	83.3
30	33.3	80.0	86.7	86.7	93.3	100	80.0
Mean	27.9	69.9	79.9	83.7	89.2	93.7	

F= 7.224 d.f.= 15 (P<0.05)

Table 3B: ANOVA of Necrotic Percentage of Callus of Varying Drought Stress (PEG) on 16 Wheat Genotypes

Model		Sum of Squares	Df	Mean Square	F	Sig.
1	Regression	6609.035	5	1321.807	7.224	.004[b]
	Residual	1829.645	10	182.965		
	Total	8438.680	15			

a. Dependent Variable: PEG (0%)

b. Predictors: (Constant), PEG 5%, PEG10%, PEG 15%, PEG 20%, PEG 25%

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Table 3C : Correlation matrix of Necrotic Percentage of Callus of Varing Drought Stress (PEG) on 16 Wheat Genotypes

		PEG0%	PEG5%	PEG10%	PEG15%	PEG 20	PEG25%
PEG 0%	Pearson Correlation	1					
	Sig. (2-tailed)						
	N	16					
PEG 5%	Pearson Correlation	.867**	1				
	Sig. (2-tailed)	.000					
	N	16	16				
PEG 10%	Pearson Correlation	.760**	.927**	1			
	Sig. (2-tailed)	.001	.000				
	N	16	16	16			
PEG 15%	Pearson Correlation	.418	.631**	.759**	1		
	Sig. (2-tailed)	.107	.009	.001			
	N	16	16	16	16		
PEG 20%	Pearson Correlation	.477	.586*	.642**	.576*	1	
	Sig. (2-tailed)	.062	.017	.007	.019		
	N	16	16	16	16	16	
PEG25%	Pearson Correlation	.657**	.796**	.790**	.508*	.610*	1
	Sig. (2-tailed)	.006	.000	.000	.044	.012	
	N	16	16	16	16	16	16

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed)

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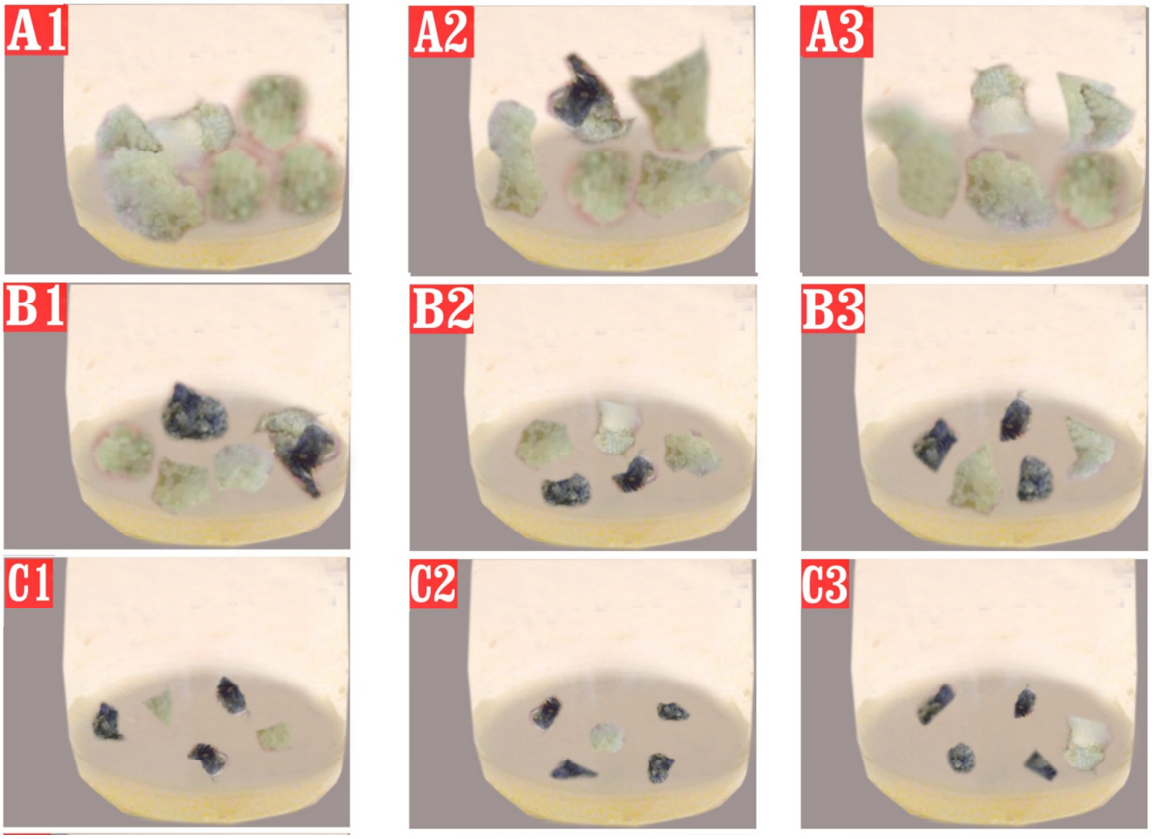

Plate 3: Showing callus of genotype 2 as effect by varying concentrations of polyethylene glycol (PEG) and 5mg/l 2,4-Dichlorophynoxy acetic acid (2, 4-D).

a. Control 0% PEG = Number of survived callus A1: 5, A2: 4 and A3:5
   Number of dead callus A1: 0, A2: 1 and A3: 0
b. 5% PEG = Number of survived callus B1: 3, B2: 3 and B3:2
   Number of dead callus B1: 2, B2: 2 and B3: 3
c. 10% PEG = Number of survived callus C1: 2, C2: 1 and C3:1
   Number of dead callus C1: 3, C2: 4 and C3: 4

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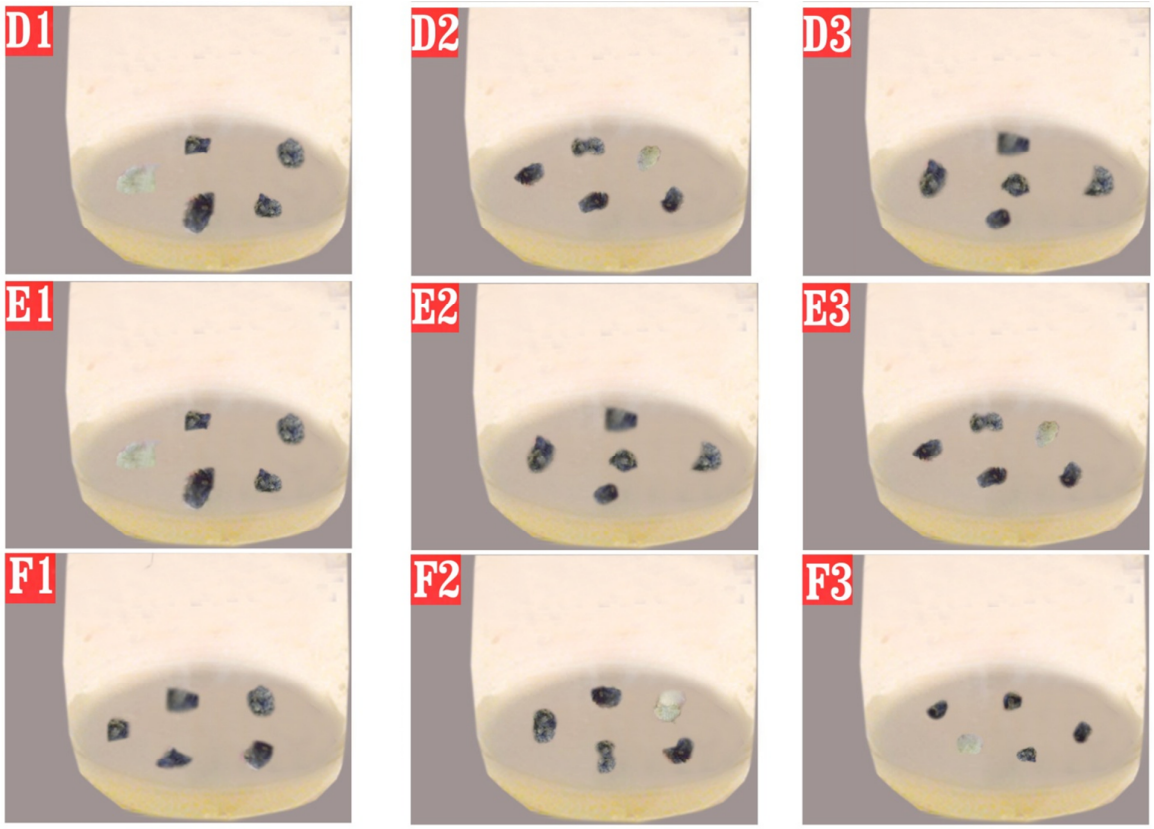

Plate 4: Showing callus of genotype 2 as effect by varying concentrations of polyethylene glycol (PEG) and 5mg/l 2,4-Dichlorophynoxy acetic acid (2, 4-D).

D. 15% PEG = Number of survived callus D1: 1, D2: 1 and D3:0
Number of dead callus D1: 4, D2: 4 and D3: 5

E. 20% PEG = Number of survived callus E1: 1, E2: 0 and E3:1
Number of dead callus E1: 4, E2: 5 and E3: 4

F. 25% PEG = Number of survived callus F1: 0, F2: 1 and C3:1
Number of dead callus F1: 5, F2: 4 and C3: 4

CONCLUSION

After subjecting the 16 genotypes of promising callus to varying concentrations of polyethylene glycol (PEG). The callus was observed to decrease in sizes with increase in levels of PEG, also necrotic percentage increased with increase in the levels of PEG, while lower percentage mean of necrosis were observed in genotypes 8(53.3%), 7(60%) and 28(61.1%) (Table 2) while maximum total numbers of callus were noticed in genotypes 8(38), 7(34), 2(32) and 6(31) (Table 1).

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