The Pharmaceutical and Chemical Journal, 2019, 6(3):69-76

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

In vitro Callus Induction Potentials of Wheat Genotypes using Mature Embryo as ex-plant source under different Levels of 2,4-Dichlorophenoxyacetic Acid (2,4-D)

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Abstract This work was carried out at Jigawa Research Institute Biotechnology Laboratory Kazaure to determine callus induction potentials of 30 wheat genotypes using matured embryo as ex-plant source under different 2,4-Dichlorophenoxyacetic Acid (2,4-D) levels. The experiment had six treatments (control, $1mgl^1$ 2,4-D, $2mgl^1$ 2,4-D, $3mgl^1$ 2,4-D, $4mgl^1$ 2,4-D, and $5mgl^1$ 2,4-D,) including control with each replicated three times and the percentage taken with callus induction frequency which ranged from 32% to 80%. Meanwhile maximum callus among the genotypes were noticed in varieties 8 followed by varieties 27, 28 and 7 with respective callus induction percentages of 79.99%, 79.99% and 77.33%, with minimum callus frequency of 32% was recorded by varieties 19, while 13 had callus induction potential of 36%. The best callus production was recorded at the treatment levels of $5mgl^{-1}$ (78.878%), callus recorded at $5mgl^{-1}$ were more granular and morphogenic in nature. However higher mean values for days to callus were at varieties 5 (6.60), 4 (6.40), 21 (6.40) and 26 (6.40), with the least mean values for days to callus at varieties 8 (5.40) and 7 (5.60), while the least mean values for days to callus was recorded at $5mgl^{-1}$ (4.69) and levels of 2, 4-D, while the higher mean values for days to callus was scored at $2mgl^{-1}$ (7.06) levels of 2, 4-D. The weight ranged from 62.24mg mean value to 85.14mg mean values. Genotype 8 had the maximum mean weight across treatments (85.14mg) followed by genotypes 7 (82.5mg), 28 (81.56mg) and 2 (81.46mg) respectively while the minimum weight values were noticed in genotypes 3 (62.24mg), 26 (64.48mg) and (64.9mg). However the maximum weight of callus according to treatments was observed at the level of $5mgl^{-1}$ of 2, 4-D with mean value 105.7mg f. while the minimum mean weight values were recorded at $1mgl^{-1}$ (31.1mg) 2, 4-D.

Keywords Wheat; Callus; Mature seeds; 2,4-dichlorophenoxyacetic acid

1. Introduction

The yield and quality of wheat have been gradually improved during the past several decades by traditional breeding methods. However, these methods have some limitations such as long time required and rather limited gene pool available for wheat breeders. For this purpose, a group of activities was focused on *in vitro* culture and regeneration as a tool of cereal breeding in the recent years. Further, it is also well documented that the genetic engineering of cereals currently depends on the use of tissue culture and plant regeneration [1]. In wheat, immature embryos have been the most widely used explants to initiate callus cultures [2-5] but they are inconvenient because of their temporal availability [1, 7]. Mature seeds are readily available throughout the year, hence can be used effectively to

initiate callus cultures in wheat. Ozgen *et al*. [8] reported high frequency of callus induction through mature embryos, however, they found it low while working with seven durum wheat genotypes in one of their previous studies, where mature embryos were used as explant source [8]. The callus is a rapidly proliferating undifferentiated mass of cells, which can be obtained by culturing explants on nutrient media containing specific growth hormones [9]. 2,4-D (2,4-dichlorophenoxyacetic acid) is a synthetic auxin and is the most commonly used growth regulator in cereal tissue culture [9-13]. In wheat, 2,4-D alone or in combination with cytokinins has been used for callus initiation [14-15]. Different genotypes are reported to respond differently to callus *in vitro* under different 2,4-D concentrations [16]. The focus of this study was to optimize cultural conditions and to find suitable 2,4-D levels for efficient callus induction and growth in thirty wheat genotypes. Starting material being available throughout the year can be efficiently used as initial explants for induction of calli and subsequent growth. The protocol will further be used to regenerate whole plants.

2. Materials and Methods

2.1 Place of Research

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

2.2 Seed source

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

2.3 Seed Sterilization Procedure

Matured explants from a totipotent plants (mature seeds) of 30 wheat accessions (genotypes) (*Triticum eastivum* L) were surface sterilized with liquid detergent and two drops of tween 20 using tap water times, 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 [17].

2.4 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 hood [18].

2.5 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 [18].

Table 1: 30 Wheat Genotypes used for the Research

Genotype NO	Name /Wheat Pedigree
1	F L O R K W A - 2 / 6 / S A K E R (S) / 5 / R B S / A N Z / 3 / K U Z / H Y S / /
2	INQALAB91*2/TUKURU//WHEAR
3	SERI.IB*2/3/KAUZ*2/BOW//KAUZ/4/KAUZ/FLORKWA-1
4	SAUA-/3/C80.1/3*BATA VIA//2*WBLLI/4/SAUAL#*1
5	ZAKIA-5
6	WBLLI/4/BOW/NKT/CBRD/3/CBRD/5/WBLLI*2……..
7	HUBARA-2/QAFZAH-21//DONIN-2
8	ATTILA 50Y//ATTILA/BCN/3/STAR*3/MUSK-3
9	ATTILA*/PBW 65*2/4/BOW/NTK/CBRD/3/CBRD
10	NEJMAH-12

11	KAUZ'S' SERI/3/KAUZ//KAUZ/STAR
12	VEE7/KAUZ/3/SERI82/SHUSHA'S'/GRUGGO-204782
13	KAUZ//MON/CROW?/4/SERI-1B//KAUZ/HEVO/3/AMAD
14	HUBARA-16/2*SOMAMA-3
15	ATTILA*2/PBW65*2/5/BOW/NKT//CBRD/3/CBRD…..
16	KACHU#1/4/CROC1/AE*SQUARROSA (205)//KAUZ/…
17	KHALIFA
18	FERROUG-2/POTAM*2KSB112,+511ZEMAMRA-8
19	PASTOR-2/HUBRA-5
20	WALLI/FRET2/PASTOR*2/3/MURGA
21	ATTILA GAN ATTILA
22	WBLLI/4/BOW/NKT/CBRD /3/CBRD/5/WBLLI*2/…
23	HOOSAM-8//CHAM-6/FLORKWA-2
24	KACHU#1/6/NG8201/KAUZ/4/SHA7/PRL/VEE#6/3/…
25	INQALAB91*2/TUKURU//WHEAR
26	KAUZ'S'/FLORKWA-1//GOUMAARIA-3
27	FRET2/TUKURU/FRET2/3/MONIA/CHTO/AMEL/4/…
28	WEAVER/WL3928//SW89.3064/3/KAUZ//MON/CROW'S'
29	ATTILA 50Y//ATILLA/BCN/3/STAR*/MUSK-3 (AISBW05-0043-9AP-OAP-OAP-2AP-OAP)
30	ATTILA 50//ATTILA/BCN/3/STAR'S'/KAUZ'S' (AISBW05-0006-2AP-OAP-OAP-2AP-OSD)

2.6. Callus Production

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±3 °C to callus. The callus induction medium (CIM) consisted of mineral salts of Murashige and Skoog (MS) [19], 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 innoculants (contained in three bottles with 5 embryo each) in each treatment of the genotype [17]

2.7. Proliferation of callus

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

3. Results

The data shows that the genotypes were different from each other with respect to callogenesis. However callus induction frequency ranged from 32% to 80%. Meanwhile maximum callus among the genotypes were noticed in genotypes 8 followed by genotypes 27, 28 and 7 with respective callus induction percentages of 79.99%, 79.99% and 77.33%, with minimum callus frequency of 32% was recorded by genotype 19, while 13 had callus induction potential of 36%. Every other genotype responded within the range. Hence all the wheat genotypes produced callus at all the levels of 2, 4-D except control. The best callus production was recorded at the levels of $5mgl^{-1}$ 2, 4-D and least was recorded in $1mg^{-1}$ per liter 2,4-D (2, 4-Dichlorophynoxy acetic acid) (Table 4A). The callus recorded at $5mgl^{-1}$ was more granular and morphogenic in nature. However interaction between genotypes and 2, 4-D indicated different percentage of callus at different 2, 4-D levels. It ranged from 13.33-100 percent. Genotypes 5, 22, 27 and 28 showed higher induction frequencies, while least callus of 13.33% were produced by genotypes 13 and 17. Maximum callus were observed in genotypes 5 and 28 at $5mgl^{-1}$ 2, 4-D, while same were recorded in genotype 27 and 28 at $4mgl^{-1}$ with genotype 22 at $3mgl^{-1}$ 2,4-D (Table 2A).

The data obtained in Table 3 revealed that the genotypes differed from each other in their days to callus, which ranged from 5.40 to 6.60 mean days to callus. Higher mean values for days to callus were at varieties 5 (6.60), 4 (6.40), 21 (6.40) and 26 (6.40), with the least mean values for days to callus at genotypes 8 (5.40) and 7 (5.60). The data also revealed that all the wheat genotypes had their respective days to callus at all levels of 2, 4-D, with the

promising mean for days to callus at 5mgl^{-1} (4.69) levels of 2, 4-D, while the higher mean values for days to callus was scored at 2mgl^{-1} (7.06) levels of 2, 4-D. Interaction between genotypes and 2, 4-D showed different days to callus at various 2, 4-D levels, which ranged from 4 to 8 days. Genotypes 2, 3, 4, 5, 6, 21, 22, 23, 26 and 27 at 1mgl^{-1} 2, 4-D level while genotypes 4, 26, 27 and 29 at 2mgl^{-1} 2, 4-D level had higher values for days to callus. The genotypes with the least values for days to callus were 7 and 27 at 4mgl^{-1} 2, 4-D level while 5mgl^{-1} 2, 4-D level had genotypes 2, 3, 8, 21, 23, 27, 28and 29 (Table 3A).

The effect of 2, 4-D on fresh weight of callus in milligrams showed the mean of wheat genotypes to differ from each other in weight. The weight ranged from 62.24mg mean to 85.14mg mean. Genotype 8 had the highest mean weight (85.14mg) followed by genotypes 7 (82.5mg), 28 (81.56mg) and 2 (81.46mg) respectively. While the lowest mean weight were noticed in genotypes 3 (62.24mg), 26 (64.48mg) and (64.9mg). However the highest mean weight of callus was observed at the level 5mgl^{-1} of 2, 4-D with mean of105.7mg. While the lowest mean weight was recorded at 1mgl^{-1} (31.1mg) 2, 4-D.

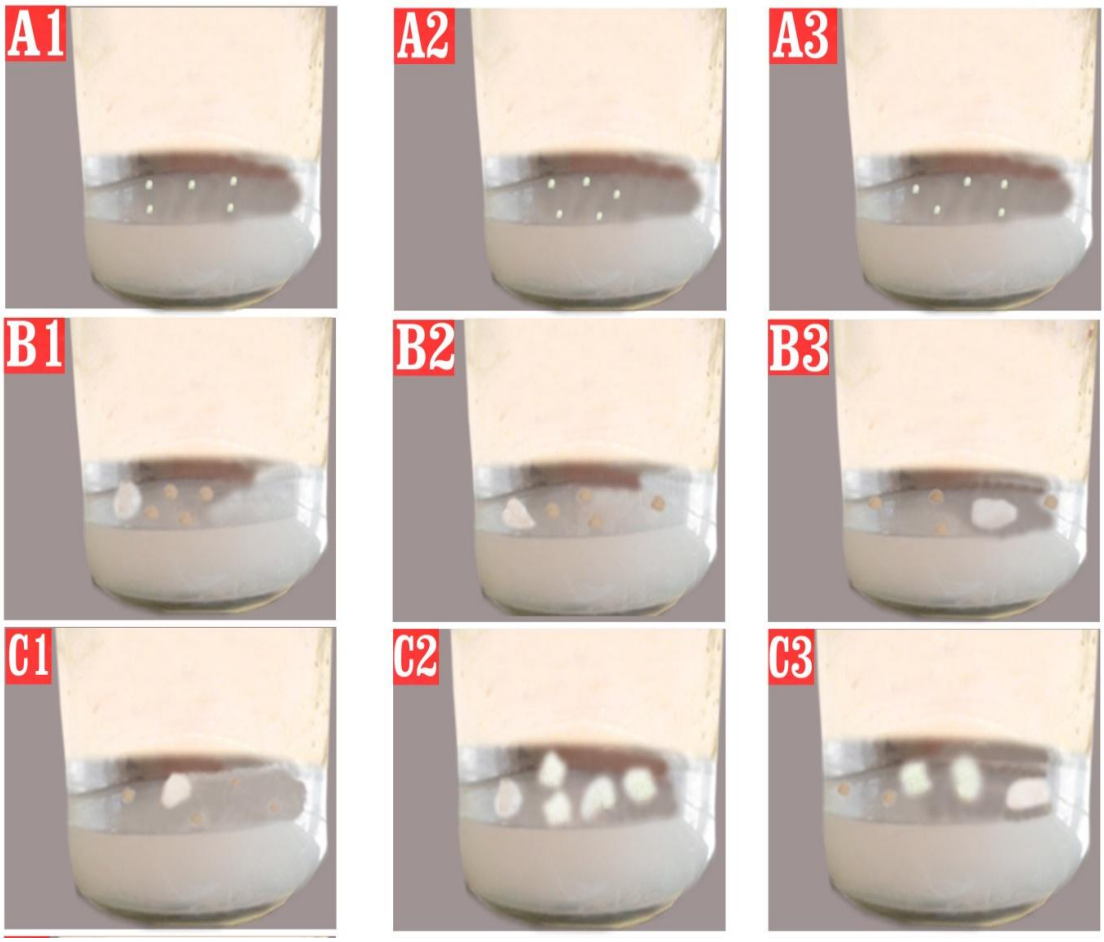

Plate 1: Callus formation of genotype 2 as effect by varying concentrations of 2,4-Dichlorophynoxy acetic acid (2, 4-D).

a. Control= Number of callus forming embryo A1: 0, A2: 0 and A3: 0.
Number of callus forming embryo A1: 5, A2: 5 and A3: 5
b. 1mg/l 2, 4-D = Number of callus forming embryo B1: 1, B2: 1 and B3:1
Number of non callus forming embryo B1: 4, B2: 4 and B3: 4
c. 2mg/l 2, 4-D = Number of callus forming embryo C1: 1, C2: 5 and C3: 3
Number of non callus forming embryo C1: 4, C2: 0 and C3: 2

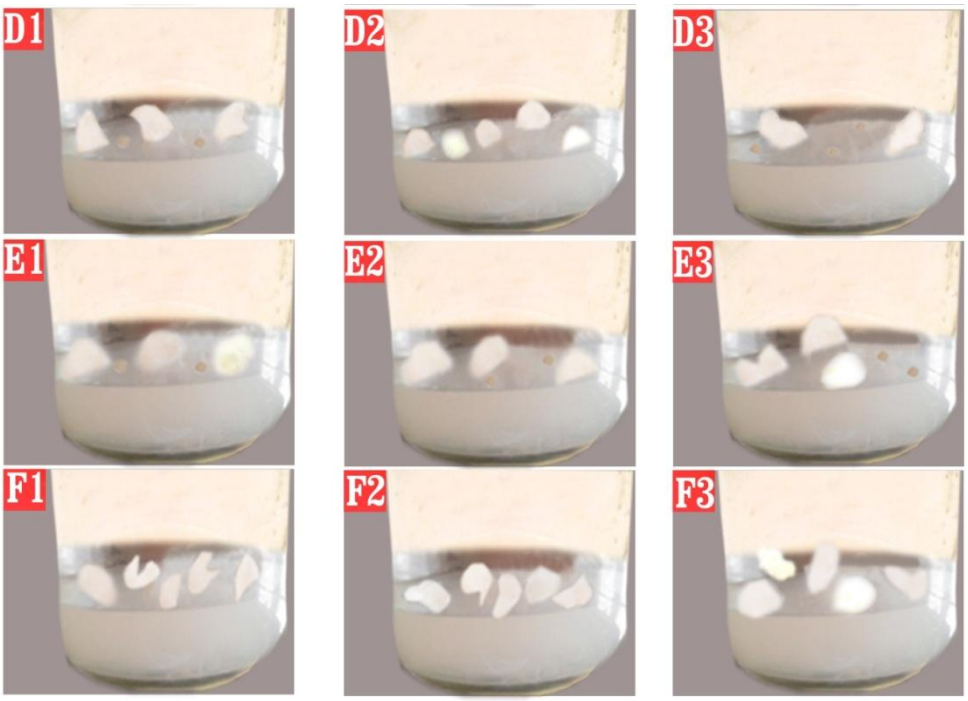

Plate 2: Showing callus formation of genotype 2 as effect by varying concentrations of 2,4-Dichlorophynoxy acetic acid (2, 4-D).

a. 3mg/l 2, 4-D = Number of callus forming embryo D1: 3, D2: 5 and D3:2
Number of non callus forming embryo D1: 2, D2: 0 and D3: 3

b. 4mg/l 2, 4-D = Number of callus forming embryo E1: 3, E2: 3 and E3:3
Number of non callus forming embryo E1: 2, E2: 2 and E3: 2

c. 4mg/l 2, 4-D = Number of callus forming embryo F1: 5, F2: 5 and F3:5
Number of non callus forming embryo F1: 0, F2: 0 and F3: 0

Table 2: Callus Induction Potentials of 30 Wheat Genotypes using Mature Embryo as ex-plant source under **different 2,4-Dichlorophenoxyacetic Acid (2,4-D) levels**

Genotype	2,4-D					Means
	1Mg L^{-1}	2 Mg L^{-1}	3 Mg L^{-1}	4 Mg L^{-1}	5 Mg L^{-1}	
1	26.67	33.33	66.67	46.67	80.00	50.68
2	20.00	60.00	66.67	60.00	100.00	61.33
3	26.67	40.00	60.00	60.00	86.67	54.67
4	33.33	40.00	73.33	73.33	53.33	54.67
5	26.67	93.33	53.33	86.67	86.67	69.33
6	33.33	86.67	46.67	73.33	60.00	60.00
7	26.67	93.33	93.33	86.67	86.67	77.33
8	33.33	60.00	100.00	73.33	86.67	80.00
9	40.00	86.67	93.33	93.33	86.67	70.67
10	33.33	60.00	53.33	46.67	73.33	53.32
11	26.67	40.00	46,67	66.67	86.67	53.34
12	20.00	33.33	26.67	40.00	66.67	37.33
13	13.33	26.67	33.33	46.67	60.00	36.00
14	26.67	40.00	53.33	66.67	80.00	53.33
15	20.00	40.00	46.67	73.33	73.00	49.33
16	20.00	33.33	40.00	80.00	86.67	52.00
17	13.33	20.00	33.33	53.33	73.33	38.66

18	20.00	26.67	33.33	46.67	60.00	37.33
19	6.67	20.00	26.67	40.00	66.67	32.00
20	26.67	33.33	40.00	60.00	66.67	45.33
21	33.33	40.00	46.67	80.00	80.00	56.00
22	26.67	53.33	60.00	73.33	66.67	55.90
23	26.67	46.67	60.00	80.00	86.67	60.00
24	20.00	33.33	53.33	60.00	66.67	46.70
25	26.67	40.00	46.67	60.00	73.33	49.40
26	26.67	40.00	53.33	93.33	86.67	60.00
27	33.33	80.00	73.33	86.00	93.33	71.98
28	40.00	66.67	93.33	100.00	100.00	79.99
29	33.33	80.00	93.33	100.00	93.33	79.98
30	40.00	46.67	80.60	86.67	93.33	69.33
Mean	28.666	47.777	58.221	69.554	78.878	

Table 3: Effect of Varying Concentration of 2, 4-D on Days to Callusing for 16 Wheat Genotype

Genotype	**2,4-D**					
	1mL^{-1}	**2mL^{-1}**	**3mL^{-1}**	**4mL^{-1}**	**5mL^{-1}**	**Mean**
2	7	7	6	5	4	5.80
3	8	7	6	6	4	6.20
4	7	8	6	5	6	6.40
5	8	7	7	5	6	6.60
6	8	7	6	5	5	6.20
7	7	6	6	4	5	5.60
8	7	6	5	5	4	5.40
9	7	6	7	5	5	6.00
21	8	7	7	6	4	6.40
22	8	7	6	5	5	6.20
23	8	7	6	5	4	6.00
26	7	8	5	6	6	6.40
27	8	8	7	4	4	6.20
28	8	7	6	5	4	6.00
29	7	8	5	7	4	6.20
30	7	7	7	5	5	6.20
Means	7.50	7.06	6.13	5.19	4.69	

Table 4: Effect of 2,4-D on fresh weight of callus in milligram (mg) of 16 wheat genotypes

Genotype	**1mL^{-1}**	**2mL^{-1}**	**3mL^{-1}**	**4mL^{-1}**	**5mL^{-1}**	**Mean**
2	35.5	70.4	84.1	102.1	115.2	81.46
3	22.6	43.3	62.4	85.5	97.4	62.24
4	22.4	49.6	67.3	91.4	98.3	66.00
5	26.2	46.5	60.5	89.1	102.4	64.9
6	30.3	63.4	78.6	98.3	106.2	75.36
7	37.6	66.1	84.3	105.4	119.1	82.5
8	40.4	73.2	82.5	107.2	122.4	85.14
9	33.5	72.3	86.2	104.3	103.4	79.94
21	31.7	69.5	77.6	95.5	107.1	76.28
22	30.6	66.5	78.2	99.2	105.3	75.96
23	34.8	68.6	80.1	101.1	113.4	79.60
26	23.5	48.5	64.4	88.4	99.4	64.48
27	34.4	69.4	85.3	97.3	110.2	79.32
28	38.2	73.3	89.1	99.1	108.1	81.56
29	29.5	55.7	68.5	72.4	86.4	62.50
30	25.6	53.6	71.7	80.2	97.3	65.68
Means	31.1	61.8	76.3	94.7	105.7	

4. Discussion

The preliminary investigation of 30 wheat genotypes for callus formation using different levels of 2, 4-D. The genotypes varies in their production of callus, sixteen wheat genotypes (2, 3, 4, 5, 6, 7, 8, 9, 21, 22, 23, 26, 27, 28, 29 and 30) were selected based on their best mean callus percentage across the five treatments which ranged from 54.67- 80.00%, while the interaction between genotypes and treatments revealed that 5mg/l 2, 4-D level performed better with 78.878% callus production potential. These results are in accordance with Mohmand [20] who found good callus from mature seed embryos of spring and winter genotypes of wheat and In contrast to Raziuddin *et al*. [21] discovered, that the use of a lower concentration of 2,4-D (2 mg/L) had beneficial effects on callus induction from immature zygotic embryos (ZEs). Cultivars Inqilab-91, hakwal-97 and Manthar formed most callus from mature seeds in the presence of 3 mg/L 2,4-D (83.3%, 77.8% and 95.2%, respectively) while 2 mg/L 2,4-D was optimal, which are quite opposite to the present result which may be due to difference in experimental conditions and equipments. Shah *et al.* [22] with best callus formed at 3and 5 mgL-1 2,4-D also supported the finding of present studies who achieved best callus at 5 mgL-1 2,4-D.

The result obtained on days to callus indicated that genotypes 8 (5.40 days), 7 (5.60 days) and 2(6.00 days) produced callus earlier based on the mean taken for all the genotypes across the treatments, while the mean for all the genotypes with respect to treatments had it that genotypes with the least values for days to callus were 7 and 27 at $4mgl^{-1}$ 2, 4-D level while $5mgl^{-1}$ 2, 4-D level had genotypes 2, 3, 8, 21, 23, 27, 28and 29. The current study differ from the work of Rahman *et al.*, [18] which revealed that maximum number of small size callus were produced in high concentration of 2, 4-D but took more time for callus initiation this may be as a result of genetic difference.

The effect of 2, 4-D on fresh weight of callus presented heavier callus mean weight at the level of $5mg/l^{-1}$ 2, 4-D with mean of 105.7mg. The higher callus fresh mean weight across all treatments were produced by genotypes 8 (85.14mg), 7 (82.5mg), 28 (81.56mg) and 2 (81.46mg) respectively. The present study corroborate the work of Sarkar and Biswas [23] who reported that the MS medium supplemented with 6.0 mg/L of 2, 4-D showed the best response for callus induction from mature wheat embryos. Also Tomar and Punia [24] took side with the current study that increase of 2, 4-D concentration in culture media produced good callus from mature embryo of wheat.

5. Conclusion

In the determination of callus induction potentials of 30 wheat genotypes using different levels of 2,4-Dichlorophynoxy acetic acid(2,4-D) ranging from 0mg/l 2,4-D to 5mg/l 2,4-D. The result shows an increase in the weight of callus due to increase in the levels of 2,4-D, with the promising callus recorded at the level of 5mg/l 2,4-D though there were no callus growth at 0 mg/l 2,4-D level, it encourage early production of callus . While 16 genotypes with total mean percentage across treatments which ranged from 54.67- 80.00% were selected for somaclone.

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