Analysis of *invitro* regeneration potential of local Pakistani wheat genotypes using mature and immature zygotic embryos as explants

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Abstract: We investigated the *invitro* regeneration potential of thirty two local wheat genotypes for genetic transformation purposes by using reported callus induction as well as regeneration media. Mature and immature zygotic embryos of these genotypes were used as explants. To study callus induction from mature embryos two callus induction media (CIM) were used. CIM1 contained MS salts with 2 mg/l 2, 4-D and 30 g/l sucrose while CIM2 contained 200 mg/l casein hydrolysate and 100 mg/l myoinositol in addition to CIM1. Thirty days old calli induced on CIM1 from mature embryo was shifted to Regeneration Medium-1 (RM1) which contained 0.1 mg/l 2,4-D along with MS salts and 30 g/l sucrose. Calli induced on CIM2 from mature embryos were shifted to Regeneration Medium-2 (RM2) having 0.5 mg/l BAP, 0.02 mg/l NAA, 200 mg/l Casein hydrolysate and 100 mg/l Myoinositol for regeneration. Calli were incubated at 26±1 ^{0}C for 8-16 hrs dark and light condition. For callus induction from immature zygotic embryos only CIM1 was used. Thirty days old calli induced on CIM1 were shifted to RM1 and incubated at 26±1 °C for 8-16 hrs dark and light condition for regeneration. Our results showed that *invitro* regeneration is generally poor in most of Pakistani wheat genotypes tested. Luckily some genotypes showed better callus induction and regeneration ability. Among these genotypes AARI-2011 produced maximum, embryogenic calli at CIM1 from both mature and immature zygotic embryos and produced maximum number of shoots per explants on RM1. Next to AARI-2011 were 9372 and Lasani 2008. Regeneration from immature zygotic embryos was comparatively better than mature embryos.

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Abbreviations: **BAP:** Benzyl Amino Purin; **NAA:** Nephthalene Acetic Acid; **2, 4-D:** 2, 4-Dichlorophenoxyacetic acid; **MS Salts**: Murashige & Skoog Salts; **RM:** Regeneration Medium; **CIM:** Callus Induction Media; **CRD:** completely randomized design; **DMRT:** Duncan's Multiple Range Test; **ANOVA**: Analysis of variance

INTRODUCTION:

Wheat is a cereal grain, originated in the Middle East and Ethiopian Highlands. Presently, it ranks third in the world after maize and rice in terms of production and covers most area among all crops. 651 million tons of wheat yield was recorded worldwide in 2010. Globally it is the biggest traded commodity in comparison with other crops. Wheat grains are source of protein, vitamins and minerals whereas the refined wheat grains are typically starch. The milled wheat grain is used as white flour. Wheat has high protein contents in its grain and these proteins, being easily digestible are the most important source of protein in human food. Up to a limited extent wheat is also used as forage crop for livestock. Wheat straw can also be used for thatched roofing. Yield of this crop has been decreasing due to various biotic and abiotic stresses.

A lot of work has been done for the improvement of wheat by using different breeding techniques. The emerging fields of biotechnology have revolutionized the whole circumstance of crop improvement including wheat. Biotechnology has been rising as a novel tool in agricultural research and is contributing in the development of new techniques to modify the plants genetically and control the development of plants leading to higher yield potential and better field performance (Patnaik & Khurana, 2001). Genetic manipulation is the most excellent approach to boost up wheat production (Ijaz and Khan, 2009). Genetic transformation is a biotechnological tool that can help to integrate genetic resources from across the phyla into the plant of interest. Optimization of established in vitro regeneration system is necessary to apply molecular approaches in crops (Anjum et al., 2012). Various methods have been identified to transform plant material with gene(s) of interest. For the purification of transgenic material after transformation event a reproducible and putative *invitro* plant regeneration system is a prerequisite. In

general, particle bombardment has been engaged successfully to develop transgenic plants in cereals including wheat. Embryogenic callus cultures are best for micro-projectile-mediated transformation; because regenerable cells are not very stable (Ijaz *et al.*, 2012). Successful development of genetic transformation protocols or techniques requires good regeneration systems (Ijaz *et al.,* 2012).Immature zygotic embryos have been used extensively for *invitro* regeneration and purification of transgenics after performing the transformation event in wheat. The availability of immature zygotic embryos is limited and season specific in many parts of the world. Many other sources like mature embryos and young inflorescences have also been tried but the success to is not economically feasible. Frequency of plantlets regeneration additionally depends on the composition of media and the genotype to be used.

Callogenesis and *invitro* regeneration for spring and winter varieties of wheat have been reported extensively. In wheat callus induction and regeneration of shoots affected mainly by composition of tissue culture media, explants and particularly by the genotype (Ozgen *et al*., 1998; Bommineni and Jauhar 1996; He *et al*. 1988 and Maddock *et al*. 1983). Under the normal culture environment, somatic embryogenesis or callus induction response from best wheat varieties has been frequently lower than model wheat genotypes. (Viertel *et al*., 1998 and Fennell *et al*., 1996). Genetic transformation in wheat is very difficult and mainly depends on some model genotype and even these genotypes in general have poor efficiency compared to model plants like tobacco and Arabidopsis (Shewry & Jones, 2005). Such genotypic variation limits the use of genetic transformation of best and cultivated wheat genotypes. Normally, if we need to improve some genotype by transformation, we have to transform model wheat genotypes and then bring the transgene into our genotype of interest by cross hybridization. This needs additional years to get stable gene integration in our genotype of interest. Genetic transformation of wheat genotypes mainly depends upon embryogenesis from somatic cells and regeneration capability of explants. Genotypic variations in embryogenesis from somatic cells and regeneration of plantlets are measured to be related with differences in genetic makeup response (Tyankova and Zagorska, 2001).

Under these circumstances we felt a dire need to identify new wheat genotypes from the prevalent wheat germplasm in Pakistan those can be used for transformation. Therefore we used at least 32 genotypes to study callus induction and regeneration. Additionally, we tried at least two callus induction and two regeneration media. Our results showed that in general immature zygotic embryo explants have an edge over mature embryo for regeneration, callus induction media with synthetic auxin 2,4-D at 2mg/l is better when compared with the medium with same auxin pluss growth enhancers. Same was the case with regeneration. Our genotypes preferred a medium for regeneration which is simple and contained only low level of 2, 4-D compared to a complicated one. Importantly, the genotypes have dominating role and any media gets ineffective once the genotypes do not respond for callus induction or regeneration. Fortunately we were able to find some genotypes which responded well to *in vitro* regeneration from local wheat germplasm of Pakistan

Materials and Methods:

In this study the *invitro* regeneration potential of 32 wheat genotypes (Table 1) was checked using mature and immature zygotic embryos as explant on a couple of callus induction and regeneration media (In this research study, we checked the response of our local 32 wheat genotypes for *in vitro* regeneration potential on reported callus induction as well as regeneration media because for genetic transformation, an expedite and proficient in vitro regeneration system is required and pre-requisite). This work has done for providing the baseline for genetic transformation of local wheat genotypes, because internationally two genotypes, bob white and Florida are being used commonly and routinely for genetic transformation purposes.

Table 1: List of Wheat Genotypes to be studied

List of Wheat Genotypes							
Sr. no	**Genotype**	**Sr. no**	**Genotype**	**Sr. no**	**Genotype**	**Sr. no**	**Genotype**
1	SH-95	**9**	9405	**17**	9494	**25**	9417
2	SH-2002	**10**	Chenab-2002	**18**	9611	**26**	MH-97
3	9408	**11**	Fareed-2006	**19**	Manthar-2003	**27**	9403
4	9381	**12**	Ufaq-2002	**20**	9438	**28**	9211
5	9437	**13**	Bhakar-2002	**21**	9476	**29**	4881
6	9511	**14**	9406	**22**	9411	**30**	9372
7	9407	**15**	PB-96	**23**	9311	**31**	Lasani-2008
8	GA-2002	**16**	9436	**24**	9428	**32**	AARI-2011

Mature embryos were cultured on callus induction medium-1 (CIM1) and callus induction medium-2 (CIM2) detailed in table-2 and table-3. While for immature zygotic embryos just CIM1 was used for callus induction and proliferation. pH of callus induction media was adjusted to 5.7-5.8. Gellan gum powder was used to solidify media. A scale was defined to collect callus data (Table 4). Data was collected in the form of callus scores, given on the basis of callus mass induction and proliferation.

Table 2: Callus Induction Medium 1 (CIM1)

Callus Induction Medium 1 (CIM1) reported by Rana, IA (2009)	
MS salts	4.33 g/l
2, 4-D	2 mg/l
Sucrose	30 g/l
Gellan gum powder	2.6 g/l

Table 3: Callus Induction Medium 2 (CIM2)

Callus Induction Medium 2 (CIM2) reported by Patnaik and Khurana, (2003); Patnaik *et al*., (2006)	
MS salts	4.33 g/l
2, 4-D	2 mg/l
Casein Hydrolysate	200 mg/l
Myoinositol	100 mg/l
Sucrose	30 g/l
Gellan gum powder	2.6 g/l

Table 4: SCALE

SCALE	
+	Low proliferation
++	Good proliferation
+++	Very good proliferation
++++	Excellent proliferation

For shoot regeneration calli of mature embryos, induced on CIM1and CIM2, were shifted to RM1 (Table 5) and RM2 respectively (Table 6). Similarly the calli of immature zygotic embryos, induced on CIM1, were shifted to RM1 only (Table 5). Regeneration data were collected in terms of plantlets recovered after the tissue culture experiments.

Table 5: Regeneration Medium 1 (RM1)

Regeneration Medium 1 (RM1) (reported medium by Rana, IA 2009)	
MS salts	4.33 g/l
2, 4-D	0.1 mg/l
Sucrose	30 g/l
Gellan gum powder	2.6 g/l

Table 6: Regeneration Medium 2 (RM2)

Regeneration Medium 2 (RM2) (reported by Patnaik and Khurana, (2003); Patnaik *et al*., (2006)	
MS salts	4.33 g/l
BAP	0.5 mg/l
NAA	0.02mg/l
Casein Hydrolysate	200 mg/l
Myoinositol	100 mg/l
Sucrose	30 g/l
Gellan gum powder	2.60 g/l

Mature seed sterilization and excision of mature embryos:
Sterilization of mature seeds was done first by rinsing the seeds for 2-3 times with distilled water. The rinsed seeds were soaked in sodium hypochlorite/commercial bleach for 15 minutes, followed by washing with distilled water for 2-3 times. These seeds were then soaked in distilled water for 3 hours to imbibe them before embryo isolation. Imbibition is required to soften the seed coat, it makes embryo isolation easier. Seeds were placed on an autoclaved Petri plate and the embryos were excised with the help of sterile scalpel, blades and forceps and cultured on Petri plates, containing callus induction media. All these operations were done under aseptic conditions in laminar air flow cabinet. Before the start of these operations, all scalpels, blades and forceps were sterilized.

Sterilization of immature seeds and excision of immature zygotic embryos
Sterilization of immature seeds was done in the same way as was of mature seeds, except soaking them in distilled water for 3 hours to imbibe. There is no need of Imbibition as immature zygotic embryos are isolated from young seeds, 15 days after fertilization. The immature zygotic embryos were excised in the same way as mature embryos were excised and cultured under aseptic conditions in Petri plates having callus induction media.

Callogenesis and shoot induction:
Mature as well immature zygotic embryos were cultured on callus induction media and placed in the dark for callus induction for 30 days. The data were taken based on defined scale given in table 3 and four observations were taken at different time points starting from week 1 to week 4 and the average callus proliferation per observation was noted. This experiment was repeated 3 times. Thirty days old calli induced on CIM1 and CIM2, in case of mature embryos were shifted to RMI and RM2 respectively for shoot induction. In case of immature zygotic embryos calli were induced only on CIM1 and then shifted to RM1 for shoot regeneration. For shoot induction, calli were transferred on to the regeneration media and maintained at 26 ±1 ^{0}C under 16/8 hrs light/ dark conditions. Regenerated Shoots were then shifted to ½ MS medium (Table 7) for the development of roots. *Invitro* regenerated plants were shifted into Belgium compost for hardening.

Table 7: Rooting Media (1/2 MS)

Rooting Media (1/2 MS)	
MS salts	2.165 g/l
Sucrose	30 g/l
Gellan gum powder	2.60 g/l

Experimental layout:
The experiments were conducted under completely randomized design (CRD) with three replications. ANOVA tables were constructed to analyze the variations. Duncan's Multiple Range Test (DMRT) at 5% probability level was implemented on various treatments means.

RESULTS:

Tissue culture provides a base for the genetic manipulation of plant genome. *Invitro* regeneration of plants is crucial step in transgenic development. For genetic transformation of wheat, exploitation of expedite *invitro* regeneration is necessary. This study was targeted at the optimization of *invitro* regeneration by selecting the suitable explants and tissue culture media for a genotype that contains better *invitro* regeneration ability among the many. Callus induction as well as regeneration response of these genotypes were evaluated using two sets of callus induction and regeneration media. The explants used for callus induction and *invitro* regeneration were mature and immature zygotic embryos.

Callus induction response of wheat genotypes from mature embryos:
For mature embryos, a comparative study was done. For checking the callus induction behavior of these genotypes by using mature embryos, two callus induction media (CIM1 and CIM2) were used. CIM2 was reported by Patnaik *et al*., (2006) for mature embryos. This medium (CIM2) was compared with the medium (CIM1) used by many, like Rana IA, 2009 and Becker *et al.*, (1994). Similarly, *invitro* regeneration from calli of mature embryos was achieved by using two regeneration media (RM1 and RM2). The calli induced from CIM1 were transferred to RM1 for regeneration and calli induced from CIM2 were shifted to RM2 for regeneration. The data taken using a defined scale were analyzed statistically and ANOVA table was drawn (Table-8).

Table 8: Analysis of variance (ANOVA): Analysis of variance for callus induction in wheat genotypes on callus induction media using mature embryos.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Media	1	2.083	2.083	5.47*
Genotype	31	175.813	5.671	14.91**
Media x Genotype	31	80.917	2.610	6.86**
Error	128	48.667	0.380	
Total	191	307.479		

*** = Significant (P<0.05); ** = Highly significant (P<0.01)**

Analysis of variance table depicts a considerable variation among genotypes for callus induction. The interaction between media and genotypes was also highly significant. It means the trend of genotypes for both media types is different. Behavior of genotypes also varied greatly being highly significant.

Excellent callus induction response was achieved from genotype AARI-2011 on CIM1 with mean value of 4.00 (Table 9, Fig 1& 2) followed by Bhakar-2002, 9311, 9403, Lasani-2008. On CIM2, 9436 showed best callus induction and proliferation by scoring the mean value of 3.67 callus score. Low callus induction response was observed in 9428 on CIM2 with mean value of 1.67 (Table 9, Fig 1&2). No callus was formed, in case of both callus induction media (CIM1 and CIM2) in genotypes PB-96 and MH-97 (Table 9, Fig 1). Wheat genotypes SH-95, 9211 and 4881 gave no calli on CIM1 but on CIM2 these genotypes gave good callus mass with mean values of 3.00 and 2.67, respectively. Similarly, 9511, Fareed-2006, 9494 and 9438 gave no calli on CIM2 whereas 9511 and Fareed-2006 showed good callus induction response on CIM1 with mean value of 2.33 (Table 9, Fig 1). The genotypes 9494 and 9438 also produced callus mass on CIM1 with mean value of 2.67 and 2.00, respectively.

By comparing the overall mean of callus induction media, CIM1 gave best response in general by scoring the mean value of 2.49 than CIM2 with mean value of 2.28 (Table 9). Therefore it is concluded that maximum callus induction from mature embryos was observed on CIM1 than CIM2. But this cannot be generalized as genotype factor is always there and some genotype performed better on CIM 2 than CIM1.

Table 9: Genotype into callus induction media interaction + SE: 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

Genotype	Callus induction Media		Overall Mean of genotypes
	CIM1	CIM2	
G1	0.00±0.00 f	3.00±0.00 a-d	1.50±0.67 DE
G2	3.00±0.00 a-d	2.67±0.33 b-e	2.83±0.17 ABC
G 3	3.00±0.58 a-d	2.67±0.67 b-e	2.83±0.40 ABC
G4	3.33±0.33 abc	3.33±0.33 abc	3.33±0.21 AB
G5	3.00±0.58 a-d	2.67±0.33 b-e	2.83±0.31 ABC
G6	2.00±0.58 de	0.00±0.00 f	1.00±0.52 E
G7	2.67±0.33 b-e	2.67±0.33 b-e	2.67±0.21 ABC
G8	3.33±0.33 abc	2.67±0.33 b-e	3.00±0.26 ABC
G9	2.67±0.33 b-e	3.00±0.58 a-d	2.83±0.31 ABC
G10	2.67±0.33 b-e	3.00±0.58 a-d	2.83±0.31 ABC
G11	2.67±0.67 b-e	0.00±0.00 f	1.33±0.67 E
G12	2.67±0.33 b-e	2.67±0.33 b-e	2.67±0.21 ABC
G13	3.67±0.33 ab	3.00±0.58 a-d	3.33±0.33 AB
G14	3.00±0.58 a-d	2.33±0.33 cde	2.67±0.33 ABC
G15	0.00±0.00 f	0.00±0.00 f	0.00±0.00 F
G16	3.00±0.00 a-d	3.67±0.33 ab	3.33±0.21 AB
G17	2.33±0.33 cde	0.00±0.00 f	1.17±0.54 E
G18	2.67±0.33 b-e	3.00±0.58 a-d	2.83±0.31 ABC
G19	2.67±0.33 b-e	3.33±0.33 abc	3.00±0.26 ABC
G20	2.33±0.33 cde	0.00±0.00 f	1.17±0.54 E
G21	2.67±0.33 b-e	2.67±0.33 b-e	2.67±0.21 ABC

G22	3.00±0.00 a-d	2.33±0.33 cde	2.67±0.21 ABC
G23	3.67±0.33 ab	3.33±0.33 abc	3.50±0.22 A
G24	2.67±0.33 b-e	1.67±0.33 e	2.17±0.31 CD
G25	2.67±0.33 b-e	2.33±0.33 cde	2.50±0.22 BC
G26	0.00±0.00 f	0.00±0.00 f	0.00±0.00 F
G27	3.67±0.33 ab	2.33±0.33 cde	3.00±0.37 ABC
G 28	0.00±0.00 f	3.00±0.58 a-d	1.50±0.72 DE
G 29	0.00±0.00 f	2.67±0.33 b-e	1.33±0.61 E
G 30	3.00±0.00 a-d	3.00±0.00 a-d	3.00±0.00 ABC
G 31	3.67±0.33 ab	3.33±0.67 abc	3.50±0.34 A
G 32	4.00±0.00 a	2.67±0.33 b-e	3.33±0.33 AB
Mean	2.49±0.13 A	2.28±0.13 B	

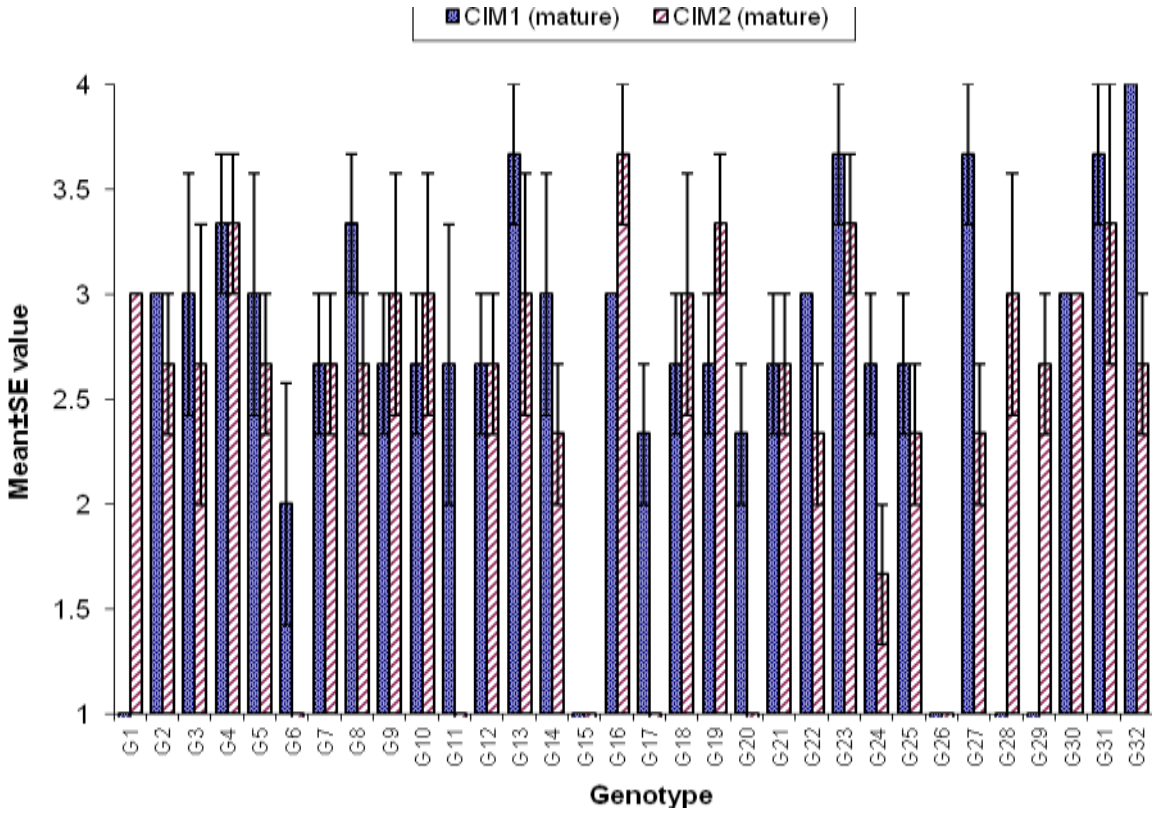


Figure 1: Callus induction in wheat genotypes from mature embryos: The genotype AARI-2011 showing maximum callus induction on both callus induction media and the genotypes PB-96 & MH-97 did not show callus induction at all on both callus induction media.

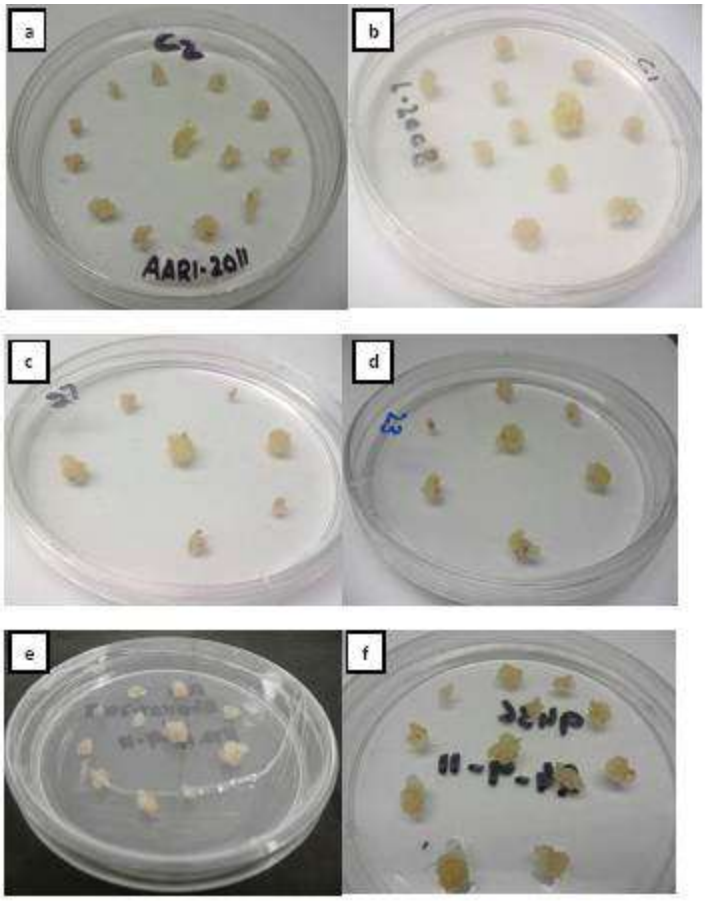


Figure 2: Callus induction response of various wheat genotypes. a. AARI-2011, b. Lasani-2008, c. 9403, d. 9311, e. Bhakar-2002, f. 9436

***In vitro* Regeneration in wheat genotypes from mature embryos:**

Calli of these 32 wheat genotypes were induced on CIM1 and CIM2. The calli induced on CIM1 were transferred to RM1 and calli induced on CIM2 were transferred to RM2 for *invitro* regeneration. Data were collected in the form of number of plants obtained and ANOVA table (Table 10) was constructed. ANOVA table revealed that significant differences were present in regeneration potential of wheat genotypes from mature embryos. Analysis of variance table showed that variation among genotypes, regeneration media (RM1 and RM2) and their interaction was highly significant.

Table 10: Analysis of variance (ANOVA): Analysis of variance table for regeneration in wheat genotypes from mature embryos on regeneration media.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Media	1	468.750	468.750	49.42**
Genotype	31	5685.813	183.413	19.33**
Media x Genotype	31	2368.917	76.417	8.05**
Error	128	1214.000	9.484	
Total	191	9737.479		

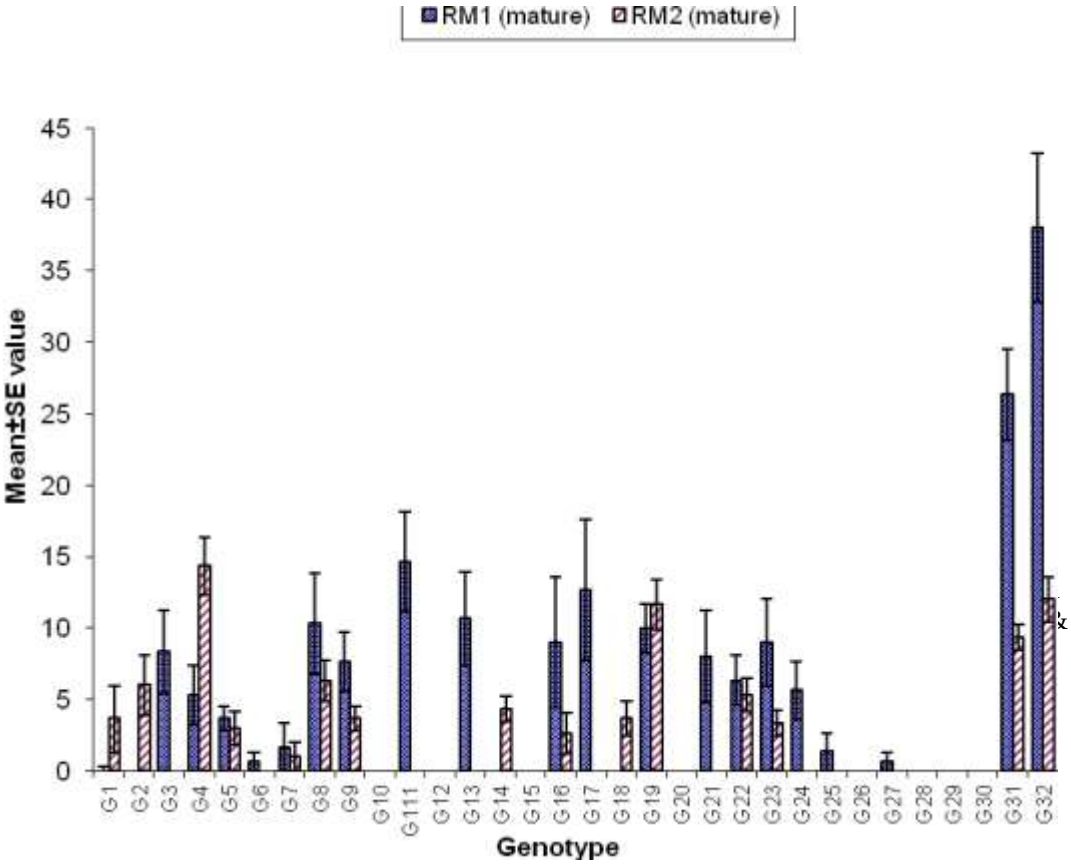
**** = Highly significant (P<0.01)**

Calli of AARI-2011, induced on CIM1 and shifted to RM1 gave maximum number of shoots. AARI-2011 gave 38 shoots per explants on RM1 followed by Lasani-2008, 26.33 shoots per explants (Table 11, Fig 3& 4). Calli of genotypes Chenab-2002, Ufaq-2002, Bhakar-2002, 9476, 9428, 9417, 9403, 9211, 4881 and 9372 induced on CIM2,

gave no *invitro* regeneration on RM2. Whereas calli of genotypes SH-2002, Chenab-2002, Ufaq-2002, 9406, 9611, 9438, 9372, induced on CIM1, gave no regeneration response on RM1. Chenab-2002, Ufaq-2002, 9372 are the common genotypes with no regeneration response in case of both regeneration media (Table 11, Fig 3&4). In case of RM1, small numbers of shoots were observed from genotypes 9511 and 9403. These genotypes gave 0.67 shoots per explants whereas on RM2, genotype 9407 gave 1.67 shoots per explants (Table 11, Fig 3& 4).

Table 11: Genotype into regeneration media interaction + SE: 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.

Genotype	Regeneration Media		Overall Mean of genotypes
	RM1 (For calli of mature embryos induced on CIM1)	RM2 (For calli of mature embryos induced on CIM2)	
G1	0.00±0.00 mn	3.67±2.33 i-n	2.00±1.29 H-K
G2	0.00±0.00 n	6.00±2.08 e-n	3.00±1.63 G-K
G3	8.33±2.91 d-k	0.00±0.00 n	4.17±2.27 F-K
G4	5.33±2.03 g-n	14.33±2.03 c	9.83±2.39 CD
G5	3.67±0.88 i-n	3.00±1.15 j-n	3.33±0.67 F-K
G6	0.67±0.67 mn	0.00±0.00 n	0.33±0.33 K
G7	1.67±1.67 lmn	1.00±1.00 mn	1.33±0.88 JK
G8	10.33±3.53 c-h	6.33±1.45 e-m	8.33±1.93 CDE
G9	7.67±2.03 d-l	3.67±0.88 i-n	5.67±1.33 E-I
G10	0.00±0.00 n	0.00±0.00 n	0.00±0.00 K
G11	14.67±3.48 c	0.00±0.00 n	7.33±3.63 C-F
G12	0.00±0.00 n	0.00±0.00 n	0.00±0.00 K
G13	10.67±3.28 c-g	0.00±0.00 n	5.33±2.80 E-J
G14	0.00±0.00 n	4.33±0.88 h-n	2.17±1.05 G-K
G15	0.00±0.00 n	0.00±0.00 n	0.00±0.00 K
G16	9.00±4.58 c-j	2.67±1.45 k-n	5.83±2.57 E-I
G17	12.67±4.91 cd	0.00±0.00 n	6.33±3.58 D-G
G18	0.00±0.00 n	3.67±1.20 i-n	1.83±0.98 IJK
G19	10.00±1.73 c-h	11.67±1.76 c-f	10.83±1.17 C
G20	0.00±0.00 n	0.00±0.00 n	0.00±0.00 K
G21	8.00±3.21 d-k	0.00±0.00 n	4.00±2.29 F-K
G22	6.33±1.76 e-m	5.33±1.20 g-n	5.83±0.98 E-I
G23	9.00±3.06 c-j	3.33±0.88 i-n	6.17±1.90 D-H
G24	5.67±2.03 f-n	0.00±0.00 n	2.83±1.56 G-K
G25	1.33±1.33 mn	0.00±0.00 n	0.67±0.67 K
G26	0.00±0.00 n	0.00±0.00 n	0.00±0.00 K
G27	0.67±0.67 mn	0.00±0.00 n	0.33±0.33 K
G28	0.00±0.00 n	0.00±0.00 n	0.00±0.00 K
G29	0.00±0.00 n	0.00±0.00 n	0.00±0.00 K
G30	0.00±0.00 n	0.00±0.00 n	0.00±0.00 K
G31	26.33±3.18 b	9.33±0.88 c-i	17.83±4.08 B
G32	38.00±5.20 a	12.00±1.53 cde	25.00±6.30 A
Overall Mean of regeneration media	5.95±0.91 A	2.82±0.43 B	



showing maximum
x 9372 did not show

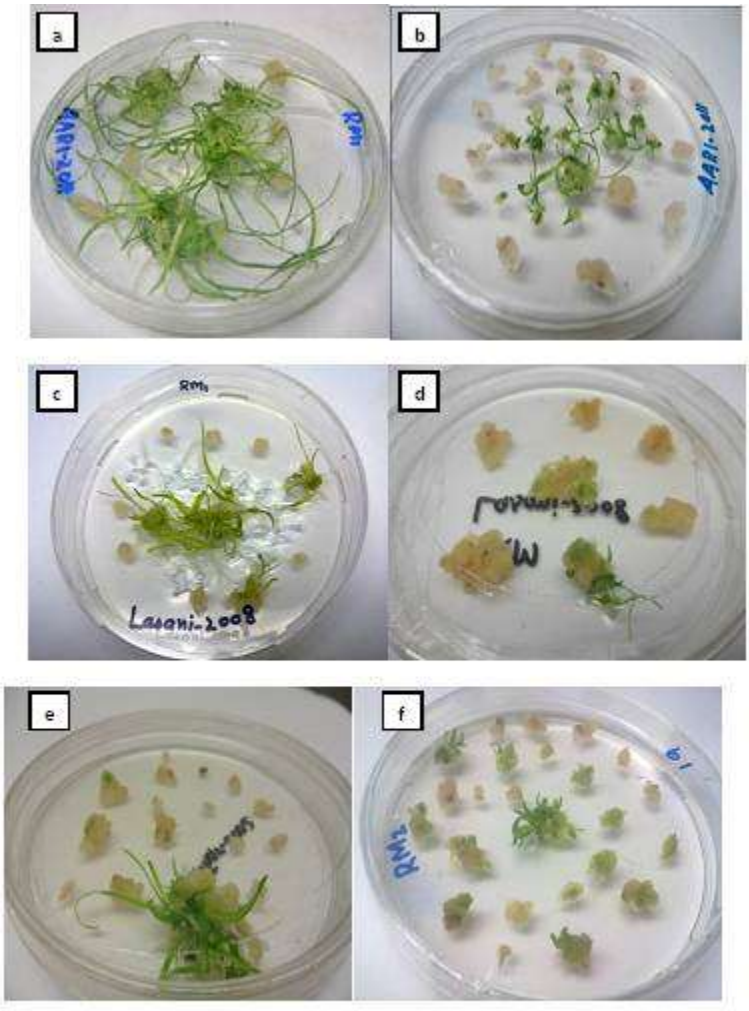


Figure 4: In vitro regeneration response of various wheat genotypes from mature embryos: a&b; AARI-2011, c& d; Lasani-2008, e& f; Manthar -2003

Callus induction and regeneration from immature zygotic embryos.

The immature zygotic embryos were isolated from 32 wheat genotypes and cultured on CIM1 for callus induction. After 30 days, calli were shifted to RM1 for regeneration of shoots. The results showed that there were significant differences among wheat genotypes for callus induction and regeneration from immature zygotic embryos (Table 12). The interaction table (Table 13) depicts that, genotypes 9411, 9372, Lasani-2008 and AARI-2011 scored mean value 4.00 and gave maximum calli mass on CIM1 followed by Ufaq-2002, 9406 and 9428 with mean value of 3.67 (Table 13, Fig 5 & 6). Low calli mass proliferation was observed in case of genotypes SH-95, Manthar-2003, 9311 and MH-97 with mean value of 1.00 (Table 13, Fig 5). When calli of immature zygotic embryos induced on CIM1 was transferred to RM1, highly significant variation was observed in regeneration response of genotypes (Table 12, Table 13). Interaction table showed that maximum regeneration potential shown by AARI-2011. This genotype gave 60.00 shoots per explant followed by 9372 and Lasani-2008, with 53.00 and 49.67 shoots per explant respectively. Whereas genotype 9405 produced minimum number of shoots and gave 0.67 shoots per explant (Table 13, Fig 5 & 7).

Table 12: Analysis of Variance (ANOVA): Analysis of variance for callus proliferation and regeneration from immature embryos

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Media	1	3283.521	3283.521	294.59**
Genotype	31	12645.813	407.929	36.59**
Media x Genotype	31	10939.479	352.886	31.66**
Error	128	1426.667	11.146	
Total	191	28295.479		

**** = Highly significant (P<0.01)**

Table 13: Genotype into media interaction + SE: 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.

Genotype	Media		Genotype Mean
	CIM1 (immature)	RM1 (immature)	
G1	**1.00±0.00 ij**	**0.00±0.00 j**	**0.50±0.22 L**
G2	**3.33±0.33 hij**	**14.33±4.10 ef**	**8.83±3.07 EF**
G3	**3.33±0.33 hij**	**5.33±0.88 hij**	**4.33±0.61 G-L**
G4	**3.00±0.58 hij**	**7.33±3.28 ghi**	**5.17±1.78 F-K**
G5	**3.33±0.33 hij**	**0.00±0.00 j**	**1.67±0.76 KL**
G6	**2.67±0.67 ij**	**1.67±1.67 ij**	**2.17±0.83 KL**
G7	**3.00±0.58 hij**	**23.33±2.33 cd**	**13.17±4.67 CD**
G8	**1.33±0.33 ij**	**0.00±0.00 j**	**0.67±0.33 KL**
G9	**3.00±0.58 hij**	**0.67±0.67 ij**	**1.83±0.65 KL**
G10	**3.00±0.58 hij**	**2.00±1.15 ij**	**2.50±0.62 JKL**
G11	**2.67±0.33 ij**	**5.00±2.65 hij**	**3.83±1.30 H-L**
G12	**3.67±0.33 hij**	**25.33±3.48 c**	**14.50±5.09 C**
G13	**3.00±0.58 hij**	**13.33±2.60 ef**	**8.17±2.60 E-H**
G14	**3.67±0.33 hij**	**11.33±0.88 fg**	**7.50±1.77 E-I**
G15	**0.00±0.00 j**	**0.00±0.00 j**	**0.00±0.00 L**
G16	**3.33±0.33 hij**	**5.00±1.15 hij**	**4.17±0.65 G-L**
G17	**3.00±0.58 hij**	**15.67±2.91 ef**	**9.33±3.13 DEF**
G18	**3.00±0.58 hij**	**4.00±1.73 hij**	**3.50±0.85 I-L**
G19	**1.00±0.00 ij**	**0.00±0.00 j**	**0.50±0.22 L**
G20	**3.33±0.33 hij**	**0.00±0.00 j**	**1.67±0.76 KL**
G21	**3.00±0.58 hij**	**0.00±0.00 j**	**1.50±0.72 KL**
G22	**4.00±0.00 hij**	**9.67±1.76 fgh**	**6.83±1.49 E-J**
G23	**1.00±0.00 ij**	**0.00±0.00 j**	**0.50±0.22 L**
G24	**3.67±0.33 hij**	**18.00±2.65 de**	**10.83±3.42 CDE**
G25	**3.33±0.33 hij**	**12.67±3.28 efg**	**8.00±2.56 E-H**
G26	**1.00±0.00 ij**	**0.00±0.00 j**	**0.50±0.22 L**
G27	**3.00±0.58 hij**	**14.00±2.65 ef**	**8.50±2.74 EFG**
G28	**2.67±0.33 ij**	**3.33±1.76 hij**	**3.00±0.82 JKL**
G29	**2.67±0.33 ij**	**0.00±0.00 j**	**1.33±0.61 KL**
G30	**4.00±0.00 hij**	**53.00±10.02 b**	**28.50±11.84 AB**
G31	**4.00±0.00 hij**	**49.67±2.73 b**	**26.83±10.28 B**
G32	**4.00±0.00 hij**	**60.00±4.04 a**	**32.00±12.65 A**

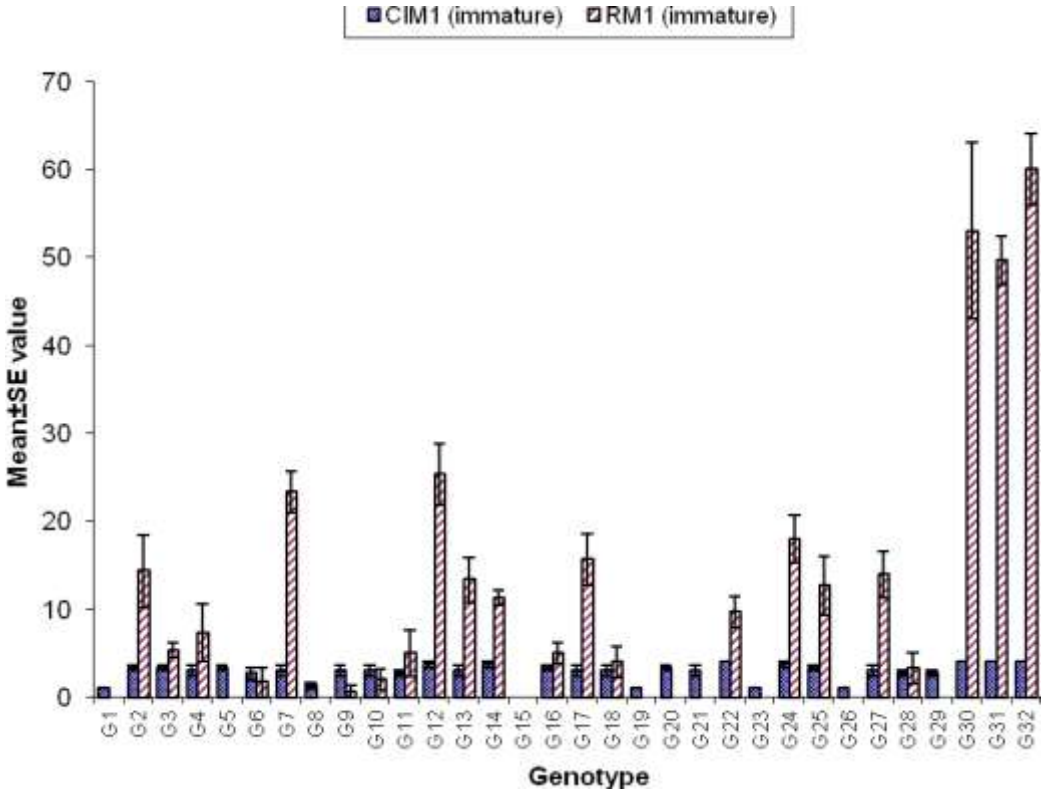


Figure 5: Response of wheat genotypes for Callus induction on CIM1 and regeneration on RM1 from Immature zygotic embryos: The genotype AARI-2011, Lasani-2008, 9372 showing maximum callus induction and regeneration potential. The genotypes GA-2002, Manthar-2003, 9438, 9476, 9311, MH-97 and 4881 showing callus induction but did not show regeneration. PB-96 did not show any response for callus induction and regeneration.

a b

c d

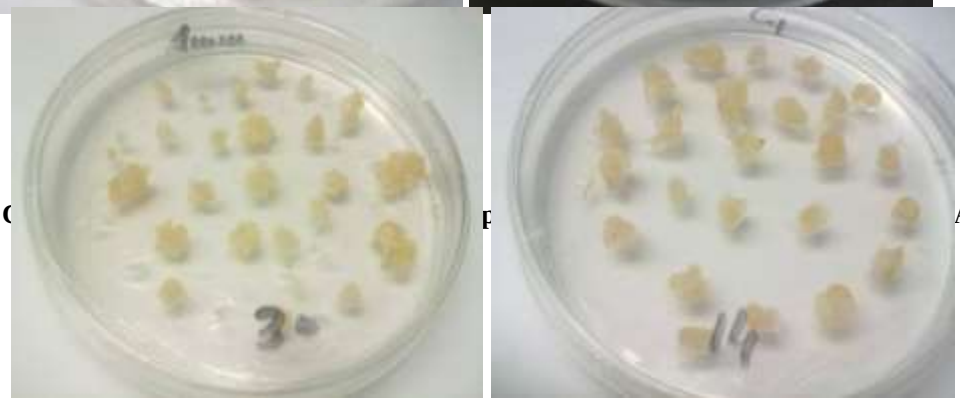


Figure 6: C **p** ARI-2011, **b.**

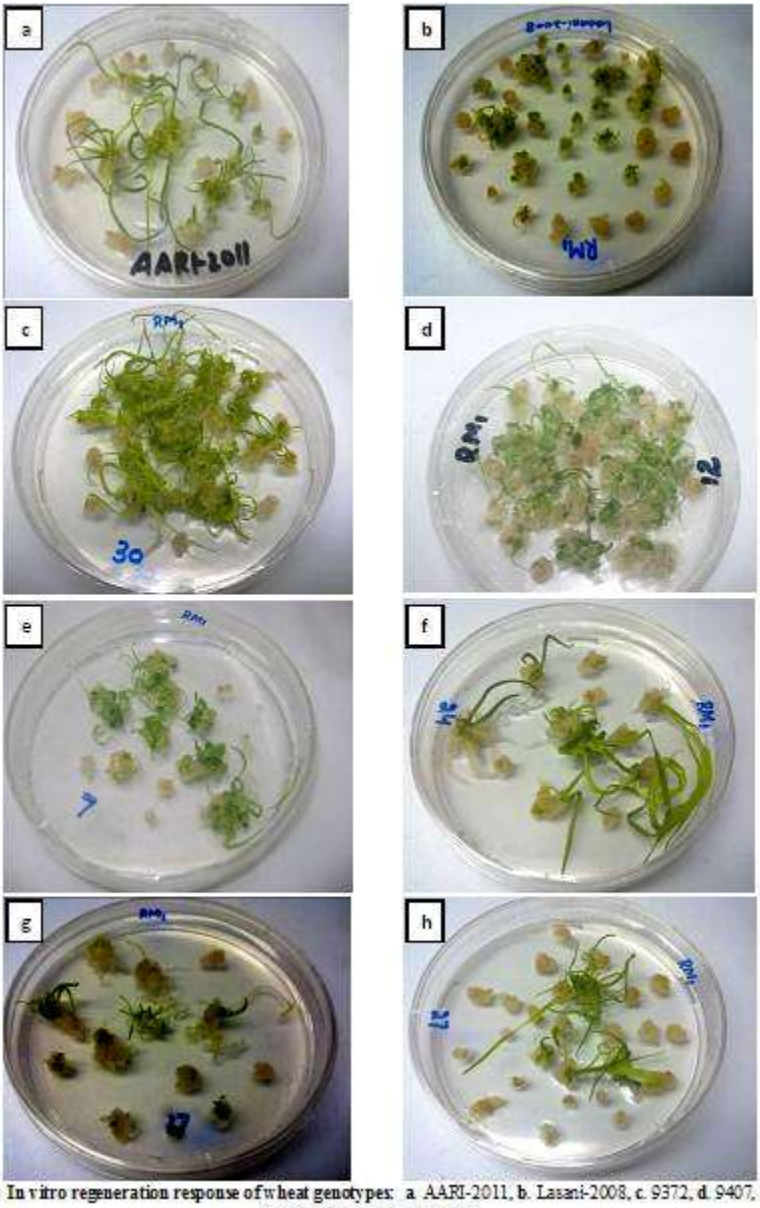


Figure 7: In vitro regeneration response of wheat genotypes: a. AARI-2011, b. Lasani-2008, c. 9372, d. 9407, e. Ufaq-2002, f. 9428, g. 9494, h. 9408,

Shoots obtained from mature and immature embryos of studied genotypes when then shifted to root induction media for getting profused rooting system (Fig 8). Subsequent to this, these plantlets were transferred to pots and were wrapped with polythene bags for acclimatization (Fig 9).

Figure 8: Root induction on ½ MS medium

Figure 9: Acclimatization of in *vitro* regenerated plants in peat moss and compost

Similarity and divergence of wheat genotypes with regards to *in vitro* regeneration potential:

The regeneration potential varied in all genotypes. Regeneration potential may vary due to various factors such as media, explants and genotypes etc. But if media, explants and all other conditions are same/ constant then genotype would only be the one factor, which would bring change. Variation in *invitro* regeneration response is due to genotype then it is directly proportional to the variation due to genetic background of genotype.

Variation in regeneration potential due to genotype α variation in regeneration potential due to genetic background of genotype

Cluster analysis of similarity and divergence in regeneration potential of wheat genotypes from mature embryos (Fig 12) revealed that straight line on genotype numbers showed that no difference in these genotypes. These genotypes showed no regeneration in this study. Among the genotypes those gave regeneration , genotypes number 32 (AARI-2011) showed maximum regeneration potential and this genotypes is closely related to genotype number 31 (Lasani-2008) with regards to its regeneration potential. Genotype number (16) 9436 and (23) 9311 are closely related with regards to regeneration potential because these genotypes showed same regeneration response. Genotype number 4 (9381) and 24 (9428) are closely related with regards to regeneration potential because these genotypes showed similar regeneration behavior. Genotypes, AARI-2011and Lasani-2008 are diverse from other genotypes in relation to regeneration potential but these are closely related to each other with similar regeneration response.

In other condition, where immature zygotic embryos were used as explants, cluster analysis of similarity and divergence with regards to regeneration potential (Fig 13) revealed that genotype number 32 (AARI-2011), genotype number 30 (9372) and 31 (Lasani-2008) are closely related and similar in regeneration response. Genotype 7 (9407) and genotype number 12 (ufaq-2002) are similar with regards to regeneration behavior. Genotype number 22 (9411) and genotype number 24 (9428) are similar and closely related to each other. Genotype number 17 (9494) and genotype number 27 (9403) are more close to each other with reference to regeneration behavior. So, these results of similarity and divergence analysis showed that variation in regeneration potential is not only due to the genotype but this could be due to the explant type.

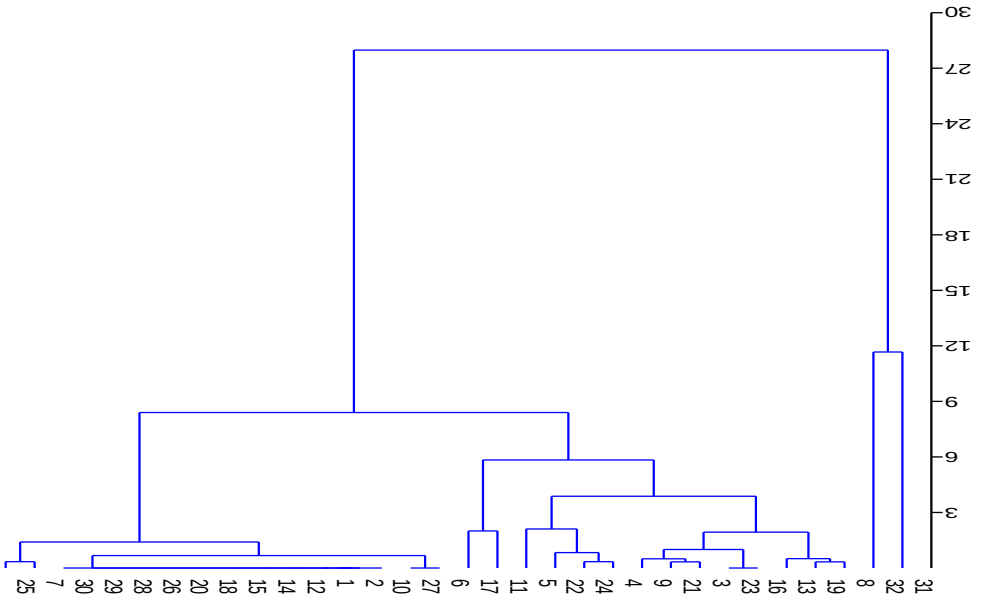


Figure 12: Cluster analysis of similarity and divergence of wheat genotypes in regeneration potential from mature embryos

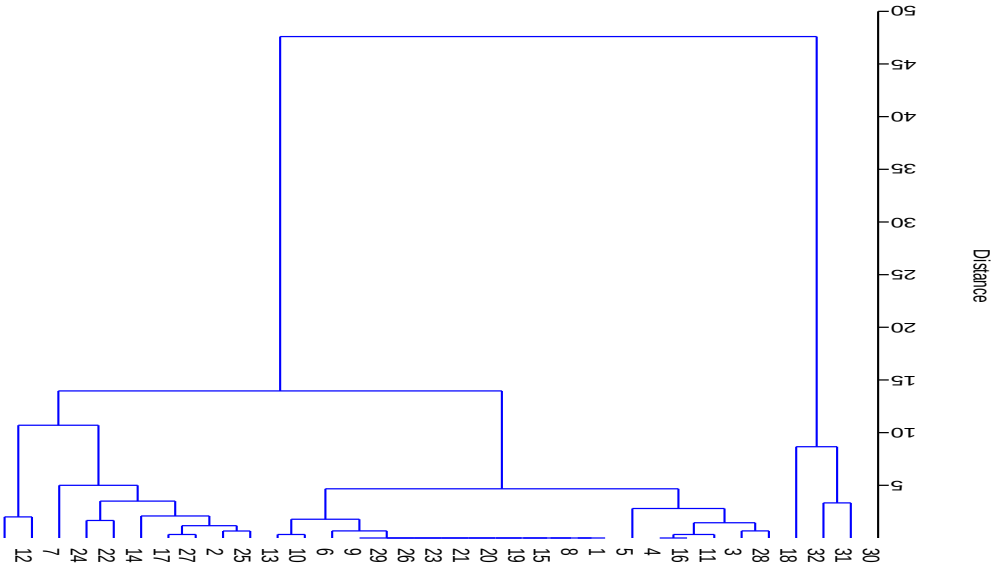


Figure 13: Cluster analysis of similarity and divergence of wheat genotypes in regeneration potential from immature zygotic embryos

Discussion:

In wheat, callus induction, regeneration of plants and transformation efficiency is mainly affected by genotype (Fennel *et al*., 1996 and Chowdhury *et al*., 1991), explants (Shewry and Jones, 2005) and culture medium to be used (Barro *et al*., 1999; Mendoza & Kaeppler, 2002 and Satyavathi *et al*., 2004). It has been reported that immature zygotic embryos have maximum potential for callus induction and regeneration of plants as compared to mature embryos (Mahesh *et al*., 1995, Yu *et al*., 1999, Qin and He, 2001). In this study, it has been confirmed that immature zygotic embryos have better potential for callus induction and regeneration of plants than mature embryos. We have observed that the genotypes which performed good with mature embryos, performed better with immature zygotic embryos. Some of the genotypes did not perform with mature embryos at all but showed good response at immature zygotic embryos, though the exceptions appeared but they were not many. The probable reason behind immature zygotic embryo to be excellent embryogenic callus producer could be softer nature of the tissues due to relatively simpler cell wall and good mitotic activity.

The role of plant growth regulators in cereal tissue culture is very important. Usually the synthetic auxin 2, 4-D in the range of 1-3 mg/l, is essential for the formation of embryogenic callus in cereal. Studies have confirmed that the use of 2, 4-D to induce callus has been a critical factor (Yu *et al*., 2003). In this study, 2, 4-D (2 mg/l) has been used for callus induction using both CIM1 and CIM2. Callus induction from mature embryos started within 4-7 days after culturing. Callus induction was found to be highly influenced by genotypes while the same *invitro* environment was provided for all genotypes. In a number of genotypes, high callus induction from mature embryos was observed but the response was still variable from genotype to genotype. These results are in accordance to those described by Bommineni and Jauhar. (1996), Maesetal *et al*. (1996), Ozgen *et al*. (1996) for callus induction in durum wheat and Fennell *et al.* (1996), Hess and Carman, (1998), Maesetal. (1996) and Sears and Deckard, (1982) in bread wheat. They examined an intense effect of genotype on callus induction and proliferation. Most of the genotypes showed good callus induction with 2 mg/l 2, 4-D. On the contrary, Turhan and Baser (2004) obtained good callus induction with 4 mg/l of 2, 4-D and 1 mg/l NAA. Sarker and Biswas (2002) used various concentrations of 2, 4-D and found 6 mg/l 2, 4-D concentration as the best for callus induction. Rehman *et al*., 2008 also used three levels of 2, 4-D to induce callus from mature embryos. They obtained best calli at 6 mg/l 2, 4-D. Noor *et al*., 2009 used various concentrations of 2, 4-D for callus induction and found greatest callus induction from mature embryos at 3 mg/l 2, 4-D. Afzal *et al*., 2010 also found best callus induction from mature embryos with 3 mg/l 2, 4-D. Our results showed similarity with the Raziuddin *et al*., 2010. They used various concentrations of 2, 4-D and observed maximum callus induction on MS medium having 2 mg/l 2, 4-D. Shah *et al*., 2003 found an excellent callus induction on MS medium having 2, 4-D (3.5 mg/l) and proliferation on MS medium having 2 mg/L 2, 4-D with 0.5 mg/L BAP. Malik *et al*., 2003 also reported that 2 mg/l 2, 4-D was best for callus proliferation from mature embryos. Minor variations in results are due to diverse genotypes used. In most of the cases CIM2 which was recommended by Patnaik et al., 2006 was poor performer than CIM1. The difference between both the media was casein hydrolysate and myoinositol. Our results showed that wheat likes only simple 2, 4-D for callus induction and proliferation compared to 2, 4-D with growth enhancers.

Callus induction from immature zygotic embryos was initiated within 3-5 days. Maximum genotypes showed callus induction from immature zygotic embryos with 2 mg/l 2, 4-D. On the contrary, Yasmin *et al*., 2009 studied various combinations of plant growth regulators (2, 4-D, NAA, Kinetin) and found best callus induction from immature zygotic embryos at media having 1 mg/l 2, 4-D, 1 mg/l kinetin, 2 mg/l NAA and 4 mg/l 2, 4-D, 1 mg/l Kin, 2 mg/l NAA. Maddock *et al*., 1998 also found best callus induction from immature zygotic embryos with 1mg/l 2, 4-D while at higher levels of 2, 4-D (2.5 and 5 mg/l) they found relatively vigorous callus but less regeneration frequency. Similarly, good callus induction from immature zygotic embryos on MS medium having 2, 4-D (2 mg/l) was also reported by Tanzarella and Greco, (1985) and Batrok and Sagi (1990). Dodig *et al*., 2001 also reported best callus induction at 2 mg/l 2, 4-D along with vitamins and 100 mg/l Casein Hydrolysate.

It was observed that regeneration potential was also genotype dependent. It may be due to the fact that regeneration of plants may be controlled by genetic makeup (Kamil *et al*., 2005). In this study a considerable variation among genotypes for callus induction and regeneration was found. The genotype AARI-2011 showed maximum potential for callus induction and plant regeneration both from mature and immature zygotic embryos. For shoot regeneration from mature embryo derived calli, two regeneration media were used (RM1 and RM2). RM1 was having 0.1 mg/l 2, 4-D with MS salts and 3% sucrose while RM2 had BAP (0.5 mg/l), NAA (0.1 mg /l), Casein hydrolysate (200 mg/l), myoinositol (100 mg/l) and MS salts plus 3% sucrose. It was found that maximum regeneration was observed on RM1 than RM2 which means simpler medium with little auxins is better for regeneration. Alizadeh *et al*. (2004)

reported 1 mg/l of BAP, 0.2 mg/l IAA and 0.2 mg/l 2, 4-D best combination for regeneration. Rehman *et al*., (2008) obtained highest regeneration frequency on MS medium having 1 mg/l of kinetin and 200 mg/l Casein Hydrolysate. Noor *et al*. (2009) and Rashid *et al*. (2002) reported best regeneration at a combination of 0.5 mg BAP and 0.1 mg/l IAA. Afzal *et al*., (2010) used various concentrations of IAA and BAP to optimize the regeneration in wheat genotypes and observed maximum regeneration at combination 0.1 mg/l of IAA and 0.5 mg/l BAP.

Regeneration from immature zygotic embryos started after 2-3 weeks of incubation on regeneration medium. AARI-2011 regenerated maximum number of plants. The regeneration response of Lasani-2008 and 9372 was also very good. We used only 2,4-D, as growth regulator in regeneration medium and here our results are matching with the results of Gul *et al*., (2006). They also achieved maximum regeneration from immature zygotic embryos on MS medium having only 2, 4-D in certain wheat lines. Cui *et al*. (2012) used 0.1 mg/l 2, 4-D for shoot regeneration. On the contrary, Bhaskaran & Smith (1990) reported that in certain cases, media supplemented with 0.5mg/l BAP without auxin also showed shoot and root differentiation. Mahesh *et al*., (1995) reported that immature zygotic embryos have great potential for callus induction and plant regeneration; this was confirmed in our study.

The genotypes 9372, Ufaq-2002 and 9406 showed callus induction from mature embryos on CIM1, with mean value 3.00, 2.67 and 3.00 respectively (Table 9), but did not show any regeneration on RM1. From immature zygotic embryos these genotypes showed callus induction on CIM1 with mean value 4.00, 3.75 and 3.67 respectively (Table 13), and showed regeneration on RM1 with 53.00, 25.33 and 11.33 shoots per explant, respectively. The genotypes SH-95 and MH-97 did not show callus induction on CIM1 and regeneration on RM1 from mature embryos but from immature zygotic embryos showed little callus induction with mean value 1.00 (Table 13) on CIM1 but showed no regeneration on RM1. Ozgen *et al*. (1998) reported that mature and immature zygotic embryos of durum wheat when cultured on MS medium supplemented with 2, 4-D, the mature embryos had low frequency of callus formation but a high regeneration capacity as compared to immature zygotic embryos. We also found that low callus formation frequency in case of mature embryo but high regeneration potential from this little calli as compared to immature zygotic embryos. The overall production of plants from immature zygotic embryos was better that mature ones. In our study, the genotypes Manthar-2003 and 9311 showed callus induction from mature embryos on CIM1 with mean value of 2.67 and 3.67 (Table 9), respectively and showed regeneration on RM1 with 10.00 and 9.00 (Table 11) shoots per explant, respectively. While from immature zygotic embryos these genotypes showed poor callus induction on CIM1 with mean value 1.00 (Table 13) and showed no regeneration on RM1. The genotype 9405 showed callus induction from mature embryos on CIM1 with mean value of 2.67 (Table 9) and produced 7.67 shoots per explant (Table 11). From immature zygotic embryos the same genotype showed callus induction on CIM1 with mean value of 3.00 and produced 0.67 shoots per explant (Table 13). This genotype produced higher callus mass from immature zygotic embryos but less number of shoots, whereas less calli mass produced from mature embryos on CIM1 but produced more number of shoots than immature zygotic embryos.
The conclusion and finding of this study is that callus induction and regeneration in wheat is dependent upon genotypes, explants and media to be used. The wheat genotypes showed high ability for plant regeneration on RM1 from Calli of immature zygotic embryos induced on CIM1. This combination seems to be the most suitable for further work concerning wheat improvement through biotechnological approaches and especially genetic engineering of crop plants for agronomic traits. Next finding is that AARI- 2011, Lasani- 2008 and 9372 are the potential genotypes to become model for transformation studies.

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