

Enhancement of Somatic Embryogenesis by Mature and Immature Seeds in Wheat (*Triticum aestivum* L.)

Supria Saha

Plant Genetic Engineering Lab., Institute of Biological Sciences,
University of Rajshahi, Rajshahi-6205, Bangladesh

Zohorul Islam

Department of Genetic Engineering and Biotechnology,
University of Rajshahi, Rajshahi-6205, Bangladesh

Sadequl Islam

Department of Zoology, University of Rajshahi,
Rajshahi-6205, Bangladesh

Mirza Fida Hassan

Plant Genetic Engineering Lab., Institute of Biological Sciences,
University of Rajshahi, Rajshahi-6205, Bangladesh

Md. Shahadat Hossain

Department of Genetic Engineering and Biotechnology,
University of Rajshahi, Rajshahi-6205, Bangladesh

S. M. Shahinul Islam (Corresponding author)

Plant Genetic Engineering Lab., Institute of Biological Sciences,
University of Rajshahi, Rajshahi-6205, Bangladesh

E-mail: shahin_ibsc@ru.ac.bd

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Abstract

A suitable plant regeneration system has been established using 3-4 weeks old calli derived from immature and mature seeds of four wheat varieties *viz.* Pavon 76, Akbar, Barkat, and Kanchan. As plant growth regulators, various auxins (2,4-D, BAP and IAA) either single or in combination were used in MS medium. The variety Pavon 76 showed maximum (72.25%) callus induction and Akbar exhibited the lowest (37.78%) from calli derived from immature seeds. Hormonal effects on callus induction were evaluated and significant results were found in case of genotypes at $P < 0.01$. Out of four genotypes, the highest frequency of plant regeneration was recorded in Pavon 76 (67.00%) and lowest in Kanchan (43.10%) when 1.5 mg/l BAP and 0.5 mg/l IAA was added in the medium. It was observed that Pavon 76 produced highest number of green plants than others. For mature seeds all of the mentioned genotypes showed significant difference with maximum frequency of callusing in Pavon 76 (69.57%) in MS + 2.5 mg/l 2,4-D followed by Kanchan (60.84%), Barkat (52.73%), and Akbar (47.19%). For plant regeneration, Pavon 76 also showed best performance (64.36%) in MS + 2.0 BAP + 1.0 mg/l IAA, using calli derived from mature seeds. The other genotypes Barkat, Kanchan and Akbar exhibited 59.44, 52.71 and 52.32% regeneration in the same medium respectively. Here, the lowest regeneration (40.63%) was found in Akbar. In this case, it was aimed to establish a suitable protocol for *in vitro* callus induction and regeneration for advance biotechnological research on wheat in Bangladesh.

Keywords: Embryogenesis, *Triticum aestivum*, callus, *in vitro*, plant growth regulators, plant regeneration.

1. Introduction

Biotechnological approaches have the potential tools to complement conventional methods that include many factors responsible for the frequencies of callus induction and plant regeneration in wheat and other cereal crops (Polumahanthi *et al.*, 2014; Haque and Islam, 2014; Siddique *et al.*, 2014; Mohammad *et al.*, 2014; Islam, 2010). Plant growth regulators and optimization of their proper dosages are also very important for success in *in vitro* culture (Kouassi *et al.*, 2017; Bhattacharjee and Islam, 2014; Morshed *et al.*, 2014). The somatic and gametic embryogenesis on major cereal crops report that regeneration capability depends on genotype (Ksia *et al.*, 2008; Islam *et al.*, 2001). Some methods like mutation and haploid breeding, somaclonal variation, genetic engineering etc. also have been gaining importance for the creation of new variability (Saika and Toki, 2010; Croughan and Chu, 1991; Ho and Vasil, 1983). Among them, the genotype and nutrient composition are major sources of variation in *in vitro* culture (Delporte *et al.*, 2014; Khatun *et al.*, 2003; Khanna and Raina, 1998). Callus culture through mature and immature embryo has often been applied to solve some practical problems in wheat breeding systems. However, callus induction frequencies

vary from genotype to genotype (Najat *et al.*, 2014; Mahmood *et al.*, 2009) and also vary much dependent on a proper combination and concentration of plant growth regulators are reported by Shafquat *et al.* (2009). Establishment of reliable tissue culture protocols for callus induction on somatic embryogenesis and its subsequent regeneration has been described in wheat (Noor *et al.*, 2009) and for maize Morshed *et al.* (2016). Direct organogenesis has also been studied in wheat (Li *et al.*, 1992) using shoot tips (Viertel and Hess, 1996); glumella and lemma (Lu *et al.*, 1988); mature and immature wheat embryos (Sarker *et al.*, 2007; Ozgen *et al.*, 1996). Works on immature embryos have been reported by various researchers to be the most responsive to tissue culture conditions and showed relatively high rate of callus induction as compared to mature embryos (Shah *et al.*, 2003). Moreover, mature embryos are readily available throughout the year and being used for advance biotechnological research (Morshed *et al.*, 2014; Patnaik *et al.*, 2006), thus necessitating the need for optimization of tissue culture protocols. There are some reports on growth and morphogenesis is remarkably affected by supplementing tissue culture medium with plant growth regulators including auxins and cytokinins. Various studies have investigated the factors which affect the plant regeneration in tissue culture including composition of culture medium, genotype and environmental factors (Fennel *et al.*, 1996; Uppal *et al.*, 1996; Mathias and Simpson, 1986). In this study, calli were used that derived from immature and matured seeds of four wheat varieties and also evaluated various culture conditions for obtaining high frequencies of somatic embryogenesis and plant regeneration in *in vitro* systems. The current study was carried out to develop a suitable protocol for *in vitro* callus induction and regeneration using mature and immature seeds of four local wheat genotypes and compared various explants and their potency on regeneration.

2. Materials and Methods

2.1 Plant Materials

Four wheat genotypes *viz.* Akbar, Barkat, Kanchan and Pavon 76 were sown during the growing season of November 2015 to January 2016 at the research field of Institute of Biological Sciences, University of Rajshahi, Bangladesh.

2.2 Explants, Sterilization and Culture

The explants were green spikes, harvested from the field grown plants that contained immature seeds (8-10 weeks old). Experiment was conducted using immature and mature seeds. Immature seeds were collected from spikes of selected wheat varieties 14 to 18 days after anthesis. At first, seeds were rinsed several times with distilled water, and then surface sterilized with 70% ethanol (v/v) for 2-3 min. Then seeds were washed 3-5 times with autoclaved distilled water in the laminar air flow cabinet.

2.3 Immature Seeds

The immature seeds were sterilized with 40% sodium hypochloride (v/v) + 1 drop Tween 20 + 1-2 drops savlon for 15-20 min by continuous shaking. Then seeds were thoroughly washed by autoclaved distilled water 4-5 times for complete removal of clorex and finally the seeds were ready for inoculation.

2.4 Mature Seeds

Mature seeds were washed by running tap water and then surface sterilized with 70% ethanol and washed 3-4 times in distilled water. Then seeds were treated with 50% of sodium hypochlorite + 1 drop Tween 20 + 1 drop savlon; and rinsed 3-4 times with sterile distilled water. Finally the sterilized seeds were plated directly in semi-solid MS medium for callus induction.

2.5 Regeneration

After callus formation, they were rescued aseptically and transferred to regeneration medium (RM). Then the regenerated shoots (approximately 3.0 cm long) were rescued carefully from the culture vessels and placed on rooting medium (RT) to develop sufficient roots. The rooted plants were cultured in pots after acclimatization and hardening.

2.6 Statistical Analysis

The average or mean values of callus induction and plant regeneration rate in wheat of mature and immature seeds were computed from three replicates with standard error (SE) and each experiment was repeated thrice. Analysis of variance (ANOVA) and list significant difference (LSD) was done by SPSS 20.0 software and MS Excel 2013.

3. Results

From immature seeds, the variety Pavon 76 showed maximum callus induction (72.25%) in MS + 2.5 mg/L 2,4-D; and the lowest value (65.50%) was recorded in MS + 2.0 mg/L for the same variety (Table 1). The variety Akbar exhibited the lowest callusing (37.78%) from immature embryo in MS + 3.0 mg/L. The effect of media and hormonal levels on CI, the varieties showed significant difference at $P < 0.01$. Three types of hormonal combinations were tested to observe regeneration efficiency of studied varieties and significant variations were found among them at $P < 0.01$ (Table 3). Out of four genotypes, the highest frequency of plant regeneration was recorded in Pavon 76 (67.00%) in combination treatment MS+ 2.0 mg/L BAP + 1.0 mg/L IAA (Table 2). On the other hand, lowest performance was found in Kanchan (43.10%) for treatment 1.5 mg/L BAP and 0.5 mg/L IAA. Besides, it was observed that Pavon 76 produced plants with highest frequency in each treatment than other genotypes. For mature seeds the efficiency of four wheat genotypes to callus induction was observed by using three hormonal combinations. All the genotypes showed significant difference with maximum frequency of callusing in Pavon 76 (69.57%) in treatment combination of MS + 2.5 mg/L 2,4-D followed by Kanchan (60.84%), Barkat (52.73%), and Akbar (47.19%) in combination MS + 2.5 mg/L 2,4-D (Table 1). The minimum value was recorded for Akbar (34.45%) in combination MS + 3.0 mg/L 2,4-D. It was observed that Pavon 76 showed highest callusing than other genotypes in all the hormonal combinations tested. Overall, the best performance with respect to callus induction was observed in Pavon 76 with 2.5 mg/L 2,4-D. On the other hand, efficient regenerations of four varieties were evaluated on MS medium with different concentrations of BAP and IAA. The results showed that Pavon 76 performed with the highest frequency (64.36%) in MS + 2.0 BAP + 1.0 mg/L IAA, when the calli were derived from mature embryos (Table 2). The other genotypes Barkat, Kanchan, and

Akbar exhibited 59.44, 52.71 and 52.32% regeneration respectively in the same medium with desired phyto-hormones. The lowest regeneration (40.63%) was recorded for Akbar in MS +1.5 BAP + 0.5 mg/l IAA. From comparison between somatic embryogenesis calli derived from immature and mature seeds of four wheat varieties it is clear that for callus induction and regeneration in both cases, immature seeds showed better performance over mature seeds (Fig. 1).

Table 1. Effect of 2,4-D on callus induction from immature and mature seeds of four wheat varieties in MS

| Explants (seeds) | Concentrations 2,4-D (mg/L) | Variety and Callus induction (% ±SE) | | | |
|---------------------|--------------------------------|--------------------------------------|---------------|---------------|---------------|
| | | Pavon 76 | Akbar | Barkat | Kanchan |
| Immature | 2.0 | 65.50 ±0.87* | 45.10 ±0.67** | 45.41 ±0.70** | 61.89 ±0.65 |
| | 2.5 | 72.25 ±0.75** | 48.35 ±0.68 | 55.95 ±0.86** | 65.76 ±0.70* |
| | 3.0 | 68.79 ±0.81** | 37.78 ±0.89* | 42.80 ±0.39 | 52.59 ±0.99** |
| Mature | 2.0 | 58.21 ±0.89 | 42.22 ±0.67 | 39.20 ±0.83 | 57.88 ±0.98* |
| | 2.5 | 69.57 ±0.76** | 47.19 ±0.78 | 52.73 ±0.76** | 60.84 ±0.79 |
| | 3.0 | 64.02 ±0.71** | 34.45 ±0.64 | 40.60 ±0.82** | 49.46 ±0.81 |

2.0 mg/l 2,4-D considered as control. Significance of mean difference from controls: $p < 0.05^*$, $p < 0.01^{**}$ according to LSD analysis.

Table 2. Overall efficiency of BAP and IAA concentrations on plant regeneration in four wheat varieties in MS medium

| Explants (seeds) | PGR's | | Variety and Plant regeneration (% ±SE) | | | |
|---------------------|---------------|---------------|--|------------|------------|------------|
| | BAP (mg/L) | IAA (mg/L) | Pavon 76 | Akbar | Barkat | Kanchan |
| Immature | 1.5 | 0.5 | 56.14±0.99 | 45.18±0.71 | 53.05±0.83 | 43.10±0.78 |
| | 2.0 | 1.0 | 67.00±0.91 | 55.22±0.93 | 63.35±0.99 | 58.63±0.85 |
| | 2.5 | 1.5 | 58.27±0.88 | 43.82±0.97 | 54.20±0.85 | 48.34±0.61 |
| Mature | 1.5 | 0.5 | 54.12±0.87 | 40.63±0.88 | 50.27±0.98 | 41.26±0.69 |
| | 2.0 | 1.0 | 64.36±0.83 | 52.32±0.82 | 59.44±0.79 | 52.71±0.63 |
| | 2.5 | 1.5 | 56.94±0.78 | 41.75±0.72 | 48.71±0.82 | 46.10±0.75 |

Values are average of three replicates (percentage); SE= Standard error; BAP= 6-benzylaminopurine; IAA= Indole-3-acetic acid.

Table 3. ANOVA on the effect of variety, callus induction and plant regeneration rate of transplantation in wheat

| Source of data | Source of variation | df | Mean sum of square of immature seed | Mean sum of square of mature seed |
|------------------|---------------------|----|-------------------------------------|-----------------------------------|
| Callus induction | Variety | 5 | 164.4844** | 182.5777** |
| | 2,4-D level | 2 | 4685.453** | 5200.853* |
| | Error | 36 | 45.83483 | 50.87666 |
| Regeneration | Variety | 5 | 148.036** | 164.32** |
| | BAP + IAA level | 2 | 4216.908* | 4680.768** |
| | Error | 36 | 41.25135 | 45.789 |

df = Degrees of freedom, Significance: $p < 0.05^*$, $p < 0.01^{**}$

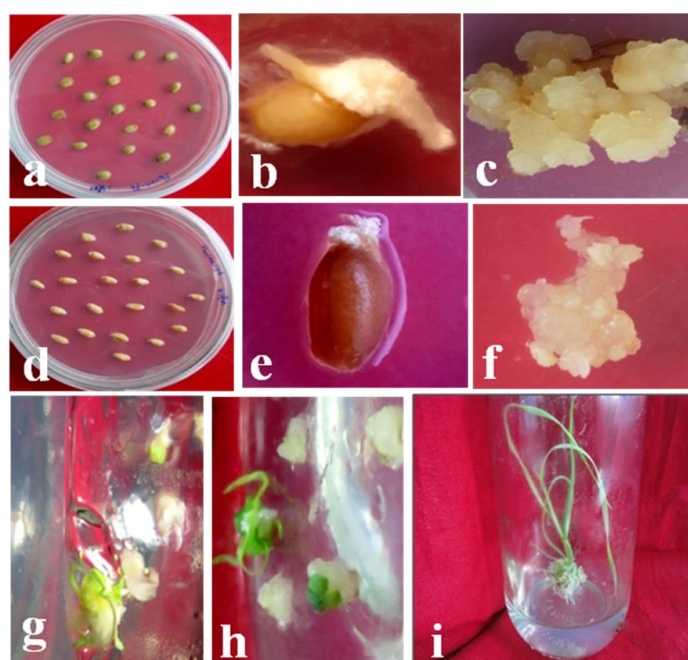


Fig. 1. Somatic embryogenesis through immature and mature seeds of wheat. a) immature seeds, b) calli initiated after 7 days of inoculation, c) 3-4 week's old calli, d) mature seeds, e) calli after 7 days of inoculation, f) calli derived from mature seeds, g) calli initiated greenish and regeneration, h) regenerated green plantlets, i) green plants with root and shoot.

4. Discussion

In this case immature and mature seeds derived calli were evaluated on regeneration efficiency in wheat. Significant effect on genotype and media including hormonal levels were found to callus induction (Table 3). The range of CI was 37.78-72.25% (immature seed) and 34.45-69.57 (mature seed) in MS medium supplemented with three concentration of 2,4-D. In both cases, Pavon 76 showed maximum callusing. Kahriz *et al.* (2017) reported that in *in vitro*

SE using plumule and radicle explants of two wheat cultivars Cakmak and Kunduru on MS induction medium amended with varying concentrations of 2,4-D. Both plumule and radicle explants were regenerative and induced variable number of somatic embryos per explants. Another study carried out by Kowalska and Arseniu (2016) to improve callus induction and plant regeneration from mature embryos of five winter wheat cultivars with various resistance levels to *P. nodorum*. For that purpose they used three type of auxins e.g. 2,4-D; 3,6-dichloro-2-methoxybenzoic acid (Dicamba); NAA, and the effect of maltose vs. sucrose were also evaluated. The results demonstrated relatively high embryogenic potential of all winter wheat cultivars they studied. Islam (2010) studied on embryoids age, size and shape for improvement of regeneration efficiency from microspore-derived embryos in wheat and reported significant effect of media and phytohormones on callus induction and regeneration that derived from anthers in wheat. The effect of different concentrations of 2,4-D for successful callus induction was reported by Munazir *et al.* (2010). They found that 4.0 mg/l 2,4-D was optimum to induce efficient callus induction for the wheat varieties of GA-02 (82.60%) and Sahar (71%). However, in some cases the frequencies of callus induction were lesser than previous reports, and it could have occurred due to different varieties of wheat. He *et al.* (1999) and Ganeshan *et al.* (2003) have documented the effect of genotypes and age of embryos on callusing. However, high (> 5 mg/L) and low (< 3 mg/L) concentrations of 2,4-D, reduced callus induction frequency and subsequent growth of calli reported by Munazir *et al.* (2010). No callus formation occurred at lower concentrations (< 2.5 mg/l) of 2, 4-D in mature wheat embryos (Yasmin *et al.*, 2001). So it could be mentioned that a suitable concentration of 2,4-D is necessary to efficiently induce callus and subsequent proliferation in tissue culture. Several authors reported optimization of 2,4-D concentrations from 2.0 mg/L to 6.0 mg/L for different wheat varieties, source of explants and culture conditions (Farooq *et al.*, 2004; Rahman *et al.*, 2008). Alizadeh *et al.* (2004) reported that gradual increase in concentration of 2,4-D, frequency of direct shoot regeneration decreased and the tendency for callus induction increased gradually. However, it was observed that higher or lower concentrations (>2.5 mg/L) of 2,4-D produced lower values of callusing than their maximum performance for all studied varieties. In this research, it has been observed that in response to plant regeneration the calli derived from immature seeds, showed significant variability on plant regeneration. The range of regenerations was recorded as (43.10-67.00%) for immature seeds and (40.63-64.36%) for mature seeds which expressed the wide range variations among the varieties. In this case maximum performances of plant regeneration were recorded in MS supplemented with 1.0 mg/L IAA + 2.0 mg/L BAP. This highest frequency (67.00 and 64.36) of plant regeneration was recorded in Pavon 76 in case of immature and mature seeds. Hence, MS supplemented with 2.0 mg/L BAP + 1.0 mg/L IAA is regarded as optimal medium for regeneration. Pathi *et al.* (2013) reported 35-90% plant regeneration by using immature seeds in MS with growth regulators. A maximum 6.36% regeneration using MS + 1.0 mg/L BAP, and 3.65% in (MS + 0) was recorded i.e. without any PGRs (Rahman *et al.*, 2008). In this study the values were higher than the previous reports. It may occur due to positive effect of optimal concentrations and combinations of PGR's along with the different genotypes.

5. Conclusion

Food security is the burning issue in the world and also in Bangladesh. Hence, it is necessary to improve food crops as well as wheat cultivars with the application of biotechnological approaches. In most cases, wheat varieties are generally recalcitrant to *in vitro* tissue culture. Hence, the present study could be exploited as a standard protocol for efficient callus induction and its subsequent regeneration. Out of four varieties, Pavon 76 showed the best callusing, and the medium MS + 2.5 mg/L 2,4-D influenced callus induction more effectively than other concentrations. On the other hand, for plant regeneration MS + 1.0 mg/L IAA + 2.0 mg/L BAP showed better performance especially for Pavon 76 (67.00% and 64.36%) from immature and mature seeds respectively. The developed system might be helpful to *in vitro* culture of wheat and related advance research on biotechnology.

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