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A N I N T E R N A T I O N A L J O U R N A L

Efficient *In Vitro* Somatic Embryogenesis and Plant Regeneration from Mature and Immature Embryos of Wheat (*Triticum aestivum* L.)

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ABSTRACT

An efficient regeneration system is a pre-requisite for the application of genetic transformation and functional genomics study of important plants. In this study, the effect of different factors (plant growth regulators, casein hydrolysate, aspartic acid and ascorbic acid) on in vitro embryogenesis and regeneration of Arta, Bahar and Zagros cultivars from mature and immature explants were investigated. Immature and mature embryos were dissected from disinfected seeds 20-25 days after pollination and imbedded mature seeds, respectively, and cultured on MS (Murashige and Skoog) *medium supplemented with different compounds. The results showed that immature embryos expose high capacity of embryogenesis and regeneration in comparison with mature embryos. There were significant differences between cultivars in terms of the percentage of callus induction and regeneration. Plant growth regulators had significant effect on percentage of callus induction in mature explants and percentage of regeneration from both explants. In immature explants, the highest percentage of regeneration (65%) was achieved with the Arta cultivar calli derived from MS medium supplemented with 1mg/L 2,4-D, 2 mg/L Picloram and 200 mg/L casein hydrolysate, and subcultured on MS medium. Also, the highest percentage of regeneration (52.38%) from mature embryo explants was achieved in the Arta cultivar with callus induction on MS medium supplemented with 1 mg/L 2,4-D, 2 mg/L Picloram and 200 mg/L casein hydrolysate and regeneration on MS medium containing 0.05 mg/L NAA.*

Key words: In vitro culture, Plant growth regulators, Somatic embryogenesis, Wheat

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INTRODUCTION

Wheat is one of the most important cereals. It cultivates on approximately 17% of the cultivatable lands and plays a key role in economic development, food security and supply, human nutrition and is an important source of calories and proteins for human [1]. While, genetic engineering of wheat provides an efficient strategy for improvement of agronomic traits such as quality and disease resistance, but the graminaceous species are recalcitrant to *in vitro* regeneration and manipulations [2, 3]. An efficient regeneration system is the main pre-requisite for the application of genetic transformation and functional genomics studies in the primomovente of important plants [4]. Somatic embryogenesis is also an important step in transformation, regeneration and rapid large scale production of healthy true-to-type plants [1, 5]. Somatic embryogenesis provide a perfect system for plant regeneration because embryogenic calli can be maintained for a long time and supply high multiplication rates [6]. Wheat is a hexaploid plant with a large genome, high copy number of DNA repeat sequences, low *in vitro* regeneration capability and difficult transformation features [7]. Therefore, one of the main limiting factors for application of biotechnological techniques in the genetic improvement of wheat is the lack of an efficient *in vitro* regeneration system for broad range of cultivars and genotypes [3]. On the other hand, establishment of an efficient regeneration system is necessary for the genetic engineering of wheat.

The explants source, genotype and medium composition were shown to influence the frequency of callus induction and regeneration in the wheat tissue culture [8-11]. In particular, genotype and explants type are important factors for embryogenic callus production and regeneration [12]. The studies showed that, the successful *in vitro* regeneration of wheat plantlets is possible from different explants such as immature embryos, mature embryos, inflorescences, anthers, and microspores [13-15]. Immature embryos were used in several regeneration and transformation studies and it has better regeneration potential than the other explants such as inflorescence, mature embryo and apical meristem [16]. But the production of immature embryo is limited by environmental conditions such as light, temperature, water, diseases, pests, growth periods and etc. The use of mature embryo has several advantages including easy handling, no time limitation and its availability in large scale [3]. Therefore, mature embryos suggested as a suitable explants for *in vitro* regeneration and genetic manipulation of cereals. [4]. Medium compositions such as plant growth regulators are important factors affecting callus induction and *in vitro* regeneration. The auxin 2,4-D (2,4-Dichlorophenoxyacetic acid) is commonly used for callus induction in cereals as well as wheat [7, 17]. The aim of this study was optimization of *in vitro* regeneration of Iranian different wheat cultivars using immature and mature embryos. Therefore, the effect of different plant growth regulators on callus induction, somatic embryogenesis and plant regeneration from three wheat cultivars were investigated.

MATERIALS AND METHODS

Plant materials

Seeds of wheat cultivars (Arta, Bahar and Zagros) were obtained from the Seed and Plant Improvement Institute, Karaj, Iran. For immature embryo explants preparation, the seeds were planted in the pots and maintained in a greenhouse at 21 ± 2 °C with a 16 h photoperiod. The immature seeds were collected 20-25 days after pollination and surface sterilized with 70% (v/v) ethanol for 30-45 seconds, 2% (w/v) sodium hypochlorite solution for 13-15 min and rinsed three times with sterile distilled

water. Immature embryos were excised from sterilized immature seeds and cultured on medium.

For mature embryo explants preparation, the mature seeds of wheat were surface sterilized with 70% (v/v) ethanol for 3 min, 2% (w/v) sodium hypochlorite for 20 min and rinsed three times with sterile distillated water. The seeds were soaked with sterile distillated water overnight at 25°C in the dark and then, mature embryos were excised and cultured on medium.

Embryogenic callus induction

In order to embyogenic callus induction, the immature and mature embryos were cultured scutellum up on MS (Murashige and Skoog) medium supplemented with different concentrations of 2,4-D, Picloram, and organic additives such as casein hydrolysate, aspartic acid and ascorbic acid (Table 1). Twenty five embryos were cultured in each Petri-dish. The cultures were maintained in a growth chamber at 25 ± 2 ˚C and dark for three weeks. Then, the percentage of callus induction, embryogenic callus and callus fresh weights were recorded.

Table 1. Culture medium used for callus induction from mature and immature embryos of wheat.

Induction medium	Composition
IM1	MS+2 mg/L 2,4-D+150 mg/L Aspartic acid
IM2	MS+2 mg/L 2,4-D+100 mg/L Ascorbic acid
IM3	MS+2 mg/L 2,4-D+200 mg/L Casein hydrolysate
IM4	MS+1 mg/L 2,4-D+2 mg/L Picloram+150 mg/L Aspartic acid
IM5	MS+1 mg/L 2,4-D+2 mg/L Picloram+100 mg/L Ascorbic acid
IM6	MS+1 mg/L 2,4-D+2 mg/L Picloram+200 mg/L Casein hydrolysate
IM7	MS+2 mg/L 2,4-D+1 mg/L Picloram+150 mg/L Aspartic acid
IM8	MS+2 mg/L 2,4-D+1 mg/L Picloram+100 mg/L Ascorbic acid
IM9	MS+2 mg/L 2,4-D+1 mg/L Picloram+200 mg/L Casein hydrolysate
IM10	MS+4 mg/L 2,4-D+150 mg/L Aspartic acid
IM11	MS+4 mg/L 2,4-D+100 mg/L Ascorbic acid
IM12	MS+4 mg/L 2,4-D+200 mg/L Casein hydrolysate

Plant regeneration

After 3 weeks, the calli obtained from immature and mature embryos were sub-cultured on regeneration medium. The regeneration medium consisted of MS medium without hormone (RM1, Table 1) and supplemented with 0.05 mg/L NAA (Naphthalene acetic acid) (RM2, table 1). The cultures were maintained in a growth chamber at 25 ± 2 ˚C and 16 h photoperiods. Percentage of rooting and regeneration of plantlets were recorded after three weeks after culture. For further growth, the regenerated plantlets were transferred to MS medium for three weeks and then to pots containing sterilized Peat Moss, and were kept in a high humidity conditions under plastic bags for adaptation. In all cases, the pH of medium was adjusted to 5.8, solidified with 8 g/L plant agar and autoclaved at 121.5 °C for 15 min. The explants and calli were cultured on 30 ml of medium in 100 × 25 mm Petri plates and wrapped with Parafilm.

Experimental design and data analysis

All experiments were performed as factorial experiment based on completely randomized design (CRD) with three replications and 25 explants per replicate. Data analyses were performed using IBM SPSS Statistics (Version 22.0 (Armonk, NY, USA)). Mean comparisons were carried out using Least Significant Difference (LSD) and Duncan's multiple range test at probability level of 0.05.

RESULTS

Callus induction from immature embryo explants

Callus induction response was quick, so that most of the explants exhibited rapid swelling and cell proliferation 3-4 days after culture leading to the formation of friable embryogenic yellow callus within 1-2 weeks (Fig. 1C). Analysis of variance (ANOVA) indicated that there was significant differences (p<0.01) between the wheat cultivars in percentage of callus induction. But callus induction medium and its interaction with cultivar did not show significant effects on the percentage of callus induction. All of the tested cultivars displayed a high percentage of callus induction (97.58-100%) 21 days after culture. The Zagros cultivar showed an excellent (100%) callus induction frequency (Fig. 2A).

Callus fresh weight was significantly influenced by interaction of callus induction medium and cultivar, while medium and cultivar did not show significant effect on this trait. The mean of callus fresh weight ranged from 46.64 to 132.89 mg per callus. The highest amount of callus fresh weight (132.89 mg) was achieved with Arta cultivar on MS medium supplemented with 2 mg/L 2,4-D and 200 mg/L casein hydrolysate (Fig. 3).

Plant regeneration from immature embryo explants

About 3 weeks after culture of immature embryos, the calli were transferred to the regeneration medium. According to the ANOVA results, the percentage of embryogenic callus was significantly influenced by different callus induction medium and their interaction with cultivars. While it not influenced by cultivars, regeneration medium and their interactions. Among different callus induction medium, the highest percentage of embryogenesis achieved from the calli derived from the IM7, IM9 and IM12 media (Table 1 and Fig. 2B).

Different stages of somatic embryogenesis and plantlet regeneration were shown in fig. 1. The results indicated that the percentage of rooting were significantly (p<0.01) affected by cultivar, callus induction medium and their interaction. But there were no significant differences between different regeneration media. The percentage of rooting in the Arta cultivar (87.45%) was significantly higher than that of the Bahar and Zagros cultivars. In addition, the highest percentage of rooting in the Arta cultivar was obtained with IM1 and IM4; with IM7 in the Bahar cultivar and with IM1, IM2 and IM6 in the Zagros cultivar (Table 2). Therefore, in the Arta and Bahar cultivars the combination of low concentrations of 2,4-D with aspartic acid were more effective on rooting as compared with their combinations with ascorbic acid and casein hydrolysate.

The percentage of plant regeneration significantly affected by cultivar, callus induction medium, and cultivar × callus induction medium and cultivar × callus induction medium × regeneration medium interactions. While, there was no significant differences between various regeneration medium. The percentage of regeneration in the Arta cultivar (36.02%) was higher than that of the others. Overall, the low concentration of 2,4-D in combination with casein hydrolysate had favorable effect on regeneration, so that, MS medium supplemented with 1 mg/L 2,4-D, 2 mg/L Picloram and 200 mg/L casein hydrolysate (IM6) showed the highest percentage of plant regeneration (Table 2). The highest percentage of plant regeneration in the Arta cultivar (65%) was obtained with the calli derived from IM6 callus induction medium and subcultured on RM1 (MS medium without hormone) regeneration medium. But, the highest percentage of regeneration in the Bahar (51.59%) and Zagros (47.78%) cultivars were obtained with the calli derived from IM11 and IM1 induction medium and subcultured on RM2 (MS medium containing 0.05 mg/L NAA) regeneration medium, respectively (Table 2).

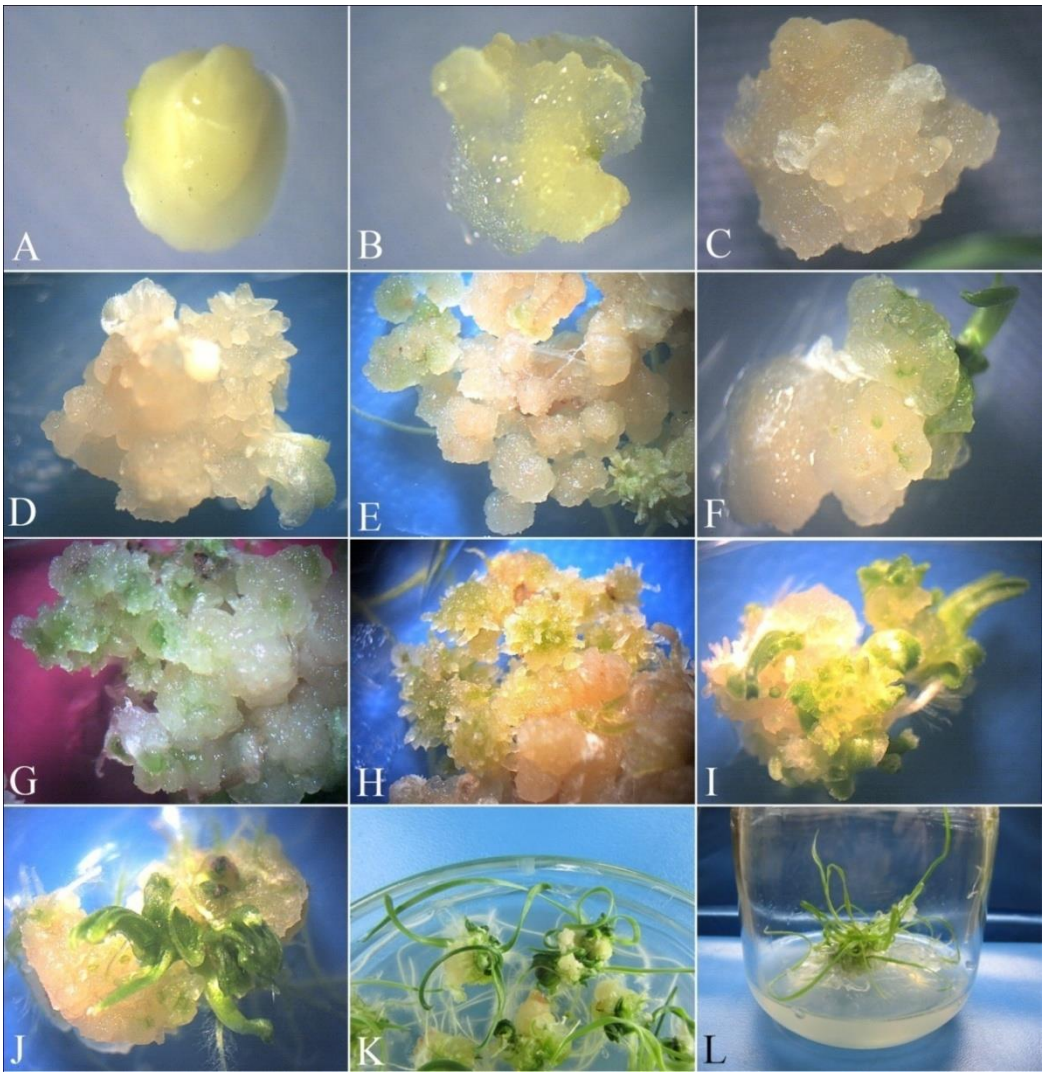

Figure 1. Different stages of somatic embryo induction and development from immature embryos of wheat. A) Immature embryo, B) Callus formation 3-4 days after culture on callus induction medium, C) Embryogenic callus, D,E,F) Different stages of somatic embryo development, G, H, I) Different stages of rooting and initiation of shoot formation, J, K) Plantlets regeneration 2 and 3 weeks after transferring to regeneration medium, L) Regenerated plantlet on MS medium

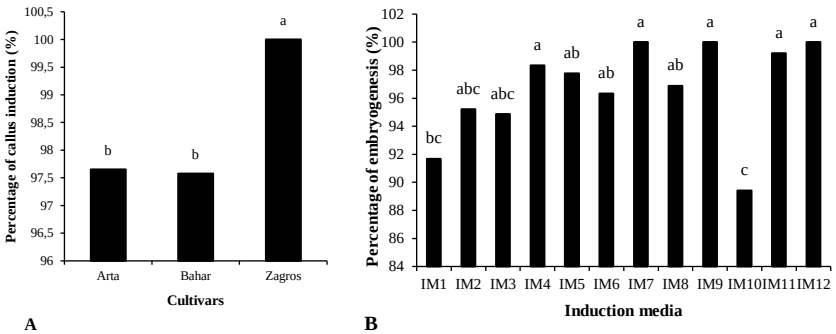

Figure 2. A) Percentage of callus induction from immature embryos of different wheat cultivars. B) Effect of different callus induction medium on percentage of embryogenesis from immature embryos of wheat

Table 2. The effect of different cultivars, callus induction and regeneration medium on plantlet regeneration and rooting of wheat immature embryos

Cultivar	Induction medium	Regeneration medium	Percentage of regeneration	Percentage of rooting	Cultivar	Induction medium	Regeneration medium	Percentage of regeneration	Percentage of rooting	Cultivar	Induction medium	Regeneration medium	Percentage of regeneration	Percentage of rooting
Arta	IM1	RM1	25.18	100	Bahar	IM1	RM1	20.83	81.55	Zagros	IM1	RM1	18.05	100
		RM2	35.61	100			RM2	23.15	87.96			RM2	47.78	100
	IM2	RM1	57.41	100		IM2	RM1	9.52	71.43		IM2	RM1	14.44	100
		RM2	49.35	95.83			RM2	9.52	73.21			RM2	35.56	100
	IM3	RM1	51.85	96.30		IM3	RM1	8.93	63.09		IM3	RM1	30.56	94.44
		RM2	42.65	87.50			RM2	28.57	76.19			RM2	31.75	84.92
	IM4	RM1	43.33	100		IM4	RM1	7.41	100		IM4	RM1	7.14	100
		RM2	35.00	100			RM2	4.17	69.05			RM2	11.11	88.89
	IM5	RM1	48.18	90.30		IM5	RM1	17.78	66.67		IM5	RM1	20.74	100
		RM2	52.42	96.97			RM2	22.22	100			RM2	6.67	96.67
	IM6	RM1	65.00	100		IM6	RM1	23.33	81.11		IM6	RM1	28.89	100
		RM2	40.00	93.33			RM2	0.00	75.00			RM2	45.45	100
	IM7	RM1	50.00	77.78		IM7	RM1	5.56	94.44		IM7	RM1	35.32	90.48
		RM2	38.89	72.22			RM2	11.11	77.78			RM2	19.25	71.82
	IM8	RM1	3.70	81.48		IM8	RM1	15.00	80.00		IM8	RM1	11.11	72.22
		RM2	25.18	64.44			RM2	26.18	75.40			RM2	4.17	72.50
	IM9	RM1	25.00	75.00		IM9	RM1	5.56	67.78		IM9	RM1	33.33	80.95
		RM2	32.50	80.00			RM2	26.67	66.67			RM2	9.52	84.13
	IM10	RM1	0.00	81.48		IM10	RM1	13.33	46.67		IM10	RM1	5.56	50.00
		RM2	0.00	53.33			RM2	8.33	61.11			RM2	16.67	50.00
	IM11	RM1	44.44	95.83		IM11	RM1	27.78	72.22		IM11	RM1	16.67	25.00
		RM2	29.17	83.33			RM2	51.59	88.89			RM2	16.67	33.33
	IM12	RM1	53.97	85.71		IM12	RM1	27.78	61.11		IM12	RM1	8.33	28.33
		RM2	15.74	87.96			RM2	25.56	65.55			RM2	0.00	38.33
$LSD_{5\%}$													23.52	25.35

Callus induction from mature embryo explants

The results indicated that, the percentage of callus induction from mature embryos significantly ($p<0.01$) affected by cultivar, callus induction medium and their interaction. The mean percentage of callus induction ranged from 96.21% in the Zagros cultivar to 100% in the Bahar cultivar. As shown in fig. 3, the callus induction of Zagros cultivar in the MS medium supplemented with 2 mg/L 2,4-D and 150 mg/L aspartic acid (IM1), and MS medium supplemented with 1 mg/L 2,4-D, 2 mg/L Picloram and 200 mg/L casein hydrolysate (IM6) were significantly lower than those of other cultivars. While, the percentage of callus induction of cultivars on the other callus induction medium did not significantly different from each others. It means that, the effect of different compounds depended upon the cultivar (Fig. 4). Although the callus fresh weight was not significantly influenced by cultivar, callus induction medium and their interaction. The mean of callus fresh weight was varied from 80.63 mg in the Arta cultivar to 93.74 mg in the Zagros cultivar. Also, the response of different cultivars to various callus induction media were different.

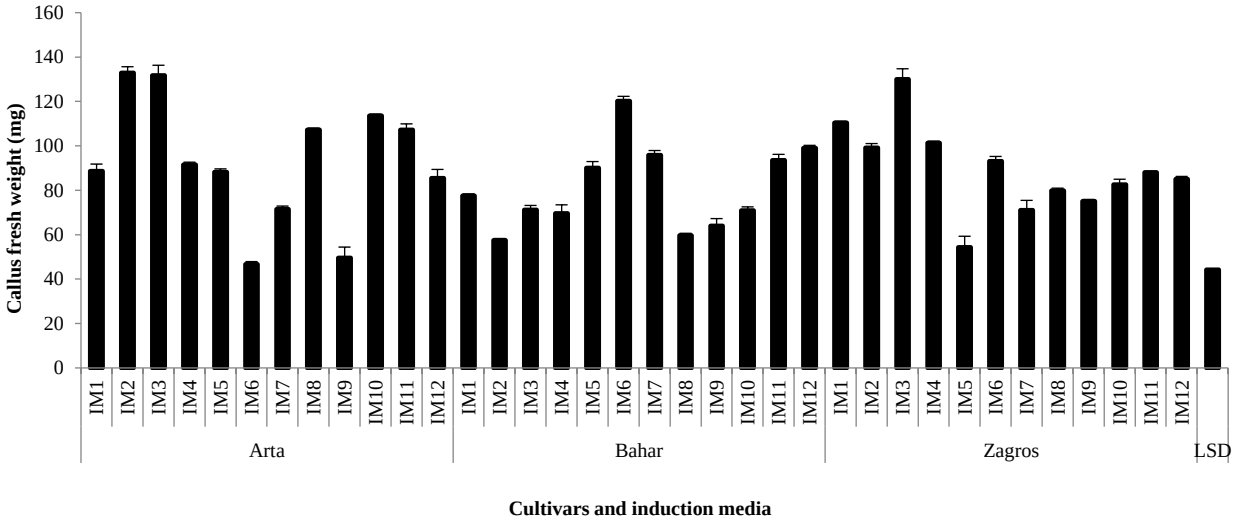

Figure 3. Effect of different callus induction medium on callus fresh weight from immature embryos of wheat cultivars

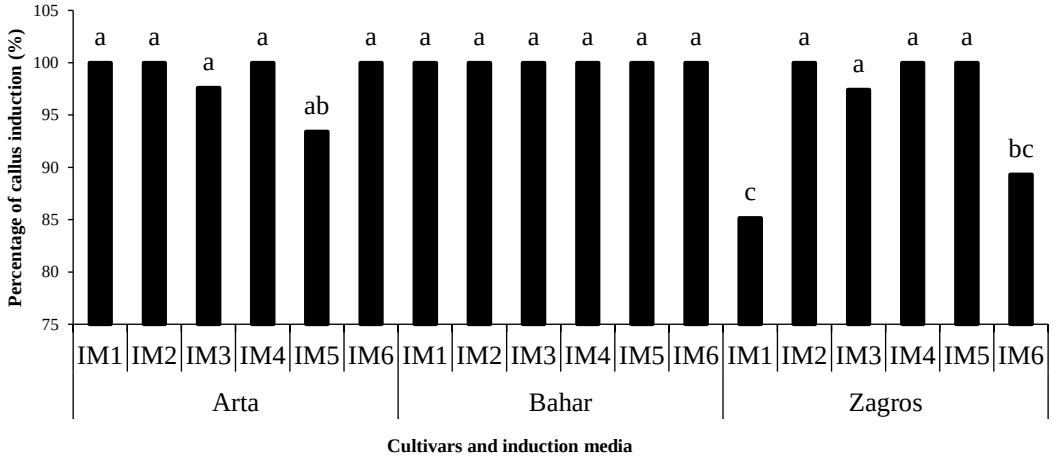

Figure 4. Effects of different cultivars and callus induction medium on percentage of callus induction from mature embryos of wheat

Plant regeneration from mature embryo explants

The results indicated that there were no significant differences in the percentage of embryogenic callus between different cultivars, callus induction and regeneration media. The percentage of embryogenic callus ranged from 86.67% to 100%. Root formation (Rooting) from the most of the embryogenic calli was occurred after transferring to the regeneration medium. According to the ANOVA, percentage of rooting after transferring to regeneration medium was significantly ($p<0.01$) affected by cultivar, callus induction medium and their interaction, but did not significantly different between regeneration media. Overall, the percentage of rooting in the Bahar cultivar was higher than that of the other cultivars. As shown in table 3, addition of casein hydrolysate to callus induction medium decreased the percentage of rooting in the Arta and Zagros cultivars. However, in the Arta cultivar the presence of picloram in the callus induction medium (IM6) compensate the negative effects of casein hydrolysate on rooting (Table 3).

The percentage of plant regeneration was only affected by callus induction medium. While, there were no significant differences between different cultivars and

regeneration medium with respect to regeneration capability. The average of regeneration percentage was varied from 6.67 to 52.38%. Overall, among the tested callus induction medium, the highest percentage of plant regeneration was occurred on MS medium containing 1 mg/L 2,4-D, 2 mg/L Picloram and 200 mg/L casein hydrolysate (Fig. 5). The highest percentage of plant regeneration in the Arta (52.38%), Bahar (44.44%) and Zagros (27.78%) cultivars were obtained on RM2 regeneration medium from the calli derived from IM6, IM4 and IM3 callus induction medium, respectively (Table 3).

Table 3. Effect of different cultivars, callus induction and regeneration medium on regeneration and rooting of wheat mature embryos

Cultivar	Induction medium	Regeneration medium	Percentage of regeneration	Percentage of rooting	Cultivar	Induction medium	Regeneration medium	Percentage of regeneration	Percetage of rooting	Cultivar	Induction medium	Regeneration medium	Percentage of regeneration	Percetage of rooting
Arta	IM1	RM1	23.81	85.71	Bahar	IM1	RM1	14.29	73.81	Zagros	IM1	RM1	10.37	100
		RM2	26.19	95.24			RM2	16.67	83.33			RM2	14.81	100
	IM2	RM1	13.33	100		IM2	RM1	25.00	100		IM2	RM1	23.21	95.24
		RM2	31.11	100			RM2	8.33	100			RM2	19.05	100
	IM3	RM1	9.52	50.00		IM3	RM1	33.33	100		IM3	RM1	15.08	95.24
		RM2	10.32	54.76			RM2	8.33	100			RM2	27.78	72.22
	IM4	RM1	20.00	100		IM4	RM1	25.00	100		IM4	RM1	20.00	100
		RM2	26.67	100			RM2	44.44	100			RM2	6.67	100
	IM5	RM1	16.06	94.66		IM5	RM1	27.98	100		IM5	RM1	13.33	100
		RM2	31.67	96.67			RM2	22.29	92.59			RM2	8.33	93.33
	IM6	RM1	33.33	100		IM6	RM1	37.30	100		IM6	RM1	20.83	71.21
		RM2	52.38	100			RM2	33.33	100			RM2	22.62	91.07
$LSD_{5\%}$													26.22	15.09

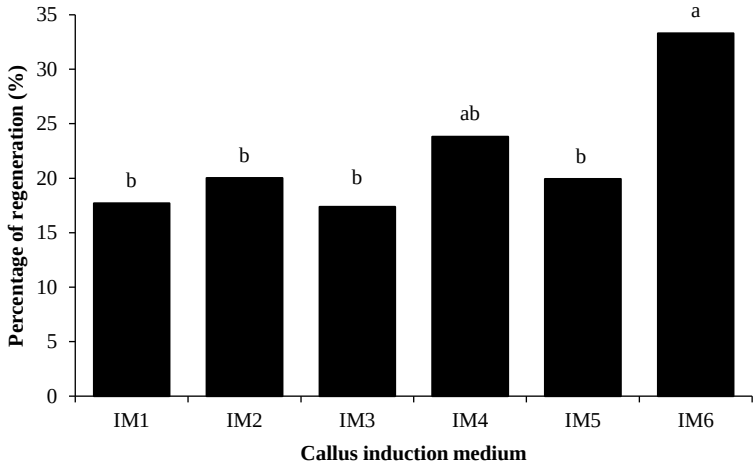

Figure 5. Effect of different callus induction medium on plantlet regeneration from mature embryo-derived calli in wheat

DISCUSSION

It has been reported that the frequency of callus induction and in vitro regeneration in wheat are highly dependent on the plant genotype and its interaction with regeneration protocol [6, 17]. Optimization of an efficient *in vitro* regeneration system is a prerequisite for the protocols which currently used for successful genetic

transformation of wheat cultivars. This situation limits the application of biotechnological techniques for the genetic improvement of wheat cultivars. In the present study, *in vitro* regeneration of three Iranian wheat cultivars was achieved using mature and immature embryos, different callus induction and regeneration medium. Different cultivars showed substantial differences in the ability of embryogenic callus induction, callus growth (fresh weight of callus), rooting and regeneration. The variation in regeneration efficiency of genotypes could be attributed to the differences in genetic programming and reprogramming of embryogenically cells, which usually influenced by different external factors [8]. Therefore, we used different external factors such as different callus induction and regeneration medium compositions to overcome the genotype influence and increase the plant regeneration efficiency. Cultivars exhibited different percentage of embryogenesis and regeneration on different induction and regeneration medium, but regeneration capacity was still genotype dependent. The Arta cultivar showed higher regeneration ability than others (Table 2 and 3). This result is consistent with the reports of Sears and Deckard [18] and Özgen et al. [19]. Filippov et al. [8] reported that overall morphogenic capacity of different genotypes of spring and winter wheat dependent on genotype. Bi et al. [20] have been compared different genotypes of wheat and reported significant difference in callus induction, embryogenic callus differentiation, plantlet regeneration and culture efficiency.

Immature embryos are most favorable explant for efficient regeneration of whole plants [21, 22]. Thus, in most reports on transgenic wheat plant production, immature embryos are utilized as explant for delivery of foreign DNA [23]. It is difficult to obtain immature embryos throughout the year, and their suitable stages for culture are also limited [19]. But, mature embryos are available at all times but they were not frequently used for embryogenic culture initiation because of their low frequencies of callus induction [24, 25]. In this study immature and mature embryos were tested for somatic embryogenesis and regeneration of the three cultivars of wheat. In general, the frequency of callus induction and callus growth (callus fresh weight) of the wheat cultivars from immature embryos were better than from mature embryos (Fig. 2, 3, 4 and 5). This result is consistent with Khurana et al. [26]. They showed that immature embryo had higher callus formation than mature embryo. On the contrary, Özgen et al. [19] reported that mature embryo had high callus induction frequency.

The overall average of plant regeneration percentage in the calli formed from immature embryos was 24.43% and in calli formed from mature embryos was 22.02%. Thus, there was no considerable significances between the overall regeneration capacity of immature and mature embryos (Table 2 and 3). High regeneration efficiency with using immature embryo in bread wheat have been reported by Rasco-Gaunt et al. [27] and Ozias-Akins and Vasil [28], while Khurana et al. [26] reported lower regeneration ability in immature embryos than mature embryos.

Plant growth regulators are other important factors affecting callus induction and somatic embryogenesis in plant tissue cultures [3, 29]. The auxin type and concentration is the most important factor of medium which determines the somatic embryogenesis in the cereals [9]. In most studies, the researchers used 2,4-D as an exogenous auxin for callus induction and somatic embryogenesis of wheat and other cereals [19, 30]. 2,4-D was used successfully for callus induction in numerous experiments but this type of auxin at higher concentrations increases chromosomal instability and cause to somaclonal variation [31]. Therefore, in subsequent studies dicamba and picloram were used instead of 2,4-D [32]. In this study, two different synthetic auxins (2,4-D and picloram) in combination and alone were used for callus

induction and somatic embryogenesis. Since higher concentration of 2,4-D had negative effect on somatic embryo development, low concentration of 2,4-D in combination with high concentration of picloram, were used for callus induction and somatic embryogenesis. As a result, the highest percentage of plant regeneration was obtained from embryogenic calli induced on MS medium supplemented with 1 mg/L 2,4-D, 2 mg/L picloram and 200 mg/L casein hydrolysate (Table 2 and 3). This results is consistent with the reports of Przetakiewicz et al. [9]. They reported that dicamba alone or in combination with picloram and 2,4-D were the best medium for embryogenic callus formation in the wheat genotypes. Kachhwaha et al. [33] compared the picloram and 2,4-D effects on somatic embryogenesis and plant regeneration of barley and showed that picloram gave much better results. Tao et al. [16] reported that substitution of 2,4-D with dicamba enhances the growth and regeneration capacity of wheat. Kordestani and Karami [34] reported that the maximum embryogenesis in strawberry was obtained by using 2 mg/L picloram.

CONCLUSION

We evaluated the *in vitro* regeneration of wheat cultivars using mature and immature embryos. In general, the frequency of plant regeneration from immature embryos was slightly higher than that of mature embryos. But, cultivation of Arta and Bahar mature embryos on embryogenic callus induction medium IM6 and IM4, respectively, and subculture of the embryogenic calli on RM2 (MS medium supplemented with 0.05 mg/L NAA) led to relatively high plantlet regeneration frequency, which was comparable with their regeneration frequency using immature embryos. Thus, with regard the availability of mature embryos throughout the year, this protocol can be used for clonal propagation and genetic transformation of wheat cultivars.

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