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RESEARCH ARTICLE

Effects of plant growth regulators and organic compounds on callus induction and plant regeneration in wheat (*Triticum aestivum* L.) using mature embryos

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ABSTRACT: To establish a highly efficient plant regeneration system for some Iranian wheat, this study was done. MS mediums supplemented with different combinations of 2, 4-D, NAA, Dicamba, and different combinations of Thidiazuron and BAP, were evaluated for callus induction and regeneration respectively. The results showed that the efficiency of mature embryo for tissue culture response was significantly influenced by the genotypes, the auxins types and concentrations. Medium supplemented with 2 mg/L Dicamba give the best response on callus inducing and also gave the highest rate of plant regeneration. Regeneration response mainly affected by auxins types and concentrations on callus induction medium. There was no significant effect of cytokinin treatments on regeneration properties. Three different organic compounds were used to improve callus induction and plant regeneration response of elite cultivars. The results showed that the Casein hydrolyzed at 3 g/L and Tryptone at 5 g/L had greatest impact on callus induction and plant regeneration of wheat respectively.

KEYWORDS: callus induction, casein hydrolyzate, mature embryo, plant regeneration, Tryptone and yeast extract

INTRODUCTION

The global production of wheat is 766.5 million tons [6], and world wheat trade was nearly $ 58 billion in 2020. Wheat is a major food staple for more than 2.5 billion people around the world, and the first source of protein and second source of calorie in the North Africa, West Asia and Central Asia [5]. Wheat with 71.2 percent carbohydrates, 12.6 percent proteins, vitamin B, and fiber play a critical role as a source of energy [10]. In Iran, same to other countries in the world, Wheat has the largest area cultivation (6).

Although production of wheat is increased in all over the world in recent years, some biotic and abiotic stresses are a major menace to its production [9, 25]. Biotechnological methods such as genetic transformation and *in vitro* selection as a new strategy can improve it productivity [30]. Many plant species including cereals have a lower response to in-vitro regeneration be study carefully, and after obtaining a reproducible method of regeneration, gene transfer can be done [31].

Gene transfer is a powerful tool that is greatly used to improve the product and molecular biology of plants such as rice, maize and wheat. However, the development of gene transfer in wheat has been associated with problems that part of it is due to poor response of plants to regeneration in *in vitro* condition [11].

The callus induction and plant regeneration in wheat affected by genotype [15, 31], explant source [21], the growing conditions of donor plants and component of culture medium [8, 17]. Different explants like immature and mature embryos [12, 22], inflorescence [12], and

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anthers [28] have been used for callus formation and plant regeneration of wheat. Therefore, optimizing of an effective callus induction and plant regeneration medium for local genotypes is a critical step in genetic transformation programs of wheat. Besides the basal culture medium and growth regulators contents, supplementation of medium with an organic nitrogen sources such as Tryptone, yeast extract and casein hydrolysate have been reported to promote callus induction and somatic embryogenesis in plants. However, to date, there have been no reports regarding the effect of these nutritional supplements on the tissue culture of Wheat. The aim of this experiment was optimization of a reliable protocol for in vitro regeneration of some Iranian cultivated wheat using mature embryos.

MATERIALS AND METHODS

Explants preparation

Seeds of four Iranian cultivated wheat varieties including Kasghogen, Pishgham, Gonbad and Aflak were obtained from Plant Breeding and Biotechnology Department of Tabriz University. Seeds were sterilized with 70% ethanol for 1 min, 2.5% sodium hypochlorite, for 10 min. The seeds were then thoroughly washed 4-5 times with sterile water and the Washing was given by continuous shaking. Sterilized seeds were soaked in sterile distilled water for 24 hours. The mature embryos were aseptically isolated from the freshly imbibed seeds with a sharp scalpel and ten mature embryos were cultured with scutellum side up in petri dish with 10 cm diameter containing 25milliliters of culture medium [19].

Callus induction

Isolated embryo from four genotype transferred to the MS medium [19] supplemented with six different concentrations and combinations of three auxins (Table 1). All media contain 30 g/L sucrose and solidified with 7 g/L agar. The media were adjusted to pH= 5.8 and autoclaved for 15 minutes at 121°C. Petri dishes were sealed with polyethylene film and were placed in a dark growth culture room, at 25°C ±2. The cultures were maintained up to 8 weeks with sub-culturing once in 15 days to fresh medium with similar growth regulators contents for further proliferation of explant as well as to proliferation of calluses. After six weeks, the fresh weight of callus and callus produced explants were recorded for all replications of treatments.

Plant Regeneration

Callus from four selected callus induction medium (Table 2) were separately transferred to regeneration medium. The regeneration media consisted of MS medium supplemented with 2 mg/L BAP or 1 mg/L TDZ and their combination for plant regeneration. The cultures were placed in a growth room under a photoperiod of 16 h light/8h, at 25°C ±2°C). Explants were transferred to fresh medium consisted of same combination of plant growth regulators every two weeks. After two months the shoot produced explant and the mean number of shoots per explant were recorded for all treatments in all replications.

For further improvement of callus induction and plant regeneration capacity, three superior cultivars according their regeneration capacity that obtained from regeneration experiment were selected and effect of three different types of organic compounds including Casein hydrolyzate (3,8 g/L), Tryptone (3.5 g/L and Yeast extract (750 mg/L and 1.5 g/L) was evaluated. In this experiment MS media along with 2 mg/L Dicamba and combination of 2 mg/L BAP plus 1 mg/L TDZ were used as a callus induction and regeneration medium respectively.

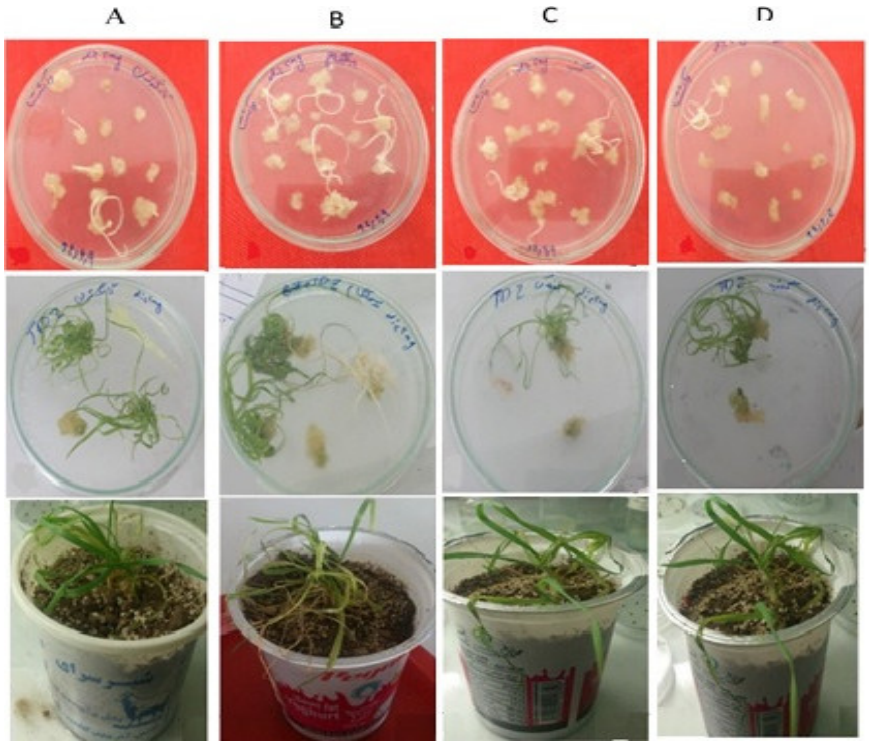
Experimental Design and Statistical Analysis

Factorial experiments based on completely randomized design with three repeat, each containing ten embryos in 10 cm Petri Dish, were used for analysis of variance for all three independent experiment. Data were statistically analyzed by MSTAT_C program. Means were separated according to Duncan's multiple range tests (Duncan, 1955) at 5% probability level.

RESULTS

Callus induction

The mature embryo cultured on all medium produced callus only after 2 week of culturing (Fig.1). After 4 weeks an optimum level of callus formation was achieved by adding 2 and 4 mg/L Dicamba. The highest amount (0.268g) of callus was observed on media supplemented with 2 mg/L Dicamba for Pishgham. When the concentration of Dicamba was increased from 2 to 4 mg/L, callus weight was decreased significantly. The lower amount of callus induction (0.058, 0.06g) was observed with Aflak in media supplemented with 2, 4-D or NAA combination with 2, 4-D (Table 1).



Figurer 1. Callus induction, plant regeneration and plantlet transferred into soil of four cultivar of wheat respectively, A: Kasghogen, B: Pishgham, C: Gonbad and D: Aflak.

Table 1. Effect of growth regulators and genotype on callus weight (g).

Combination of auxins	Cultivars			
	Ghonbad	Pishgham	Kasghogen	Aflak
2,4-D 2 mg/L	0.127fgh	0.157cdef	0.071i	0.06i
NAA 1mg/L + 2,4-D 2mg/L	0.145efgh	0.182bc	0.064i	0.058i
Dicamba 2 mg/L	0.246a	0.268a	0.255a	0.179bcd
Dicamba 4 mg/L	0.148defg	0.125fgh	0.240a	0.123gh
Dicamba 2mg/L + 2,4-D 2 mg/L	0.115h	0.196b	0.195b	0.173bcde
Dicamba 4mg/L + 2,4-D 2 mg/L	0.132fgh	0.166bcde	0.188bc	0.117gh

Means with the same letter(s) in the same column are not significantly different at 5% using Duncan multiple range test.

Table 2. Effect of auxins type and concentration on plant regeneration of mature embryos of wheat cultivars.

		Plant regeneration rate (%)				Number of shoot per explant			
		Ghonbad	Pishgham	Ksghogen	Aflak	Ghonbad	Pishgham	Ksghogen	Aflak
Dicamba 2 mg/L	BAP	100 a	66 e	50 i	55g	3.6 b-h	3.22 b-i	1 h_l	2.6 c-l
	TDZ	100a	58.33 f	55 g	44j	9.6 a	1.66 e-l	3.1 b-j	2.2 d-l
	BAP+TDZ	100 a	91.67 b	27.67 l	65e	4 b-e	5.08 bc	0.27 l	4.3 b-d
Dicamba 4 mg/L	BAP	88.67 c	58.33 f	55 g	22m	3.6 b-g	3.33 b-i	0.77 i-l	0.77 i-l
	TDZ	77.33 d	33 k	11 n	33k	5.5 b	0.45 kl	0.11 l	0.55 j-l
	BAP+TDZ	49.67 i	22 m	58f	33k	3.8 b-f	0.55 j-l	3.27 b-i	2.99 c-k
Dicamba 2mg/L + 2,4-D 2 mg/L	BAP	100 a	44 j	55g	11n	1 h-l	1.1 g-l	1.44 e-l	0.33 l
	TDZ	100 a	44 j	55g	66e	4 b-e	0.85 i-l	2.22 d-l	1.33 f-l
	BAP+TDZ	100 a	55 g	55g	66e	3 c-k	1.33 f-l	1.44 e-l	1 h-l
Dicamba 4mg/L + 2,4-D 2 mg/L	BAP	52.33 h	22 m	55g	0o	2.1 d-l	0.22 l	1.66 e-l	0. m
	TDZ	33 k	33 k	66e	20m	0.99 h-l	0.44 j-l	2.21 d e-l	0.20 l
	BAP+TDZ	55g	55.33 g	55g	11n	0.55 j-l	1.33 f-l	1.44 e-l	0.33 kl

Means with the same letter(s) are not significantly different at 5% using Duncan multiple range test.

Plant regeneration

the Callus obtained from callus induction medium transferred to MS medium supplemented with 1mg/L TDZ or 2mg/L BAP and combination of both for plant regeneration. The green spots from cultured callus was observed 2 weeks after inoculation in all treatments except for calluses from 2,4-D and NAA plus 2,4-D supplemented medium, which didn't show any signal for green organ formation. Plantlet with leaves was developed after 4 weeks. The frequency of the shoot regeneration was mainly influenced by the concentration of growth regulators and their combination in callus induction medium. There was significant interaction between genotype and plant growth regulator combination both in callus induction and regeneration media. Genotype were showed the different response to in-vitro regeneration, independent of culture media (Fig.1). Maximum regeneration response (100%) was obtained on Gonbad on MS medium supplemented with BAP, TDZ and the combination of both. Maximum number of shoots (9.6) were obtained on Gonbad on MS medium supplemented with TDZ (Table 2).

The Effect of the Organic compounds on callus induction and plant regeneration

Effect of three different of supplement on improvement of property of callus induction and regeneration were investigated in 3rd experiment. There was no interaction between genotypes and organic compound in both recorded traits, callus induction and regeneration properties. The highest amount of callus weight (0.230 gr) was obtained on MS medium supplemented with 3 g/L Casein hydrolyzat and lowest amount of callus weight (0.152) was achieved on MS medium supplemented with 1.5 g/L Yeast extract (Table 3). The highest shoot number (2.4) was achieved on MS medium supplemented with 5 g/L Tryptone (Table 3). Among the cultivar Kasghogen (0.231) has maximum callus weight and Gonbad (0.172) has minimum callus weight (Table 4).

DISCUSSION

There are many factors affecting in-vitro regeneration of wheat including type and age of explant, culture medium component, and genotype [32]. Using mature embryo as an explant for in-vitro regeneration of wheat was reported [29, 24]. According to Ozgen et al. [22] and Jasdeep et al [10] mature embryos have great advantage for wheat tissue culture, science it has high potential to callus induction and regeneration and available in all seasons. In present study efficiency of tree auxins including 2, 4-d, NAA and Dicamba was evaluated for callus induction in four cultivar of Iranian wheat. Our experiment showed that Dicamba in an average concentration had highest efficiency on callus induction of wheat but when the concentration of Dicamba increased from 2mg/L to 4mg/L, callus productivity decreased significantly. There was no significant difference between varieties and treatments for callus induction rate. Li-li et al. [14] and Baday [3] reported that although genotype had the major effect on callus induction of wheat but replacement of Dicamba for 2,4-d has better result in in-vitro culture of wheat. Mendoza and Keappler [18], Chen et al. [4], Malik et al. [16] and Yu et al. [33] reported the callus induction with high efficiency using mature embryos as an explant. Callus induction were happened in all treatments with all combination of plant growth regulators except for plant growth regulator free medium. Our result was consistent with others. [1, 7, 24].

Effect of two cytokinins, BAP, TDZ and combination of them, on regeneration response of callus was evaluated. Analysis of variance showed that there was no significant difference between them in regeneration rate and number of shoots. Percent of regeneration and shoot production was influenced significantly by plant growth regulators content in callus induction medium and genotype. Other reports showed that genotype had significant effect on callus production, somatic embryogenesis, and plant regeneration properties of wheat [10, 20]. In recent study it was observed, that comparison with other auxins, Dicamba had a great impact on the plant regeneration. Miroshnichenko et al. [17] reported that the callus was induced with Dicamba had more regeneration potential in comparison with 4-CPA.

Although 2, 4-d has been successfully use in most of the researches for callus induction, but chromosomal aberrations and somaclonal variations have been increased with this hormone. Therefore, recently 2, 4-D has been replaced with an alternative strong auxin, dicamba which is supposed to be more quickly metabolized in wheat tissue and therefore is very important for rapid development of somatic embryos from mature embryo-derived callus [7, 16]. The callus formation increased with boosting in the concentration of Dicamba while the generation of the callus derived embryo was inversely decreased with high concentration of Dicamba [7, 16]. Dicamba at the highest levels (6mg/L) reduced precocious germination of the cultured

Table 3. Effect of organic compounds concentration on callus weight and plant regeneration.

Treatment	concentration	Callus weight (gr)	Shoot per explant
Control	0	0.197 ab	1.74 ab
Casein hydrolyzate	3g	0.230 a	1.35 ab
	8g	0.204 ab	1.18 ab
Tryptone	5g	0.194 ab	2.4 a
	3g	0.194 ab	1.86 ab
Yeast extract	750 mg	0.193 ab	1.6 ab
	1.5 g	0.152 b	0.89 b

Means with the same letter(s) are not significantly different at 5% using Duncan multiple range test.

Table 4. Callus induction and plant regeneration efficiency of wheat cultivars

Cultivar	Callus weight (gr)	Number of shoot per explant
Kasghogen	0.231a	1.5a
Pishgham	0.181b	1.95a
Ghonbad	0.172b	1.55a

Means with the same letter(s) are not significantly different at 5% using Duncan multiple range test.

mature embryo and induced embryogenic callus more effectively [7, 16]. Our results showed that adding the Dicamba supplemented with 2, 4-D improved callus induction in elite genotypes. According to Li-li, et al [13] auxins differentially influenced callus induction in different genotypes and in some varieties using 2,4-D and for others using Dicamba as an uxine has a greater impact. Regeneration property was mainly influenced with callus induction mediums. Applying average amount of all two cytokines were critical for regeneration but didn't differ significantly regeneration response of wheat callus in three type of regular Cytokinins including TDZ, Kin and BAP didn't differ significantly [1]. It has been reported that TDZ is most effective in compared to BAP [9].

Supplementation of culture medium with some undefined nutrient substance can improved in-vitro organogenesis of plants. Here we evaluated effect of three different type of nutrient agent including Yeast extract, Triptone and Casein hydrolyzate on callus induction and plant regeneration of wheat. Alought callus weight were influenced significantly by using all concentration of those materials, but regeneration response of the callus didn't influence by supplemented materials except that yeast extract significantly reduced regeneration properties of callus.

Using yeast extract in culture medium have a negative effect on shoot propagation of *Asclepias curassavica* [26]. Generally, the higher concentration of the yeast extract doesn't lead to the increment of the regeneration and the shoot propagation rate, and their application at higher doses causes the tissues to become brownish [27]. Best organogenesis was achieved in presence of Trypton in booth concentrations. Casein hydrolyzate were not significantly increased shoot regeneration of wheat. Hydrolyzed casein has been improved shoot regeneration rate in barely [28]. Morover, it was reported that the highest percentage of the regeneration from wheat mature embryo was gained on MS medium supplemented with 200 mg/L casein hydrolyzate [2]. Finally, addition of yeast extract, Trypton and Caseine hydrolysate were not improved regeneration capacity of wheat considerably.

CONCLUSION

This study reveals that the callus induction and the plant regeneration can be affected by the cultivar and plant growth regulator content of medium. The study also shows that Dicamba gave the best results in callus induction. The callus that induced by this plant growth regulator has a better regeneration potential respectively, casein Hydrolyzed and Tryptone are the best organic compounds for the callus induction and the plant regeneration. Among cultivars, Gonbad has the highest regeneration frequency and it can be used in gene transformation to wheat.

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اثر تنظیم کننده‌های رشدی و ترکیبات آلی در القای کالوس و باززایی گندم (*Triticum aestivum*) با استفاده از جنین‌های رسیده

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چکیده

این تحقیق برای ایجاد یک سیستم باززایی کارآمد برای برخی ارقام گندم ایرانی انجام گرفت. محیط کشت MS تکمیل شده با ترکیبات مختلف 2,4-D، NAA، دی‌کمبا و ترکیب‌های مختلف تیدیازورون و BAP به ترتیب برای القای کالوس و باززایی مورد ارزیابی قرار گرفتند. نتایج نشان داد که کارآیی جنین‌های رسیده برای پاسخ به کشت بافت به طور معنی‌داری تحت تاثیر ژنوتیپ، نوع و غلظت اکسین مورد استفاده می باشد. محیط کشت تکمیل شده با دو میلی‌گرم در لیتر دی‌کمبا بهترین پاسخ را برای کالوس‌زایی و همچنین برترین فراوانی باززایی را داشتند. پاسخ به باززایی به طور قابل توجهی تحت تاثیر نوع اکسین و غلظت آن در محیط کشت القای کالوس بود. تیمارهای سیتوکنین اثر معنی‌داری بر اجزای باززایی نداشتند. سه ترکیب آلی برای بهبود کالوس‌زایی و باززایی هشت رقم مورد ارزیابی قرار گرفت. نتایج نشان داد که سه میلی‌گرم در لیتر کازئین هیدرولیز شده و پنج میلی‌گرم در لیتر تریپتوفان اثر قابل توجهی را به ترتیب در القای کالوس و باززایی گندم داشتند.

کلمات کلیدی: القای کالوس، کازئین هیدرولیزشده، جنین رسیده، باززایی گیاه، تریپتون، عصاره مخمر