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Callus Induction and Plant Regeneration from Immature Embryos of Two Wheat Cultivars (*Triticum aestivum* L.)

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Abstract: This study was conducted in the Plant Tissue Culture Laboratories of Genetic Engineering Institute/Baghdad University at the aim of inducing callus and regenerated plants from immature embryos of two wheat cultivars. Immature embryos were excised and cultured on Murashige and Skoog (MS) medium for callus induction. MS and doubled MS components were used with the addition of different growth regulators combinations to induce callus from immature embryos of the studied cultivars. The medium MS with 2 mg L^{-1} 2,4-D gave the highest callus fresh and dry weights compared with the other medium. Tamooz 2 was significantly higher in both, fresh and dry weight of the induced callus than cv. Al-Iraq. Plants regeneration was induced on MS media supplemented with BA of (0, 1, 2, 3, 4 mg l^{-1}). The control treatment (0 BA) was the best medium for regeneration.

Keywords: Wheat, Callus induction, Immature embryos, Plant regeneration.

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Introduction:

Wheat (*Triticum aestivum* L.) is premium crop belongs to Poaceae, the largest and globally widespread family. This crop occupies the first place in terms both, cultivated area and production, hence representing the main source for feeding more than a third of the world's population (1). Wheat crop has attracted great attention to improve both quantity and quality through the development of cultivars and hybrids with desired characteristics, using classical breeding methods or modern biotechnological tools that accelerated breeding programs (2).

Out of these, plant tissue culture is widely adopted as a precious biotechnological tool that had contributed effectively in the rapid development of new genotypes (3).

Plant regeneration is the most important stage in tissue culture practices and is the key step in application of tissue culture procedure at the aim of mediating a genetic improvement in the desired direction. Most of the genetic modification practices goes through tissue culture technique (4). In spite of the great theoretical and technical progress have been made in the studying of metabolic pathways, growth and development in plants, the regeneration step still represents a stubborn obstacle that varies greatly depending on the plant species and subjected conditions(5, 6),

thus, it still limiting the application of such technique in breeding programs, especially in monocotyledons (7).

Although the successful use of plant tissue culture in producing of several monocots variants like wheat, a continuous efforts still needed to improve efficient regeneration system guarantees a high survival rate (8).

Response for callus induction and regeneration rate in wheat depend on several factors, including the nutritional components of medium (9-11), the used tissue and it's physiological status (9, 11,12) and the genotype (11,13). At each stage of growth, differentiation stage is mainly affected by the hormonal balance in the nutritional medium. Mature and immature embryos were used successfully in in vitro regeneration of wheat cultivars. However, the frequency of regenerated plant from immature embryos was slightly higher than mature embryos (14). current study aims to induce callus and regenerated plants from immature embryos in the two local cultivars of wheat, Al-Iraq and Tamooz 2.

Materials and Methods:

In the present experiment, seeds of immature wheat (14-15 days of pollination) from two local wheat cultivars (Al-Iraq and Tamooz 2). The seeds were superficially sterilized with 70% alcohol for a minute, then washed three times with distilled water and NaClO used in a concentration of 4.2% for 15 min. followed by three times washing with sterilized water, each for 5 min.

Immature embryos were removed from the sterilized seeds and grew in a rate of 15 embryo on each Petri dish containing a different combinations of nutritional medium(15,16) (Table 1). Subsequently, Petri -dishes were placed in a dark room on 25 ± 2 ° C for callus induction. Four weeks later, the induced callus was transferred to a new medium supported with the same nutrients content. This process was repeated every 4 weeks with the exposure of the callus to a light intensity of 1000 lux for 16 hours followed by 8 hours of dark.

After 8 weeks, the induced callus was transferred to MS medium supported with 0.4 mg l^{-1} of 2,4- D and 0.8 mg l^{-1} of BA to maintenance for 4 weeks. The induced callus was cultured on MS nutritional medium to develop and regenerated plants, supported with four concentrations of growth regulator benzyl adenine (0, 1, 2, 3 and 4 mg l^{-1}), 300 mg l^{-1} of casein, 30 g l^{-1} of sucrose and solidified with 7 g l^{-1} agar.

To persuade vegetative growth, the rootless plantlets were transferred to a new nutrient medium with half of the MS free of growth regulators for rooting. Then, regenerated plants were transferred to plastic containers containing a mixture of sand and Peat Moss (1:1) and covered with transparent plastic sheets. After three days, these sheets were gradually lifted.

The *in vitro* experimental design is (CRD), using Genstst statistical program. The differences among means of groups were compared using LSD test at 0.05 level of significance.

Table (1): Media composition for callus induction from wheat immature embryos.

Medium	Medium Nutritional Components
MSA	MS; 2 mg l^{-1} 2,4- D ; 300 mg l^{-1} casein; 30 g l^{-1} sucrose and 7 g l^{-1} agar **(15)**
MSB	MS; 2 mg l^{-1} 2,4- D ; 300 mg l^{-1} casein; 0.5 g l^{-1} kinetin; 30 g l^{-1} sucrose and 7 g l^{-1} agar.
MSC	2MS; 2 mg l^{-1} 2,4- D ; 300 mg l^{-1} casein; 0.5 g l^{-1} kinetin; 30 g l^{-1} sucrose and 7 g l^{-1} agar. **(16)**

Results and discussion:

Effect of Media composition in the fresh weight of the induced callus:

The results of callus fresh weight (Table 2) showed that cv. Tamooz 2 had the maximum fresh weight of callus (217.2 mg) compared to 184.6 mg for Al-Iraq. As expressed by (Table 2), the induced callus from immature embryos was superior in their fresh weight (236.3 mg) when cultured on MSA medium, although there is no significant difference against MSB medium. The lowest mean of callus fresh weight was achieved by the MSC medium averaging 12.63 mg and despite the lowest weight it can observed that it has the best in the quality of the induced callus in terms of the formation of vegetative growths and asexual embryos.

Table (2): Effect of Media composition in the fresh weight of the induced callus from wheat cultivars.

Cultivars	Media composition			Mean
	MSA	MSB	MSC	
Al-Iraq	208.0	254.7	91.2	184.6
Tamooz 2	264.6	209.0	177.9	217.2
Mean	236.3	231.8	134.6	
LSD 0.05%	Cultivars 25.70	Medium 31.48	Interaction 41.55	

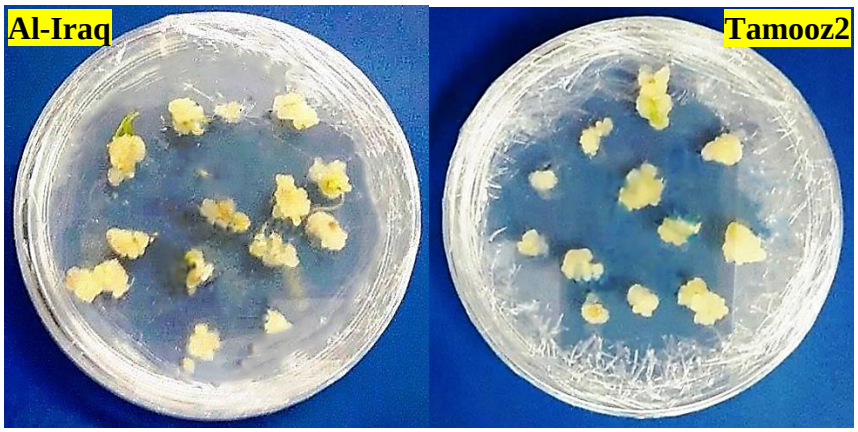


Figure (1): Callus induction of wheat genotypes on MS media supported with 2 mg. l $^{-1}$ 2,4- D ; 300 mg l^{-1} casein; 0.5 g l^{-1} kinetin; 30 g l-1 sucrose and 7 g l^{-1} agar.

Effect of media composition in the dry weight of the induced callus:

The presented results in (Table 3) cleared that cv. Tamooz 2 was produced the higher mean of dry weight reached 16.53 mg, while cv. Al-Iraqi had the minimum value of 13.90 mg. For mediums, MSA was in the lead achieving the highest mean of callus dry weight for both cultivars to be 0.0173 mg. However, MSA did not exceed the significance level compared with MSB medium. The lowest dry weight was achieved in callus grew on MSC medium reached 12. 63 mg. The results showed that there were no significant interactions between the two tested cultivars and the three used mediums. However, cv. Tamooz 2 was in higher mean for callus dry weight (18.94 mg) when grow on MSA medium, meanwhile cv. Al-Iraq had the lower mean for callus dry weight as grew on MSC medium reach 10.59 mg .

Table (3): Effect of Media composition in the dry weight of the induced callus from wheat cultivars.

Cultivars	Media composition			Mean
	MSA	MSB	MSC	
Al-Iraq	14.40	16.71	10.59	13.90
Tamooz 2	18.94	15.97	14.67	16.53
Mean	16.67	16.34	12.63	
LSD 0.05%	Cultivars 2.5	Medium 3.06	Interaction NS	

Plants regeneration:

(Table 4) expressed that cv. Tamooz 2 was owned the higher significant mean of regenerated plants which was 0.55, on the other hand cv. Al-Iraq exposed the lowest mean of 0.15. The control treatment found to be superior in respect no. of regenerated plants in both experienced cultivars of wheat.

In general, benzyl adenine found to negatively affected the no. of regenerated plants, as the later decreased in response to higher levels of benzyl adenine. The two wheat cultivars showed different ability to regenerate plants from immature embryos in response to the experienced levels of benzyl adenine. Indeed, cv. Tamooz 2 was more responsive to change in benzyl adenine levels in contrast to cv. Al-Iraq.

The detected differences between the two cultivars of wheat could be due to their different genetic background that draw the total phenotypic and physiologic performance, especially the differentiation process. These results confirmed by previous findings stated by (17) that the different levels of benzyl adenine (0, 1, 2, 3, 4, and 5 mg l^{-1}) played different role in the no. of the regenerated plants from six wheat cultivars.

Table(4): The effect of benzyl adenine in the plants regeneration rate in two wheat cultivars.

Cultivars	benzyl adenine concentrations					Mean
	0	1	2	3	4	
Al-Iraq	0.76	0.00	0.00	0.00	0.00	0.15
Tamooz 2	1.00	0.67	0.43	0.33	0.33	0.55
Mean	0.88	0.34	0.21	0.16	0.16	
LSD 0.05%	Cultivars 0.07		Con. 0.11		Interaction 0.16	

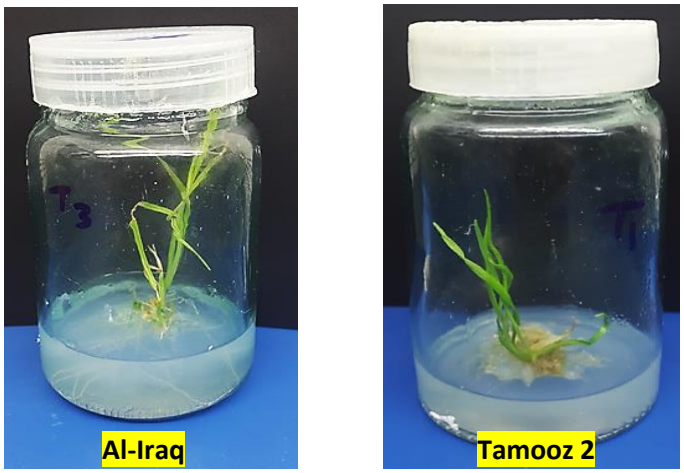


Figure (2): Plant regeneration From Immature Embryos of Two Wheat Cultivar on MS medium free hormone.

Rooting:

Growing the regenerated plants on a half MS medium has led to root formation in the vegetative growths (Figure 3). The addition of auxin (2 mg l^{-1} of 2,4-D) in callus induction stage has helped the regenerated plants to form roots.

Some of the regenerated plants failed in formation roots which may due to the opposite effect of cytokinin via inhibiting root formation, and extended the vegetative growth period.

Determination of the differentiation stage depends on the internal content of growth hormones and the external addition of the growth regulators in the culture medium. Several studies have indicated that the process of differentiation depends primarily on balancing between auxins and cytokinins to guide the differentiation in the right path.

The higher level of auxins compared to cytokinins may stimulates the root formation, while the increase level of cytokinins against auxins will participate effectively in extending the vegetative growth stage in direct or indirect way (18).

In Arabidopsis, auxins found to be responsible for path signaling with aid of specific transcriptional factors named Auxin Response Factors (ARF), for an example ARF7 and ARF19. These signaling compounds have a crucial role in activating certain genes take under control cell differentiation to root cells (19,20) An alternative mechanisms may be adopted through which auxins may targeted the encoded gene of inhibitors like KIP-Related Proteins (KRP), (21,22) .

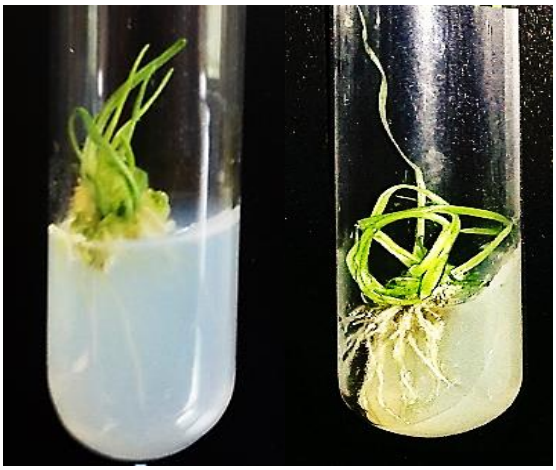
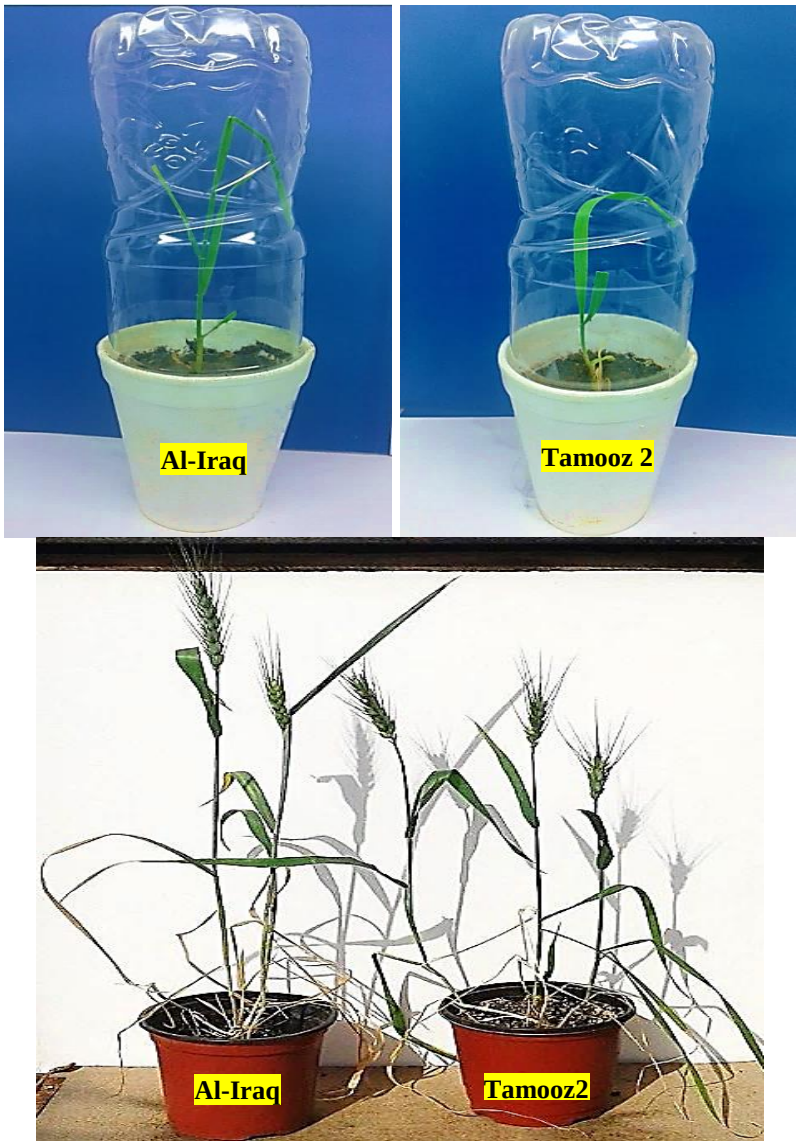


Figure (3): Root formation on media free hormone 1*l* 2 MS.

Acclimatization:

Plants that have roots in both cultivars (Al-Iraq and Tamooz 2) were removed and washed under the running water to get rid of the nutrient medium. Then, the plants were transferred to soil composed of sand and Peat Moss (1:1). The plants were covered with a transparent plastic sheet (Figure 4) to preserve moisture and reducing the water loss due to the absence of the cuticle layer on the leaves surface. The regenerated plants were irrigated with MS solution for one week, then the solution was diluted to half MS in the second week, only MS in the third week, and finally with saline-free water

in the fourth week. This helped to provide the necessary nutrients for growth at the early stages, because at this stage roots are inefficient in absorbing the required nutrients. Gradually, the plastic sheet is lifted to reduce the lost plants four weeks after they were transferred to the soil. This process has played a key role in the survival of tissue culture-derived plants because they are very sensitive to the lackness of the waxy layer, and gave plantlets enough time for adaptation.



Figure(4): Adaptation of plants resulting from the cultivation of plant tissues for two varieties of wheat which succeeded in the process of localization and living in natural conditions.

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