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Full Length Research Paper

The study of 2,4-D and 2,4,5-T effects on gene expression at early stages of embryogenesis in chickpea (*Cicer arientinum* L.)

Mahmood Ghorbani-Marghashi[1], Mansour Gholami[2]*, Reaza Maadankan[1] and Hassan Jamshidi[1]

[1]Depatment of Biology, Malayer Uiniversity, Malayer, Iran.
[2]Department of Horticultural Sciences, Bu-Ali Sina University, Hamedan, Iran.

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Factors affecting the induction of embryogenesis in somatic plant cells *in vitro* are highly variable depending on different growth regulators, explants types, culture media and stress treatments. A series of experiments were set to study the effect of some factors involve in the initiation and development of somatic embryos including genotype, growth regulator and the kind of explants in four Kabuli type chickpea genotypes (F3, Jam, Bivanig and ILC485). Mature embryos were transferred on Murashige and Skooges (MS) medium plus 3% sucrose supplemented with two kind of auxin as growth regulators in order to induce embryogenesis. The best induction media (found in the preliminary experiment) was the one supplemented with either 3 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) or 3 mg/l 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in single use or in combination, to induce globular embryos. Globular shape embryos were distinguishable 6 to 7 weeks after culture. Desiccation treatments were applied by keeping the samples for 7, 14 and 20 min in laminar air flow to induce globular embryos maturity. Total RNAs were isolated using guanidium isothiocyanate method. The polymerase chain reaction amplification (PCR) reaction was performed using three one-base-anchored oligo-dT primers, in combination with eight arbitrary primers. Two genotypes of Bivanig and ILC482 were selected as explants for gene expression studies due to their highest number of globular embryos production, then 3 mg/l 2,4,5-T and 3 mg/l 2,4-D were used to produce embryogenic and non-embryogenic calli, respectively. The results showed a similar gene expression pattern in both treatments with three eminent steps. Comparison of both similar band patterns of two auxin treatments indicated that, a number of particular genes are involved in cells determination. Different growth regulators and the genotypes created little changes in gene expression pattern through the embryogenesis process just before the globular stage, resulting that, a large number of changes and genes occur similarly in both genotypes and auxin treatments.

Key words: Chickpea, embryogenesis, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), gene expression.

INTRODUCTION

Legumes are one of the most important plants for human nutrition. Grain legumes are a major protein source for more than two billion people worldwide. Chickpea is an important grain legume for many regions like India, Africa, Southern Europe, South and Central America, Australia and Middle East including Iran. However, production of this crop has remained consistently low because of its susceptibility to several fungi such as *Fusarium aschochyta* blight and also insect pests such as *Helianthus sp.* (Kiran et al., 2005). Production of resistant crops against harsh conditions, pests and diseases via genetic engineering is a useful solution for overcoming adverse conditions. Somatic embryogenesis is one key

*Corresponding author. E-mail: mgholami@basu.ac.ir. Tel: +98 811 8221181. Fax: +98 811 4424012.

process for transgenic plant production in many economically important plants. Furthermore, it can be used to produce synthetic seeds and secondary metabolites and also in cryopreservation (Janick, 1993).

Somatic embryogenesis is the developmental restructuring of somatic cells towards the embryogenesis pathway and reflects cellular totipotancy in higher plants. Such developmental switching involves various molecular events encompassing not only differential gene expression but also various signal transduction pathways for activating or repressing numerous gene sets, many of which are yet to be identified and characterized (Denis et al., 1991).

By the time cells dedifferentiate, they gain ability to initiate a new developmental pathway. In direct embryogenesis, cells do not need to dedifferentiate and this ability is pre-existing, but in indirect embryogenesis, beginning of induction stage needs a callus induction phase. Production of callus needs an auxin. This event is not exclusively related to embryogenesis and it could be found in any tissue culture method which proceeds by callus production. This developmental switching also involves differential gene expression conferring on the somatic cells with ability to manifest embryogenic potential. Actually, auxin treatment is an intensive stress by which the cells exert some variation in gene expression in response to this stress accompanying transient increase in genes, but after this period, gene expression accords with increased callus production (Thorpe, 1995).

In recent years, embryogenesis pathways have been studied in model plants such as Arabidopsis, carrot, orchard grass and alfalfa in more details identifying many genes involved (Bhumica, 2007). However, little is known about the early inductive events underlying the transition of the somatic cells to an embryonic pattern. During the initial phases of embryogenesis, somatic cells embark on a series of developmental events referred to dedifferentiation, induction and competence acquisition (Bhumica, 2007; Lucau-Danila et al., 2010).

Factors have been utilized for inducing *in vitro* embryogenesis in somatic plant cells are highly variable, ranging from various plant hormones to stress treatments (Feher et al., 2003; Feher, 2008; Zavattieri et al., 2010). However, the most widely used inducers are auxins especially 2,4-dichlorophenoxyacetic acid (2,4-D). Auxins play important roles in embryo formation, induction and in the elaboration of proper morphogenesis during embryo development (Feher et al., 2003, Quint and Gray, 2006; Bhumica, 2007). Although, acquisition of embryogenesis competence is dependent on many circumstances, it is mainly recognized by the given physiological state of the cell which is determined by its genetic and developmental conditions and environmental cue (Feher, 2008). This sophisticated interaction of genetic and physiological factors may explain why only certain genotypes and certain cells can go through the whole process of somatic embryogenesis (Feher, 2008).

There are two types of chickpea, Kabuli and Deci, but most of the embryogenesis studies have been carried out on the Deci types. Chickpea is a recalcitrant plant for somatic embryogenesis and this event had not been induced before in type of Kabuli chickpea. Few reports are available about chickpea somatic embryogenesis, from cultured anthers and pollen (Bajaj and Gosal, 1979), leaves (Dinesh Kumar et al., 1994), immature cotyledons (Sudha and Reddy, 1994; Ramana et al., 1996), immature leaflets (Rao and Chorpa, 1989; Barna and Wakhlu, 1993; Dinesh Kumar et al., 1995), immature embryo and mature embryo explants (Sagar et al., 1995; Suhasini et al., 1994). However, the response of somatic embryogenesis has remained inconsistent and often not reproducible. Although, the effects of different factors including genotype, explants and hormone treatment have been studied on embryogenesis but to our knowledge this is the first gene expression pattern study in chickpea, investigating the early embryogenesis stages. The major object of our article is not regeneration of somatic embryos or gene isolation but the research has focused on changes in gene expression pattern under different treatments. To do this, we first assessed globular embryo formation in different genotypes and treatments in Kabuli chickpea type to select the best factors affecting globular embryo formation. Then, we chose two genotypes in which the highest rate of embryogenesis occurred. Two embryogenic and non-embryogenic hormone treatments were used to compare gene expression pattern in two selected genotypes.

MATERIALS AND METHODS

Two separate sets of experiments were conducted as follows: (a) The plant genotype explants type, growth regulator, light and desiccation effects were studied as the factors affecting embryogenesis, in order to select the best embryogenic and non-embryogenic genotypes and the more effective concentrations of auxins; (b) gene expression patterns were compared in selected genotypes in both embryogenic and non-embryogenic media.

Plant materials and the treatments

The seeds of chickpea cultivars, F3, Jam, Bivanig and ILC485), were obtained from the Dry Land Agricultural Research Center, Kermanshah, Iran and surface sterilized in a 50% commercial bleach solution (2.5% sodium hypochlorite) for 20 min followed by washing 3 times with sterile distilled water and then, soaked overnight (16 h) in sterile distilled water. Embryo axes were subsequently isolated under aseptic conditions from cotyledons (Figure 1).

In the preliminary experiment, five aseptic mature embryos we cultured on Murashige and Skooges (MS) medium plus 3% sucrose, supplemented with two kind of auxin as growth regulators, in order to induce embryogenesis. pH of the medium was adjusted to 5.8 and solidified with 0.8% agar prior to autoclaving at 1.2 atm and 121°C for 20 min. The cultured embryos were grown in a controlled growth room with a 16/8 h light/dark photoperiod at 25 ± 2°C. The experiment was repeated three times. At the end of the 6th

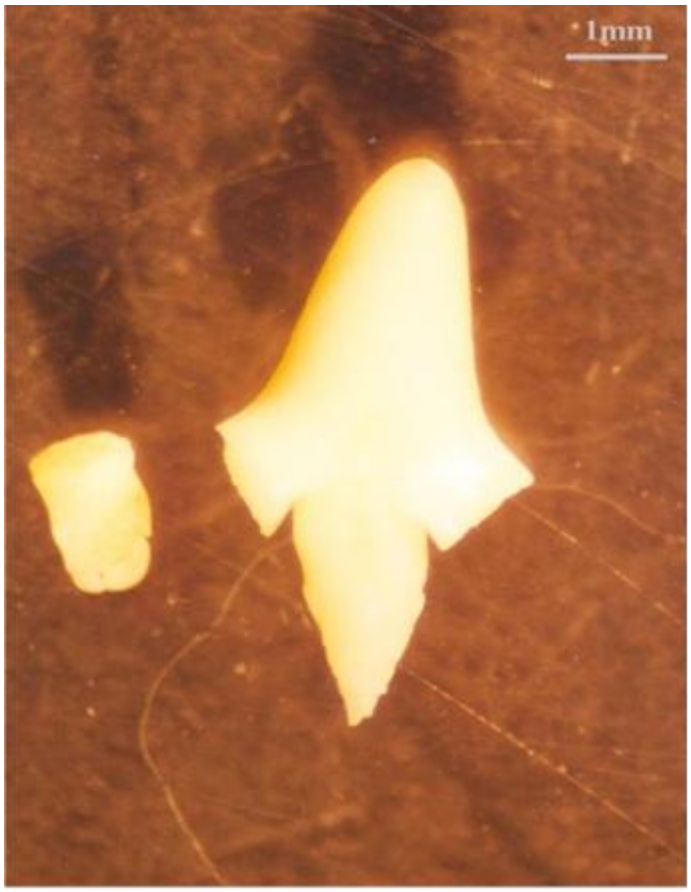

Figure 1. Mature embryo and epicotyle.

Table 1. Early treatments used for embryogenesis in MS medium.

Treatment	Medium	Growth regulator
MS1	MS	2,4,5-T 3 mg/l
MS2	MS	2,4,5-T 1 mg/l + 2,4-D 1 mg/l
MS3	MS	2,4,5-T 1.6 mg/l + 2,4-D 1.6 mg/l
MS4	MS	2,4,5-T 3 mg/l + 2,4-D 3 mg/l
MS5	MS	2,4,5-T 4 mg/l + 2,4-D 4 mg/l
MS6	MS	2,4,5-T 2 mg/l + 2,4-D 2 mg/l
MS7	MS	2,4,5-T 2.4 mg/l + 2,4-D 2.4 mg/l

week, nodular and early stage embryos were counted microscopically.

In the main experiment, the best induction media (selected from the preliminary experiment) supplemented either with 3 mg/l 2,4-D or 3 mg/l 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in single use and in combination with each other were chosen, which are listed in Table 1. In the whole experiment, the sum of 740 explants was inoculated in 168 Petri dishes.

Maturation of somatic embryos

After 6 weeks, calli derived from the preliminary experiment were transferred to the maturation media which are listed in Table 2. Desiccation treatments were also applied by keeping the samples for 7, 14 and 20 min in laminar air flow, for induction of globular embryo maturity in three replicates. Globular embryos were desiccated through three different treatments: (1) The embryos were spread on a filter paper in a Petri dish; (2) sealed with a plastic wrap and exposed to a rapid air flow at 20 to 25°C; (3) embryos were dried by placing them in desiccators containing NaCl, each in three replicates.

Histology

Histological analysis was performed on calli containing globular embryos. Samples were fixed in formalin, acetic acid, absolute

Table 2. Treatments used for globular embryos maturation with desiccation stress.

$M_1 = \frac{1}{2}MS + B_5 Vitamins + \%6 Maltose + \%3$ agar
$M_2 = MS + B_5 Vitamins + \%6 Maltose + \%3$ agar
$M_3 = MS + B_5 Vitamins + \%6 Maltose + \%5$ Active carbon $+ \%3$ agar
$M_4 = \frac{1}{2}MS + B_5 Vitamins + \%6 Maltose + \%5$ Active carbon $+ \%3$ agar

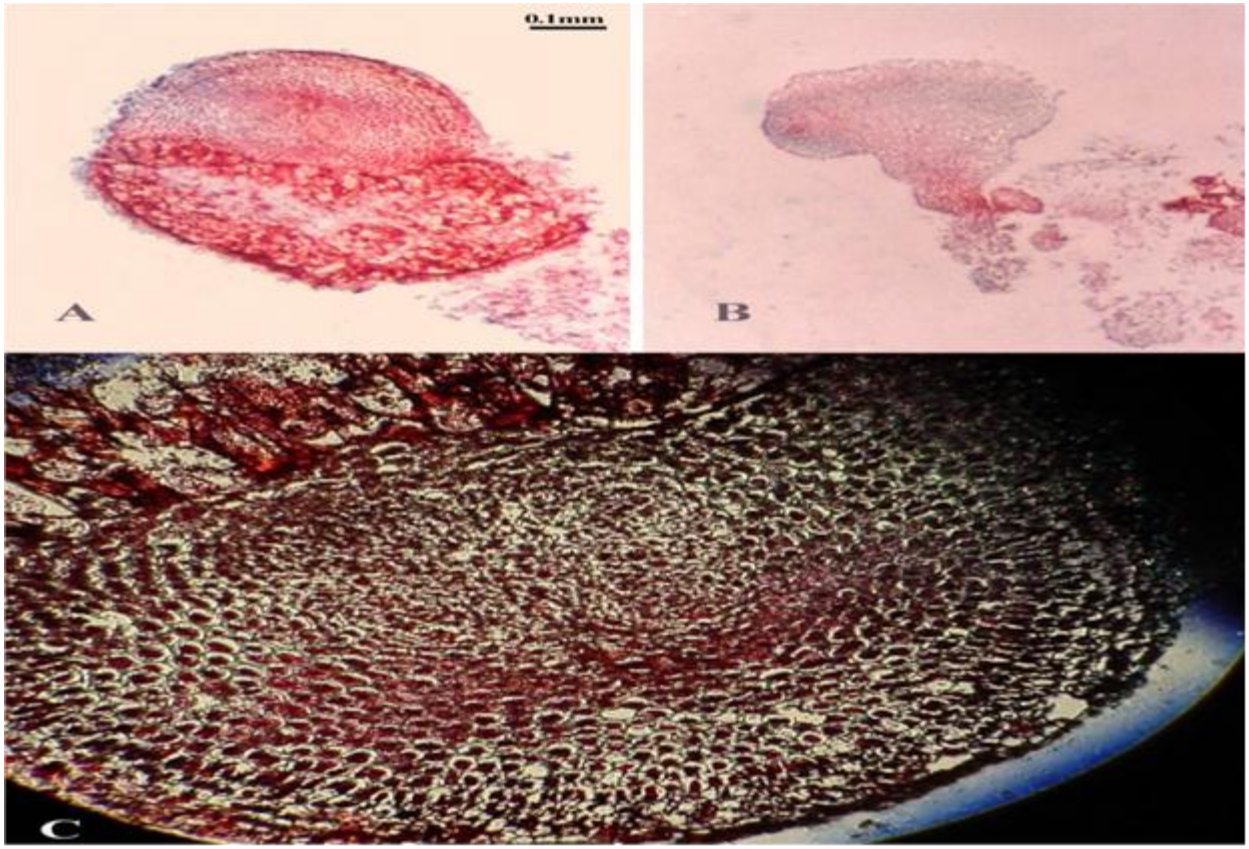

Figure 2. Embryogenic and non-embryogenic are distinguishable in A and C, globular embryo (B).

ethanol (FAA) 17:1:2, dehydrated with different concentration of alcohol, then, embedded in paraffin wax and sectioned into 8 to 10 µm thick serial sections with a microtome. Sections were mounted on glass slides and stained with safranin-fast green dye and observed under a light microscope (Figure 2).

RNA preparation and cDNA synthesis

To study the variation of preferentially expressed genes, embryogenic and non-embryogenic samples were collected over 42 days with 7 days interval and were frozen immediately in liquid nitrogen prior to grinding in a mortar and pestle. Total RNAs were isolated using guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). DNase I treatment was performed using 1.5 unit of deoxyribonuclease I at 25°C for 1 h. The DNase was inactivated by adding 1 ml of 25 mM ethylenediaminetetraacetic acid (EDTA) to the reaction mixture and heated for 10 min at 65°C. Three one-base-anchored oligo-dT primers, H-T11A (oligo2), H-T11C (oligo3) and H-T11G (oligo1), in combination with eight arbitrary primers, H-AP49 to H-AP56 (Table 3) were used for polymerase chain reaction (PCR) amplification according to the manufacturer's protocol. This method was first devised for human cells with the assumption that every cell expresses 15000 genes (Bauer et al., 1993). To display all expressed genes, the method requires at least 25 arbitrary primers to achieve a level of confidence of about 0.95 (Krassimira and Conger 2002). Assuming that chickpea cells express the same number of genes, it could be speculated that this study has covered

Table 3. Arbitrary primers used in the experiment.

Primer	Primer sequence (´3-´5)	Primer	Primer sequence (´3-´5)
H-AP49 A	AGCTTTAGTCCA	H-AP53 A	AGCTTCCTCTAT
H-AP50 A	AGCTTTGAGACT	H-AP54 A	AGCTTTTGAGGT
H-AP51 A	AGCTTCGAAATG	H-AP55 A	AGCTTACGTTAG
H-AP52 A	AGCTTGACCTTT	H-AP56 A	AGCTTATGAAGG

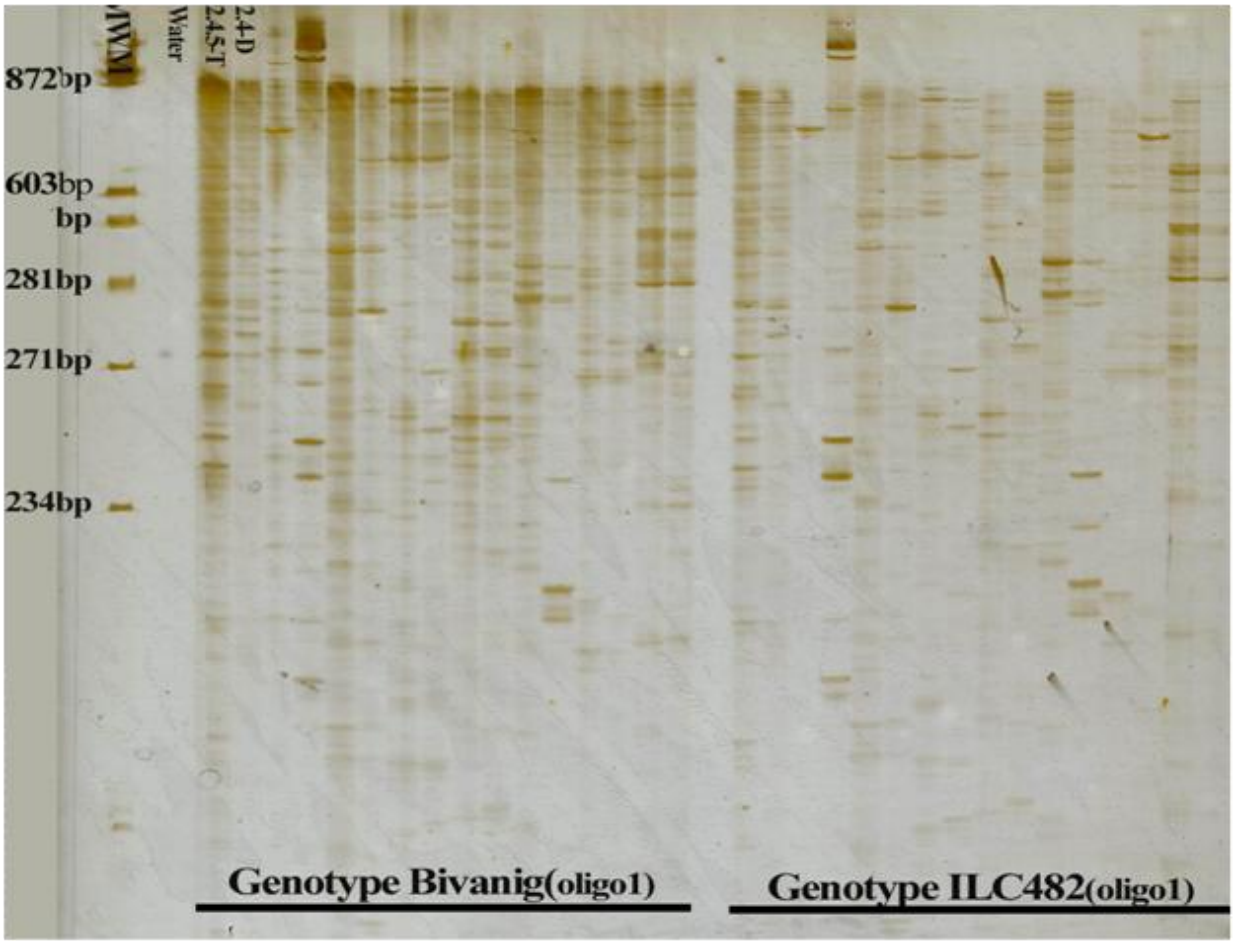

Figure 3. Isolated cDNA on poly-acrylamid gel visualized with silver nitrate staining.

one third of them. Reverse transcription was done using Boschi and Vergara (1998) method in a thermocycler (Ependorf gradiant) at 42°C for 60 min. The final volume for the reaction was 20 µl. The enzyme was inactivated by heating at 99°C for 5 min. The cDNA was stored at -80°C for subsequent use.

RNA arbitrarily primed (RAP)-PCR

The PCR reaction was performed using three one-base-anchored oligo-dT primers, HT11G, H-T11A and H-T11C in combination with eight arbitrary primers, H-AP49 to H-AP56 (Table 3) using ependorf gradiant thermocycler in a final volume of 25 µl, including 1.25 µl template from the reverse transcription reaction. After 5 min denaturizing step at 94°C, 45 cycles were performed (60 s at 94°C, 2 min at 35°C, 60 s at 72°C), followed by a 5 min final extension step at 72°C. Reverse transcriptase (RT)-PCR differential display, (Liang and Pardee, 1992), was adapted and repeated three times. The amount of cDNA was determined using UV spectrophotometer. The products were separated on a 6% (w/v) denaturing polyacryl-amide gel (0.25 mm in thickness). Bands were visualized by silver nitrate staining method (Figure 3).

Statistical design and analysis

To study the effect of genotype, type and concentration of hormones, explants, light and basal medium, a factorial experiment based on a randomized complete design was designed in this study in three replications. Data were analyzed using the Statistical

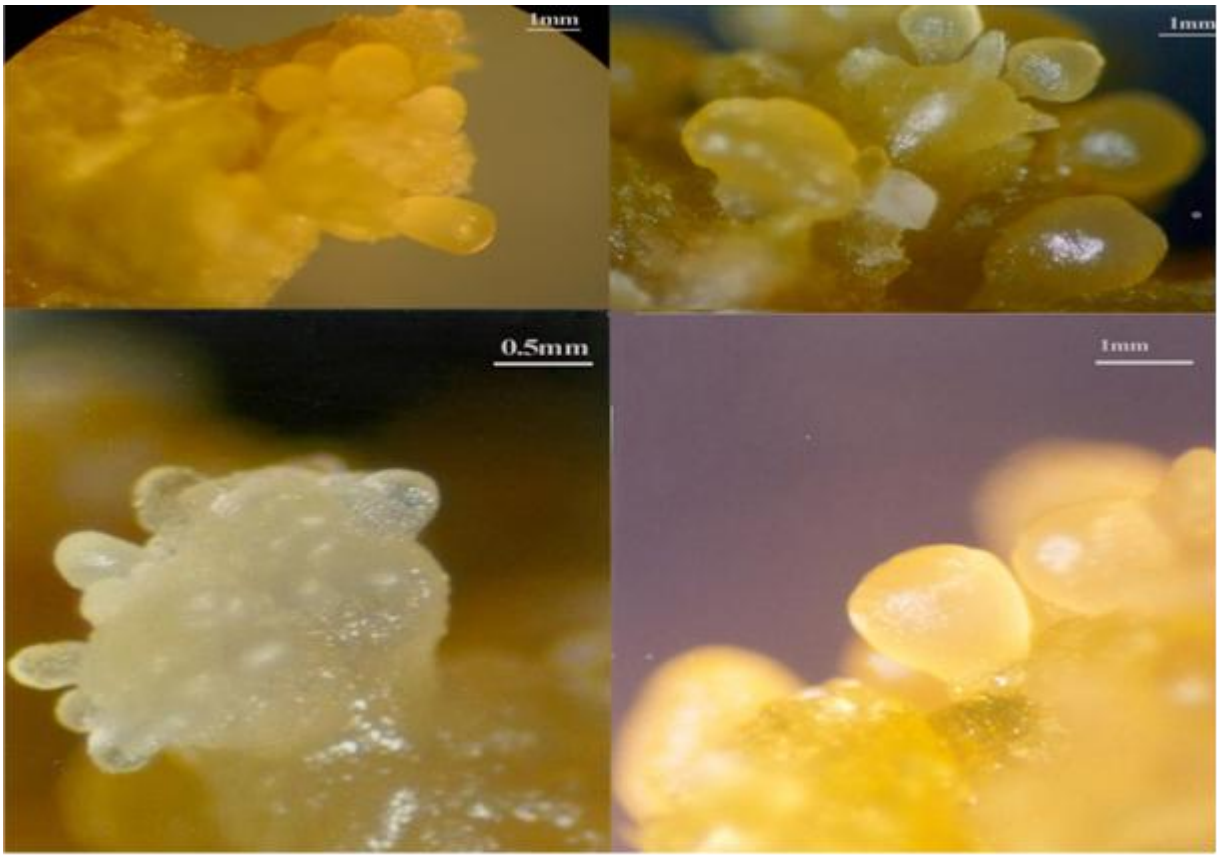

Figure 4. Different globular embryos obtained after 6 weeks from explants.

Analysis System (SAS) software version 6.2 by analysis of variance (ANOVA). The means were compared by Duncan multiple range test.

RESULTS

Selection of genotype and embryogenesis media

Somatic embryogenesis is a sophisticated process in which growth and development of regenerated plants depend on provided situation in early steps when somatic embryos form and germinate (Thorpe, 1995). Hence, the aim of this research is to evaluate various factors including light and drought stress, genotype, explants type and concentration of auxins in this experiment (Feher et al., 2003). After about 4 to 6 weeks in culture, two types of callus appeared on the explants. The first type was compact and light yellow in color and detached hardly from originated tissue. The second type was light greenish in color and watery held very loosely on the surface of calli tissue. Our observations correspond to results of Sagar et al. (1993). In our research, 4 genotypes, Jam, Bivanig, F3 and ILC482, produced callus after 7 to 8 days. According to Kumar et al. (1994) and Sagar et al. (1993) who studied various genotypes and explants and their interaction with different concentrations of growth regulators, genotype had probably no distinguishable effect on callus induction. However, the amount of calli differed in different geno-types. In the current study, Jam and ILC482 produced less calli than Bivanig and F3 (statistical data not shown). In general, globular embryos or embryogenic clusters appeared on compact and yellow calli detached easily from embryogenic tissue. Globular embryos were distin-guishable and numerable after 6 to 7 weeks (Figure 4). Histological studies exhibited differences between embryogenic and non-embryogenic tissue.

The results for analysis of variance and means comparison are shown in Table 4. Our results showed that, 3 factors including medium type (A factor), genotype (B factor) and explants type (C factor) and their interactions had significant effects on embryogenesis. These observations are in accordance with Barna and Wakhlu (1993). Considering these three factors and their interactions, it was possible to provide the most appropriate circumstances for induction of maturity and regeneration. It also opens a way for other works including gene transfer and study of gene expression pattern.

Differences in genotypes affect induction of the embryogeneic state. Each genotype also genetically has a unique ability for regeneration. In this study all four

Table 4. Analysis of variations for different factors.

Source	df	Mean square	F	Sig.
Corrected model	55	1557.708	42.020	0.000
Intercept	1	350324.167	9450.279	0.000
Genotype	3	2242.821	60.502	0.000
Genotype * explants * medium	18	880.644	23.756	0.000
Genotype * explants	3	962.901	25.975	0.000
Explant * medium	6	806.625	21.759	0.000
Genotype * medium	18	763.344	20.592	0.000
Explant	1	24450.666	659.577	0.000
Medium	6	2862.425	77.216	0.000
Error	112	37.070		
Total	168			
Corrected total	167			

R squared = .954 (adjusted R squared = .931) α=0.01

Table 5. Frequency and number of somatic embryos differentiated from epicotyle and mature embryos explants on MS medium supplement with different concentrations of growth regulators.

Genotype	Mean	Standard error	99% confidence interval		Level of significance
			Lower bound	Upper bound	
ILC482	45.821	0.939	43.359	48.282	b
Biovanig	55.614	0.939	53.152	58.076	a
JAM	42.862	0.939	40.400	45.324	b
F3	38.362	0.939	35.900	40.824	c
Medium					
MS1.00	54.580	1.243	51.323	57.837	b
MS 2.00	61.343	1.243	58.087	64.600	a
MS 3.00	50.236	1.243	46.980	53.493	bc
MS 4.00	32.101	1.243	28.844	35.358	e
MS 5.00	32.913	1.243	29.656	36.169	e
MS 6.00	47.301	1.243	44.044	50.558	c
MS 7.00	41.178	1.243	37.921	44.435	d
Explant					
Mature embryo	57.729	0.664	55.988	59.469	a
Epicotyle	33.601	0.664	31.860	35.341	b

Average number of somatic embryos per embryo from three replications with 5 explants each. Means in each row with the same letters are not significantly different at 0.01 level according to the Duncan's multiple range test.

genotypes produced embryogenic and non-embryogenic calli. Jam showed embryogenic producing ability after 26 days, while globular embryos observed in F3, Bivanig and ILC482 after 42 to 56 days. Although, there is no report about the effect of genotype on initiation time of embryogenesis in chickpea, according to these results, genotype is likely to be effective on embryogenesis initiation time.

As shown in Table 5, the highest rate of embryogenesis is related to Bivanig, while the lowest rate occurred in F3, emphasizing that genotype influences embryogenesis. It is also indicated that, MS media supplemented with 2, 4-D only ,induced callus finely, but it failed to produce globular embryos, while media supplemented with 2,4,5-T or in combination with 2,4-D induced globular embryos signally. Our findings are similar to the results of Sagar et al. (1995) and Suhasini et al. (1994). The highest rate of embryogenesis obtained in MS2 growth regulator treatment and the lowest belonged to MS4 and MS5. As much as the concentration of growth regulator (2 to 8

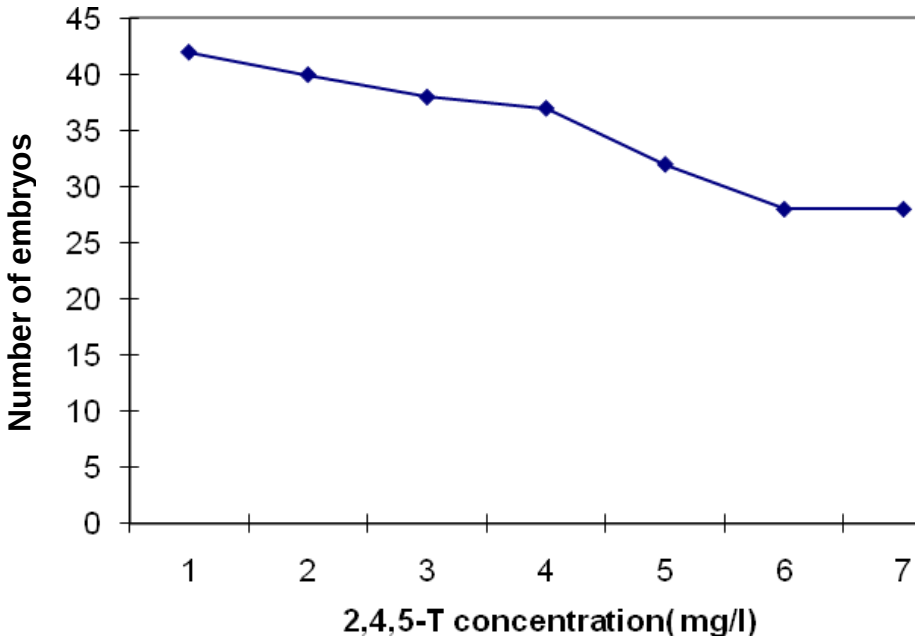

Figure 5. Relation between 2,4,5-T concentration and embryo production.

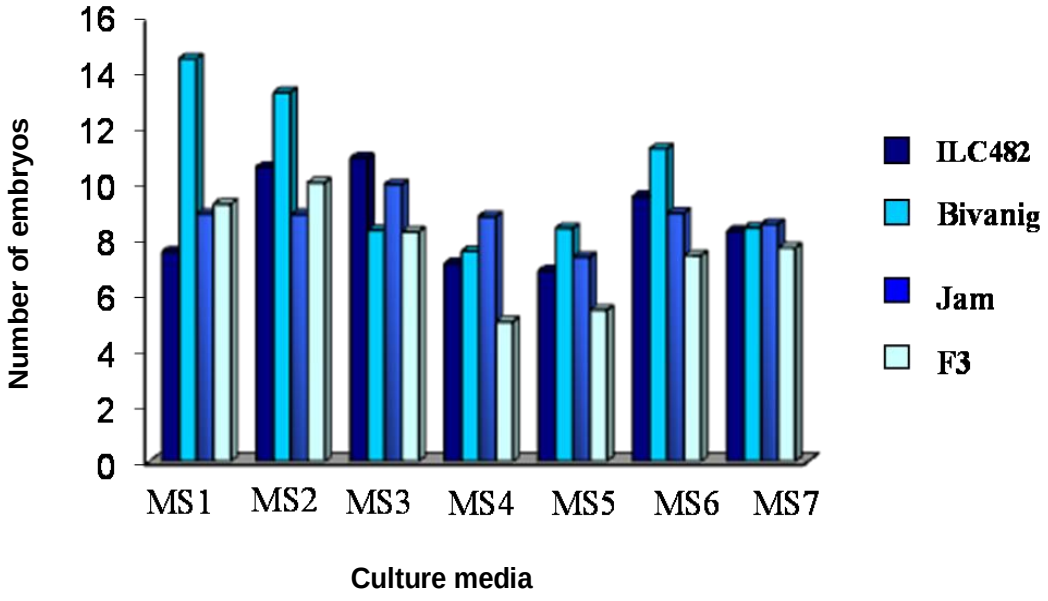

Figure 6. Interaction between culture media and genotype on number of embryos.

mg/l) was increased, the less globular embryo was induced (Table 5, Figure 5). Similar results have been reported by Suhasini et al. (1994). In somatic embryogenesis, a special tissue is superior in production of embryogenic callus and embryos. Explants also can directly affect embryogenesis. In the present study, among explants, mature embryo showed the highest frequency of globular embryo formation and there was a big difference between mature embryo and epicotyle explants concerning the globular embryo production (Table 5). Combination interaction of genotypes and plant growth regulators indicated MS1 and MS2 as the best treatments for induction of embryogenesis, while MS4 showed the lowest induction (Figure 6).

According to the results of tissue culture, two genotypes of Bivanig and ILC482 that produced the highest number of globular embryos were selected as explants for gene expression studies. The amounts of 3 mg/l

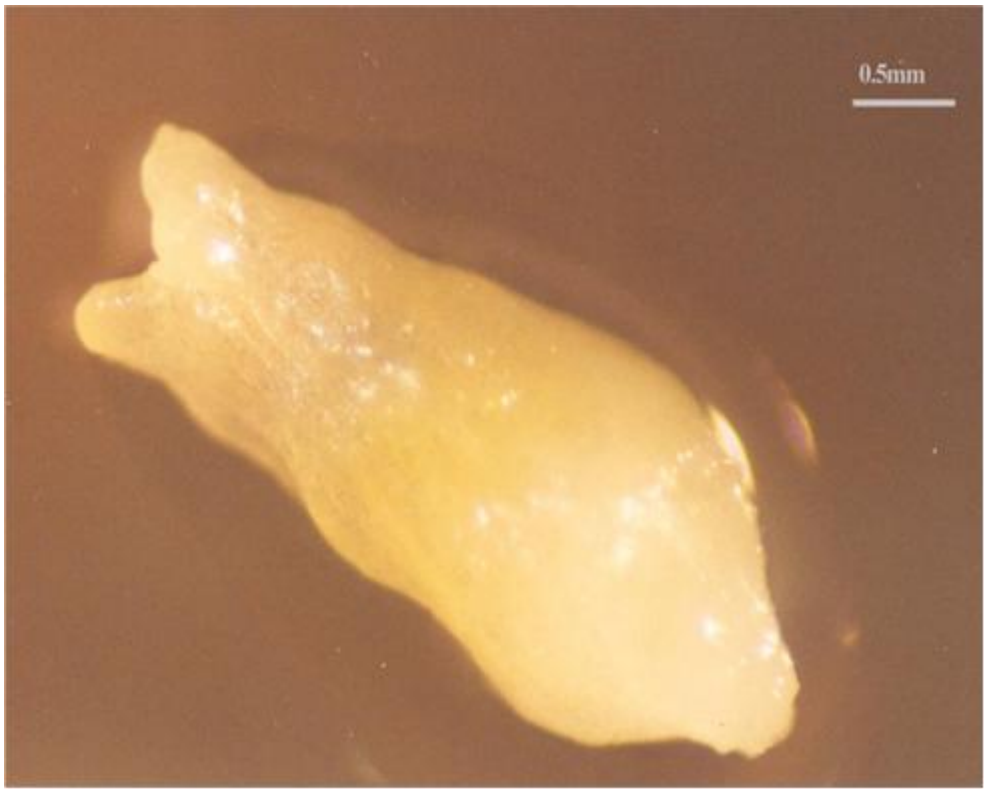

Figure 7. The embryo achieved from globular embryo on maturation medium

2,4,5-T and 3 mg/l 2,4-D was also considered as embryogenic and non-embryogenic auxin treatments, respectively.

Since the induction, development and maturation of embryos happens with difficulty in chickpea, a range of treatments including cytokinins, putresin, amino acids (proline and glutamine), hydrolyzed casein, abscisic acid (ABA) and sugars (sucrose and maltose) have been tested in this plant. Of these treatments, only 4 induced partially-developed embryos and the rest re-induced callus production and caused disappearance of globular embryos (Table 2). These results are in agreement with Sagar et al. (1993) emphasizing the effect of zeatine and reduction of auxin concentration on the induction of embryo maturity. Putresin had an effective role in efficient globular embryo development (data is not shown).

On the basis of acquired results, desiccation stress was used to stimulate cotyledon embryos. The results displayed in 6 h treatment, 50 to 70 percent of transferred globular embryos were depressed while in 2 h all embryos remained alive. 2 weeks after transferring globular embryos, M1 and M4 treatments (Table 2) induced 4 to 5 torpedo shape embryos and after 4 weeks 4 cotyledon embryos from both treatments were observed (Figure 7). Mature embryos were transferred onto embryo germination medium (1/2MS with B5 vitamins), devoid of growth regulators. Just one embryo was further developed but no plant regenerated. Our results showed desiccation and maltose have important impact on production of fully-developed embryos.

Differential expression by RT-PCR analysis

Molecular study of somatic embryogenesis is focused on regulatory mechanisms of cell cycle, emphasizing on message transduction form. Therefore, primarily, the goal of molecular studies of diverse embryogenesis culture systems is identifying particular regulatory component. In our study, gene expression patterns resulted from two growth regulators (2,4,5-T, embryogenic and 2,4-D, non-embryogenic) which were investigated using different primers and sampling at different times (every 7 days) until globular embryos formed. Since this process is affected by endogenous and exogenous factors, experimental and environmental conditions in all stages were sustained the same.

Differential Display Reverse Transcription (DDRT) is conceptually a simple method that allows detection and identification of differentially expressed genes. Although, reverse transcriptase (RT)-PCR has different problems, it is a suitable way for gene expression and gene isolation (Peng, 2002). False positives is an intrinsic feature of DDRT which was decreased by different ways including DNase treatment, anchored primers (Peng, 2002), extraction replication or length of primer (13 mer). All

these items were used to decrease false positives. To decrease serious changes in the band pattern (false positives); experiments were performed twice (including controls). RNA was extracted from five different samples in each factors (2, 4.D and 2, 4, 5-T) and a RNA mixture obtained to create the same condition. Also, two different genotypes (JAM and ILC482) were used for replication and the results and patterns in both were the same. It showed that false positive does not affect the results.
Counting of band numbers is a suitable way for understanding of gene expression pattern and we hope that our results has opened a new way to distinguish different steps during early somatic embryogenesis because it is the first experiment using RT-PCR for studying of changes in gene expression patterns.

Auxins and gene expression pattern

Numerous genes which are involved in indirect somatic embryogenesis have so far been identified in some model plants using embryogenesis culture systems. These genes consisted of LEA, cell wall, latent genes and endokinase genes (Krassimira and Conger, 2002; Chugh and Khurana, 2002; Fowler et al., 1998; Kiyosue et al., 1998; Wurtel et al., 1993). None of these genes are related to gene expression pattern in early embryogenesis. Although, some of them like endokinase are constructive genes that express in whole embryogenesis steps. But transcription factors and other regulatory genes which have major effects on early stages of somatic embryogenesis should be found out. Gene expression variation was analyzed by descriptive statistics. It just has considered statistical aspect of our study and analysis of gene sequence is necessary to investigate two growth regulators treatments. In most researches carried out on model plants including alfalfa, carrot and orchard grass, time duration for induction (incubation of medium and globular embryo formation) has been very short with a maximum of 15 days after incubation. Although, our results shows that chickpea undergoes embryogenesis hardly, but prolonged induction period of globular embryo formation in chickpea, help gene expression studies largely, at least 3 times of other model plants.

As it is shown in Figure 8, gene expression pattern under both treatments is almost similar. Three steps were distinguished in graphs. The first one is related to first week, indicating that during this week, the rate of gene expression was high and also calli formed on surface of explants by the end of this week. Studies have indicated regulating and inducing genes expression 24 h to 5 days after incubation. Suspension culture of alfalfa and carrot has clarified that genes expressed 1 to 2 and 5 days after induction by 2,4-D, respectively (Aleith and Richter, 1990; Fowler et al., 1998). Overall, although, expression pattern of regulating genes are different, it depends on various factors. Genotype is one of the most important factors.

Comparison of ILC482 and Bivanij on graphs, illustrated that the amount of expression in ILC482 was low compared with Bivanij during the first week (Figure 9).

The second phase took place during the second and third weeks in which there was a decreasing trend, so that the graphs of plant growth regulators and genotype treatments overlapped with each other in the third week. Also, gene expression was at the lowest point at the end of this step. Comparison of calli masses variations with these graphs showed that high masses proliferated slowly by the third week (Figures 8 and 9). On the other side, it is most likely that a lot of genes that appeared as a result of induction had been inactivated after the first week, but their effects had been presented by callus formation. The most activated genes are cell division inducing genes at this step that resulted in successive division of callus and preparation for achieving embryogenesis state. Amount of gene expression during the third to sixth weak (third step) was increased. In contrast to the first and second steps, there was a remarkable difference between both hormone treatments in third week in which 2,4,5-T depicted an extremely high level of gene expression compared to 2, 4-D. There is also a significant difference between both genotypes, so that proportion of embryo formation was higher in Bivanij than ILC482. Callus mass variations showed that although, callus production was at maximum rate at the end of the forth week, content of produced calli were approximately constant in 2,4,5-T after the forth week, while at the same period, 2,4-D showed a slight decrease. Globular embryos were only observed in 2,4,5-T treatment. It is found that, the globular embryos were initiated when 2,4-D concentration in the medium was low or depleted (Thorpe, 1995). It probably happens when endogenous and exogenous auxin concentrations reach at proper level to induce embryogenesis. These results imply that although, 2,4-D was able to induce competent cells as same as 2,4,5-T, it was not able to provoke determination phenomenon in the cells. It was also observed by comparing the band discrepancy. As mentioned before, in spite of the same gene expression pattern, no globular embryo was formed in 2,4-D treatment. This was also observed when the bands differences were compared. It is essential to adopt deep investigation including sequencing based on differences that helps to reach strong and noticeable reason about these events.

DISCUSSION

There are a few reports describing gene expression patterns during somatic embryogenesis (Thibaud-Nissen et al., 2003; Stasolla et al., 2004; Che et al., 2006; Lucau-Danila et al., 2010). Although, these studies provide valuable information on this unique process, almost all effects have employed zygotic and immature explants instead of somatic embryos or described the molecular

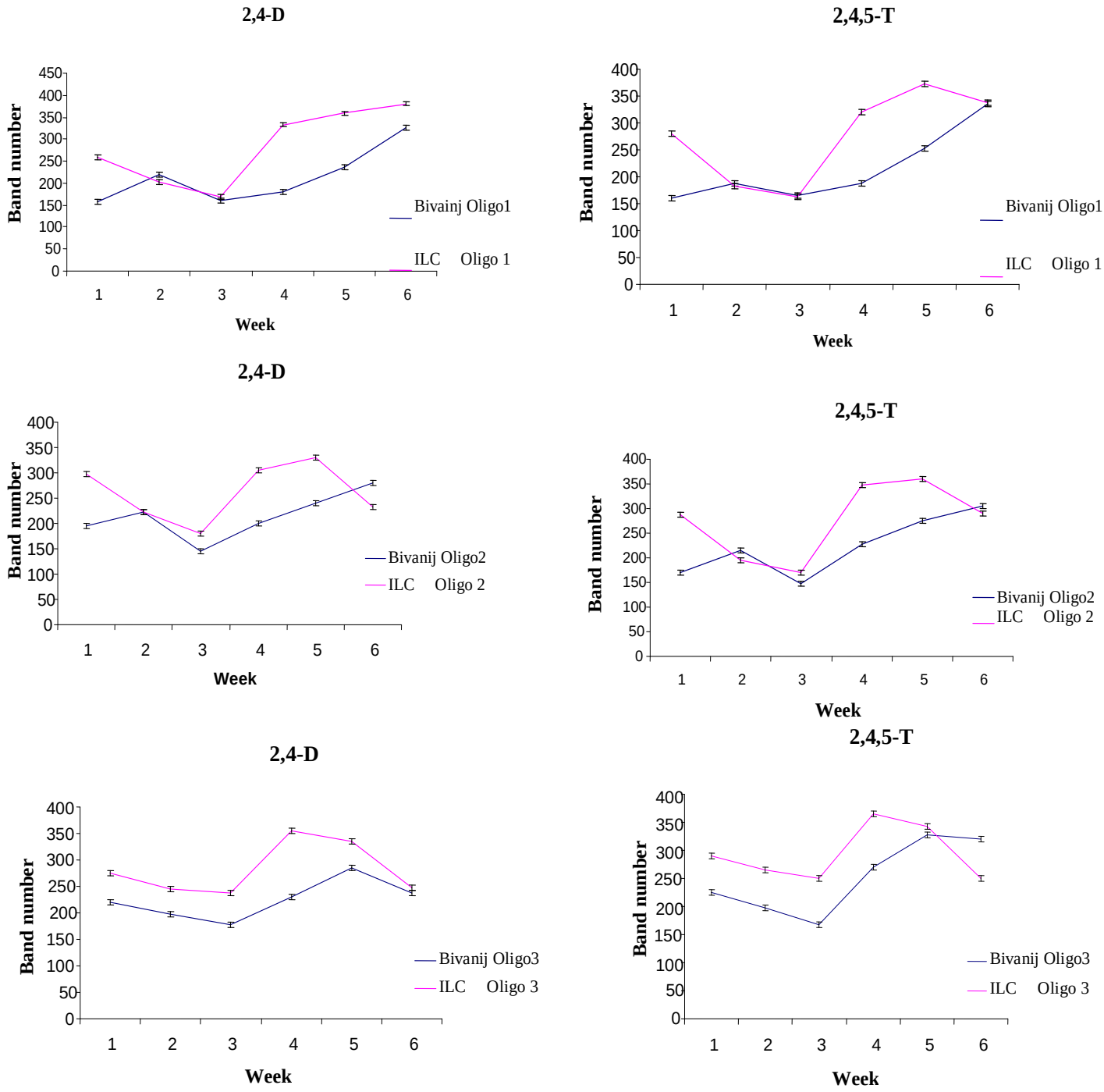

Figure 8. Somatic embryogenesis pattern and changes in gene expression under 2,4-D and 2,4,5-T, treatments using oligo1, 2 and 3.

events occurring in globular embryos as well as maturation of embryos. In order to gain a better understanding of early events of somatic embryogenesis, the concepts of competence, induction and determination used by animal biologists are now being used by botanists to interpret their observations (Thorpe, 1995).

This is the first report showing that the early embryogenesis includes three stages. Recent efforts on the gene expression of maize (Che et al., 2005), potato (Sharma et al., 2008) and wheat (Bhumica et al., 2007) have offered useful information about changes in expression of some genes, but the focus of these researches have been on the late stages of globular embryo formation or only one step before globular embryo formation. Such studies simply measured expression rate of known genes, but it seems that the first important thing in embryogenesis

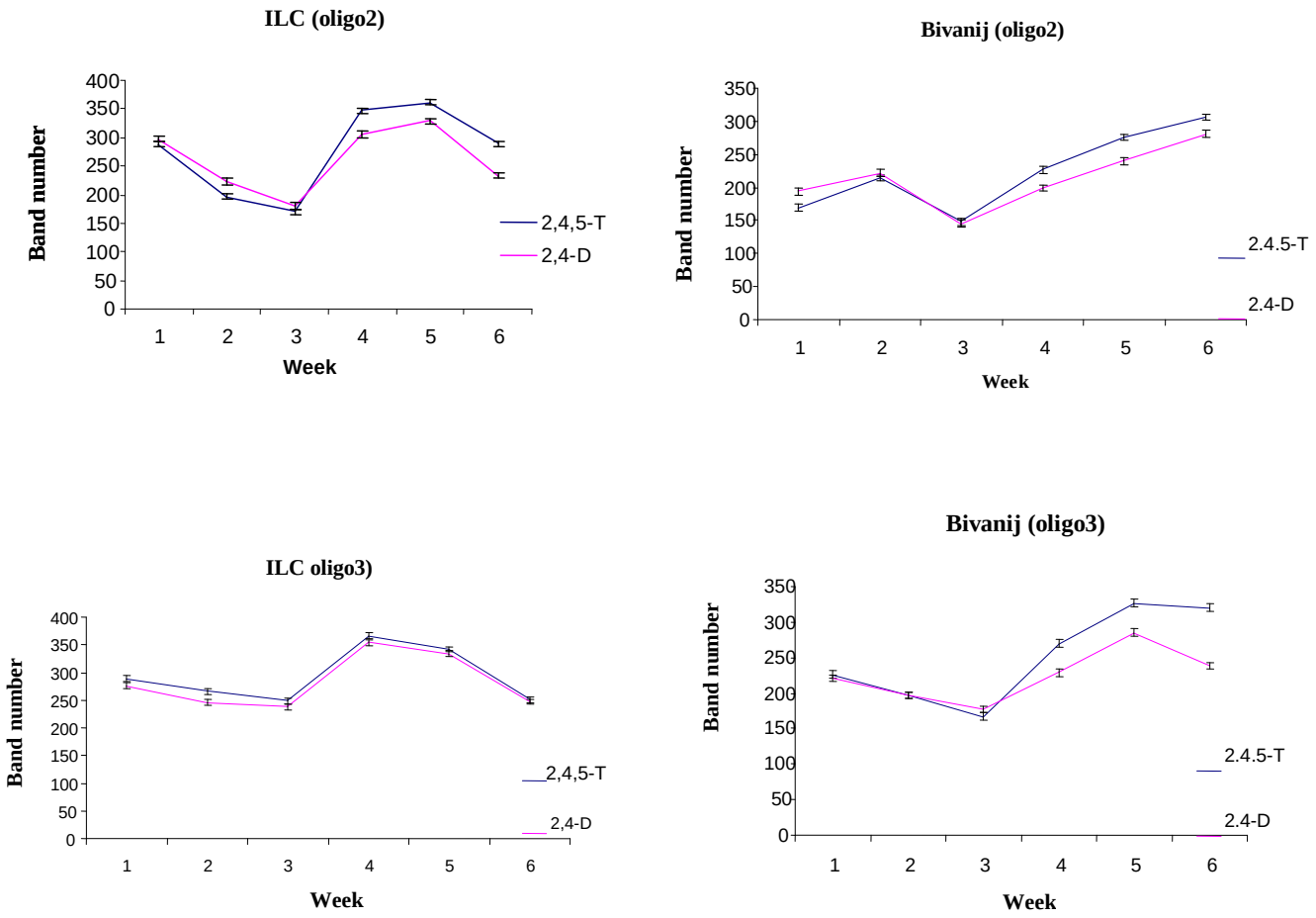

Figure 9. Somatic embryogenesis pattern and changes in gene expression of ILC482 and Bivanig genotypes using oligo1, 2 and 3.

studies is to have a model for behavioral variation of all genes. Since embryogenesis is a complex process and requires extensive changes in the gene expression pattern, focusing on some changes in genes will not help to better understand this process (Suprasanna and Bapat, 2005). But, in the majority of studied systems, the early steps of somatic embryogenesis are usually short and tightly coupled, thus, it is difficult to separate these steps in experimental studies.

Various methods have been prospered for studying these steps (Yeung, 1995; Che et al., 2005; Bhumica et al., 2007; Sharma et al., 2008), but this type of studies provides little information concerning initial events. These recently accomplished experiments are based on identification, expression rate and isolation of genes involved in globular embryos formation events and subsequent stages. The use of two different Chicory genotypes differing in their responsiveness to somatic embryo induction, together with β-GlcY-treatment represented an efficient tool to discriminate cell reactivation from the somatic embryo morphogenetic pathway. Such an approach, together with microarray analyses, permitted researchers to identify several putative key genes related to the somatic embryo morphogenetic pathway in chicory (Lucau-Danila et al., 2010).

Present study is an initial step in this area, but the overall process has shown changes in three stages: Induction, competence and determination. Early researches about changes in expression of specific genes have been carried out in the presence, absence or changed concentrations of auxin and explants types showing that changes in the expression of specific genes is depended on explants types, concentration and kind of auxin in the media. As such, researches have led to discovering some effective genes and showing pattern of these changes, it can not depict a picture from interaction of these embryogenenic factors. The present study showed that growth regulator type and the cultivar created little changes in behavioral pattern of gene expression until the globular stage.

It means that embryogenesis process with all its complexities is related to specific genes which play a key role in this process and introduced as a marker in some publications (Ikeda et al., 2006). Although, in contrast

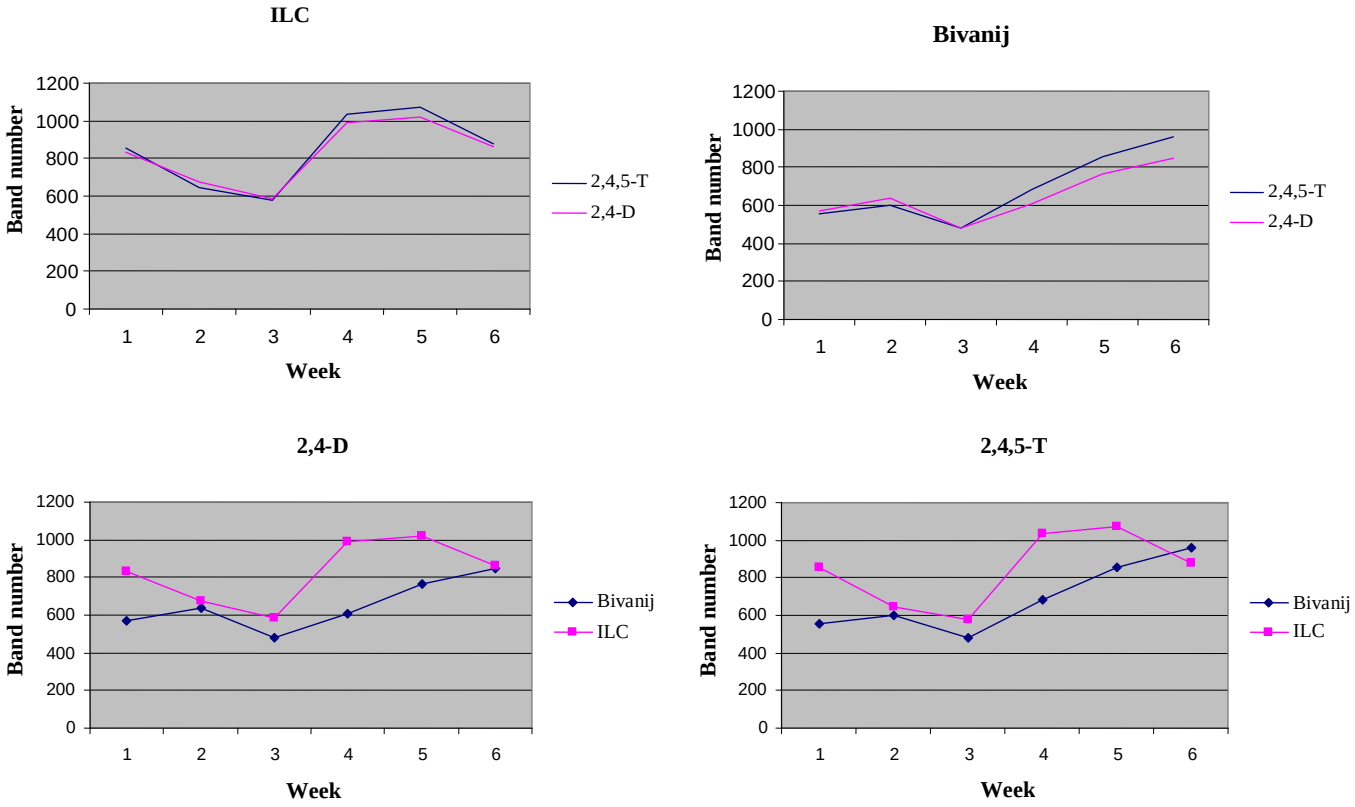

Figure 10. Total pattern of somatic embryogenesis and changes in gene expression of two genotypes under 2,4-D and 2,4,5-T treatments.

with the other studies these genes were not identified, this research is the first model that shows the pattern of genes expression changes. This model of behavior is almost constant between the two cultivars and two growth regulators until globular embryo formation. It seems that this pattern exists in other plants as well.

As Deci type of chickpea has been used for some embryogenesis studies, in this study it was ecided to study embryogenesis of the Kaboli type. In this experiment, two genotypes of Kaboli type chickpea were elucidate with high embryogenesis performance. Two different auxins, 2,4-D and 2,4,5-T, which are typically used for their embryogenic effects, caused different results. This study showed that using 2,4-D caused callus production, but it was not able to stimulate globular embryos formation, while 2,4,5-T was considerably amenable in globular embryos in tested cultivars. As results were similar to Deci type cultivars, it has raised some questions such as: why do two growth regulators from one group show such difference? Do the gene expression pathways and patterns change differently under these two growth regulators?

In the present study, gene expression pattern was investigated from callus-inductive phase to globular embryo formation during 42 days in chickpea. Our studies showed that the globular embryo formation occurred in 6 weeks time. This provides good opportunity for unraveling and separating relative events. Nomura and Komamine (1995) clearly showed that, auxins play an important role in the inductive process. Auxins can quickly accumulate large amounts of mRNA and thereby, stimulate synthesis of specific proteins (Thorpe, 1995). We studied total RNA abundance under auxin treatment during early steps of embryogenesis. Considering non-embryogenic cells or their productions including glyco-proteins may affect the fate of embyrogenic cells in a callus mass (Chugh and Khurana, 2002). This study was conducted without separating embryogenic and non-embrogenic calli. Considering long time of globular embryo production with no need to subculture, unlike most model plants, we prepared cDNA and gene expression profiles under these two treatments for two genotypes in 6 stages until globular embryo formation.

Our observation indicated a significant total RNA increase during the first week and after that a gradual decline till the third week (Figure 10). Based on the reports, total RNA abundance changing resulted of gene expression pattern and variation is capable of leading the cells towards differentiation (Dudtis et al., 1995). This finding is in accordance with our results about the geno-

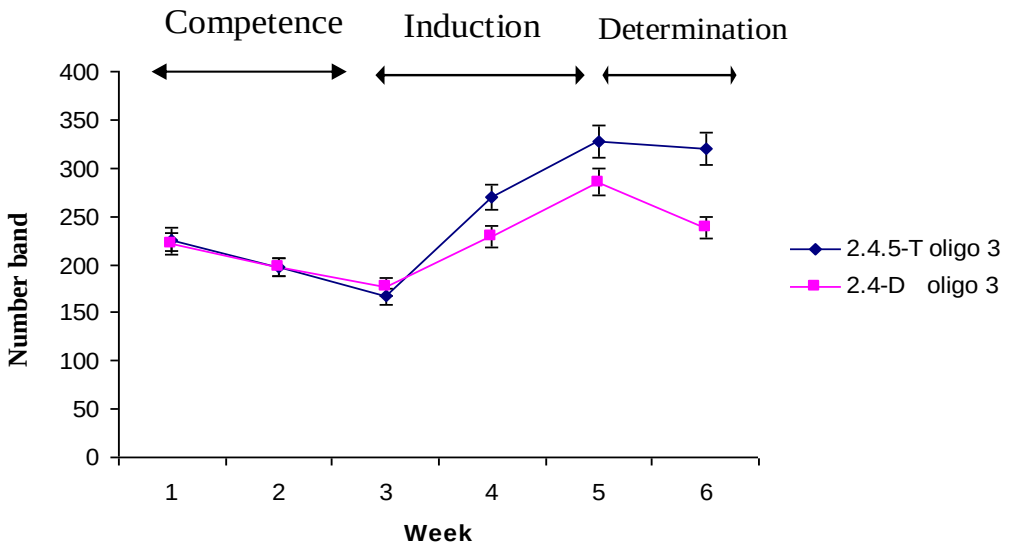

Figure 11. Competence, induction and determination.

types and auxin treatments. Although, the expression level of ILC482 was higher than Bivanig, at the beginning of the third week their expression level declined by the same trend. According to Nomura and Kumamine (1995) studies, prior to active DNA synthesis, the turnover rate of RNA and proteins increase substantially. It was also confirmed that, the activity of *de novo* and salvage pathways for pyrimidine nucleotide biosynthesis increase during embryogenesis accompanying active RNA synthesis.

Indirect somatic embryogenesis needs to form an unorganized cell mass before induction phase. The embryogenic cells are usually in the form of cluster of small sytoplasamic cells referred to proembrygensis masses (Thorpe, 1995). In the present study, from the fifth week, the RNA increasing trend stopped while callus mass was in the largest amount of its own and it did not increase any more. Beyond this phase, globular embryos appeared gradually. It could confirm that polarity and movement towards embryogenesis program has started since the 5th week. Globular embryos appeared in the fifth and sixth weeks when there were embryogenic and non-embryogenic masses on growing tissue and they were feasible to be detached from tissues. They are considered as determined cells where asymmetric divisions occur. A glimpse of 2,4-D and 2,4,5-T graphs shows that they have quite similar patterns. Observations show that 2,4,5-T can produce globular embryo, while 2,4-D cannot. Comparison of both similar band patterns of two auxin treatments indicates a number of particular genes involved in the determination and a large number of changes and genes are similar in both genotypes and auxin treatments.

A number of researches have shown that, there is an ability of embryogenesis in all cells of different genotypes and explants to be induced under proper circumstances. This means the process is different in each plant but occurs when proper situation is provided and cells display their embryogenesis potential (Feher, 2008). Two similar gene expression patterns show that, the activities of many genes are same and simultaneous but it occurs different in specified times identically. A little difference observed is related to differences in some genes that involve in changing the cell developmental path. The other important result concluded from graphs is existence of a critical point that shows a substantial step in induction of embryogenesis.

Conclusions

The first achievement that comes from such model is to help us find a proper time for cDNA preparation or study of proteome for finding specific genes and it provides a way for discovering essential genes and markers in short time. It was concluded that during early embryogenesis, the stages of competence, induction and determination are distinguishable regarding RNA changes (Figure 11).

Similar patterns obtained in two genotypes and two auxin treatments show that the variation trend is stable. Thus, if this study could be repeated for other plants, it is expected that such pattern will occur in the same trend. Occurrence of this model of changes could help researchers to reach the key genes responsible for the key stages. In this regard, chickpea offers a different experimental material for analysis of molecular changes especially during the early inductive phase. We did not investigate the genes in this research, but chickpea is an appropriate model for future investigations because it shows a continuous trend till globular embryo formation

without any need to subculture. Precise study of such model needs to repeat this method in other plants. It also requires separation and identification of genes that is determine at any stage.

Abbreviations

2,4-D, 2,4-Dichlorophenoxyacetic acid; **MS,** Murashige and Skooges; **2,4,5-T ,** 2,4,5-trichlorophenoxyacetic acid; **PCR,** polymerase chain reaction; **DDRT,** differential display reverse transcription.

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