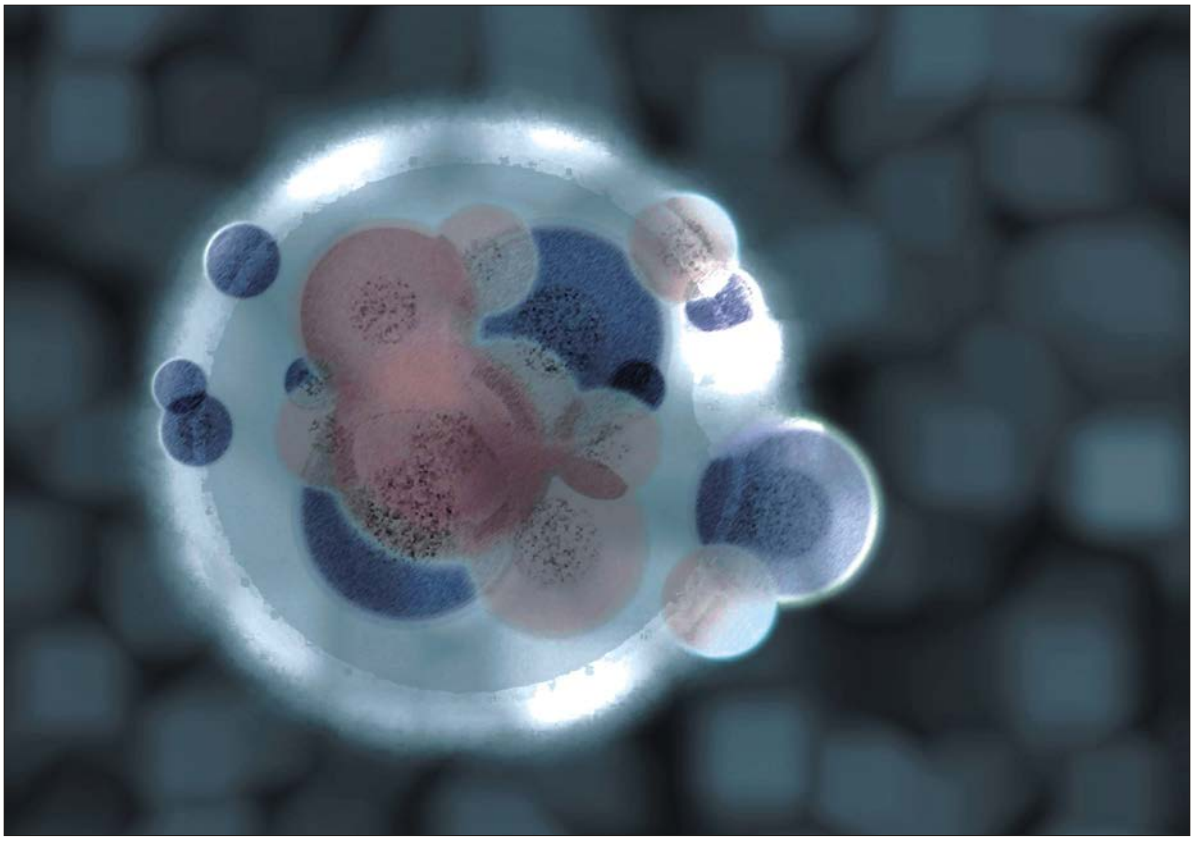

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Qualitative and Quantitative Evaluation of Proteins During Different Stages of Somatic Embryogenesis from Leaf Explant in *Hyoscyamus niger* L.

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Abstract: Because of the therapeutic effects of the tropan alkaloids contained in *Hyoscyamus* genus, fast and enormous propagation of this plant is important. Embryogenesis is the clue to this aim. Assaying protein changes during different stages of embryo growth is a way to get knowledge about embryogenesis mechanism. We assayed protein changes during different stages of embryo formation. Protein amount is very high in leaves and decreases gradually as it is minimum in callus and again increases gradually to late-embryo holding callus. Protein electrophoresis (PAGE) shows that during embryogenesis proteins with molecular weights of 114.4 and 42 KD decrease, proteins of 108.7 KD increase and proteins of 84.8, 60.4, 38.4 and 22.8 KD increase at first and then decrease. SDS-PAGE electrophoresis shows that during embryogenesis, peptides of 111.3, 107.1, 88, 82.7 and 78.3 KD decrease at first and then increase, peptides of 86.25, 76.8, 59.5 and 57.7 KD increase gradually and peptides of 38, 28.8, 21.5 and 20.7 KD increase at first and then decrease.

Key words: *Hyoscyamus niger*, embryogenesis, protein, electrophoresis

INTRODUCTION

The therapeutic effects of *Hyoscyamus* genus are attributed to their contained tropan alkaloids (Li *et al*., 2006) especially hyoscyamine and scopolamine.

Fast and enormous propagation of this plant, regarding its pharmaceutical importance has been of utmost regard. Embryogenesis is the clue to this aim. The knowledge on embryogenesis mechanism in this genus is insufficient at the moment. Evaluation of changes occurred in protein content during embryogenesis is a step forward in improving this knowledge.

An increase in 22 and 65 KD proteins, which probably are storage proteins, occurs simultaneously with the development of somatic embryos and during later maturation of embryonic axes (Roja Rani *et al*., 2005). Also, the development of embryogenic callus is accompanied by the synthesis of embryogenesis-specific polypeptides (35 KD) (Kairong *et al*., 1999). There is a high coincidence between embryogenesis stages and the molecular event related to the biosynthesis and accumulation of proteins (Novozhilova *et al*., 2004), although the storage proteins have also been found with the decrease of embryogenesis (Dahmer *et al*., 1992; Robert *et al*., 1989).

Assessment of protein phosphorylation and its induction showed that phosphorylation is important for acquiring the embryogenesis potential and this phosphorylation has occurred during the transition to embryogenesis (Tan and Kamada, 2000) and is important in biochemical signaling pathways (Rampitsch *et al*., 2006).

Late Embryogenesis Abundant proteins (LEA) possess a protective function in response to drought (Liang *et al*., 2004; Tan and Kamada, 2000), osmotic and low temperature stresses (Liang *et al*., 2004). The synthesis of LEA which is involved in embryogenesis (Gianazza *et al*., 2007), during zygotic and somatic embryogenesis is often regulated via ABA and expression of the gene induced by water stress (Champalans *et al*., 1999; Dodeman *et al*., 1997; Reinbothe *et al*., 1994). Also small heat stress proteins (sHSPs) are expressed in specific developmental stages such as embryogenesis (Lubaretz and Nieden, 2002).

The arabinogalactan proteins (AGPs) found in cell wall and plasma membranes and also in culture mediums are important for development of somatic embryos and increasing the number of embryos (Chapman *et al*., 2000; Majewsla-Sawla and Nothnagel, 2000; Thompson and Knox, 1998; van Hengel *et al*., 2001; Vroemen *et al*., 1999).

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Adding AGPs to embryonic cultures promotes somatic embryogenesis (Kreuger and van Holst, 1993) and in *Picea abies*, will result in the formation of more developed somatic embryos in cell lines (Egertsdotler and van Arnold, 1995).

Lipoketooligosaccharides (LCOs) which are signal molecules of embryogenesis are involved in regulation of somatic embryos development (Arnold *et al*., 2002) and are utilized as stimulators of somatic embryos for progression of the last stage of globulization (de Jong *et al*., 1993) and triggering the development of pre-embryonic masses from small cellular masses (Dyachok *et al*., 2000; Egertsdotter and van Arnold, 1998).

The analogues of endogenous LCOs, in the structure of rhizobiomal nodulation factors are released from AGPs with the action of endokitinases and as signal molecules of development, stimulate the formation of somatic embryos (Arnold *et al*., 2002). Protein kinase I is a receptor for somatic embryogenesis that functions in the signaling pathway in *Arabidopsis thaliana* (Araujo *et al*., 2004).

All of the points mentioned above indicate the importance of proteins in embryogenesis; hence evaluation of protein changes taken place in different stages of embryogenesis will instruct us in our way to get better knowledge about the mechanism of embryogenesis. Similarity between proteins found in regenerated maize plants via embryogenesis and zygotic embryos (Lubaretz and Nieden, 2002) gives us the hope that the results gained from somatic embryogenesis be also applicable to zygotic embryogenesis.

In this research, with qualitative as well as quantitative evaluation of proteins' changes in different stages of embryogenesis, we are about to take a step toward better knowledge about embryogenesis mechanism in *H. niger* L.

MATERIALS AND METHODS

The seeds of *H. niger* L. were collected from the region of Kandovan tunnel, Chalus road. Seed dormancy was removed with gibberlin (35 mg L^{-1}) treatment for 12 h. After 2-stage sterilization, the seeds were grown on MS medium without any growth regulators. Leaf explants collected from sterile seedlings were cultured on embryogenic medium containing BAP (1.5 mg L^{-1}) and IAA (0.2 mg L^{-1}) (Ebrahimzadeh *et al*., 2003). Collection of the explants was performed in a stage-by-stage manner from the initiation of culture to embryo formation (i.e., leaf, callogenic leaf, callus, embryogenic callus and embryo-holding callus). In every single stage the explant was first fixed in liquid nitrogen and stored in -70°C.

This study was conducted in the laboratory plant physiology in the faculty of science, University of Tehran, during 2006.

Protein extraction: The appropriate buffer for extraction is 0.2 M phosphate buffer pH = 6.8 (Bollage and Edelstein, 1991). The explants were ground in buffer with 1:1.5 w/v proportions. The homogenates were centrifuged (with HERAEUS 400 R) at 13000 rpm for 20 min in 4°C. The collected supernatants were then centrifuged again at 13000 rpm for 10 min in 4°C. The resulted new supernatants were collected for various assays.

Quantitative evaluation of soluble proteins in extracts was performed using Bradford (1976) method and the protein concentration (mg protein/1 g tissue) was determined for each explant.

Qualitative evaluation of proteins was performed with two methods of electrophoresis on polyacrylamide

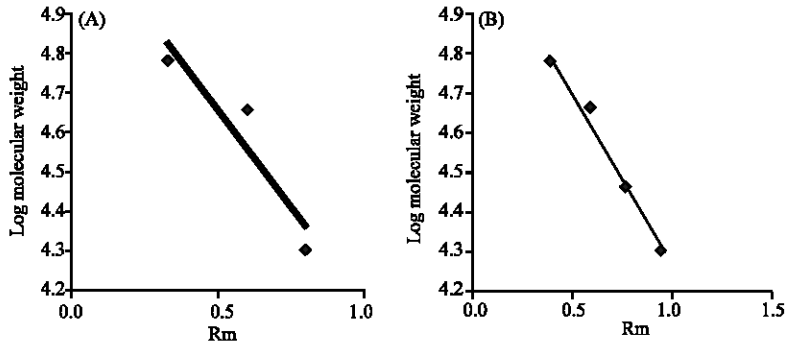

Fig. 1: Standard diagram for determination of molecular weights of polypeptides. This diagram has been drawn according to relative mobility of standard proteins with certain molecular weights in PAGE (A) and SDS-PAGE (B) gels. Rm: Relative mobility

gel in discontinued system: on reducing gel containing sodium de decil sulphate (SDS-PAGE) which is appropriate for determining polypeptide size (Crawford, 1990) and on non-reducing gel (PAGE) (Patel, 1994).

The standard curve for determining molecular the weights of peptides and proteins according to relative mobility (Rm) of standard proteins with known molecular weights in PAGE (Fig. 1A) and SDS-PAGE (Fig. 1B) gels was drawn.

RESULTS

After leaf culture (Fig. 2A) on embryogenic medium, the explants first inflate (Fig. 2B), start to form callus (Fig. 2C), after callus formation (Fig. 2D), they change to embryogenic callus (Fig. 2E) and at last embryo-holding calli (Fig. 2F) formed.

According to this Table 1, protein contents are highest in leaf (explant segment), a little decreased in callogenic leaf, is the least in callus and again gradually increases in embryogenic callus, callus holding early embryo and callus holding late embryo protein contents in callogenic leaf, callus, embryogenic callus, callus holding early embryo and callus holding slate embryo are 36.21, 60.94, 57.7, 52.54 and 43.17%, respectively lower as compaired to the leaf protein contents (Fig. 3). Kruskal Wallis test indicates that the differences are significant (Chi square (p):14.91 (0.01).

Electrophoresis on poly-acrylamide gel in discontinued system of non-reducing gel (PAGE) showed 7 different proteins in different stages of embryo formation (Fig. 4).

According to the Table 2, during embryogenesis proteins 1 and 5 decrease, protein 2 increases and proteins 3, 4, 6 and 7 show an increase followed by decrease.

Electrophoresis on poly-acrylamide gel in discontinued system on reducing gel (SDS-PAGE) showed 14 different peptides in different stages of embryo formation (Fig. 5).

Table 3 shows that during embryogenesis, peptides 1, 2, 3, 5 and 6 decrease at first and then increase, peptides 4, 9 and 11 gradually increase, peptides 12, 13, 14 and 15 increase at first and then decrease and peptides 8 and 10 decrease.

Presence or absence of peptides and proteins in different stages of embryogenesis indicate their role in this differential phenomenon. Those which are present in some stages and absent in some other, possess main role in differentiation but those present in all stages but undergo amount changes, are conservative proteins or peptides which play their role in general activities of

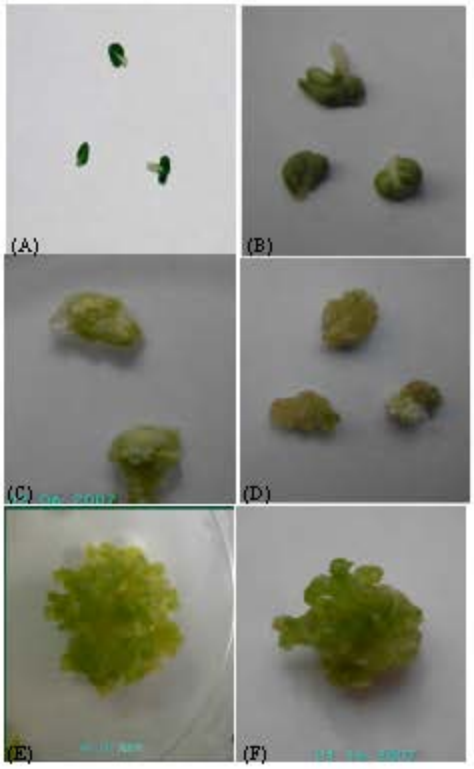

Fig. 2: Various stages of embryogenesis (A) leaf, (B) inflated leaf, (C) callus formation form leaf, (D) callus, (E) embryogenic callus and (F) embryo-holding callus

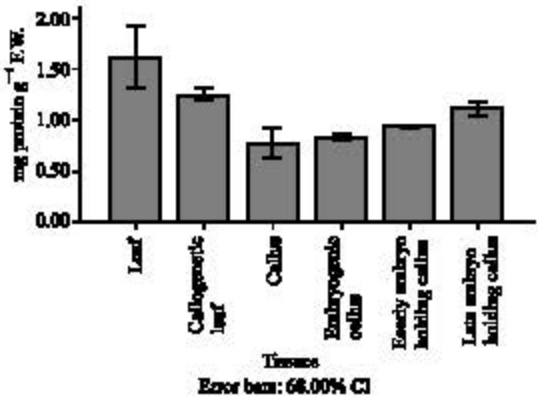

Fig. 3: Protein changes during different stages of embryo formation

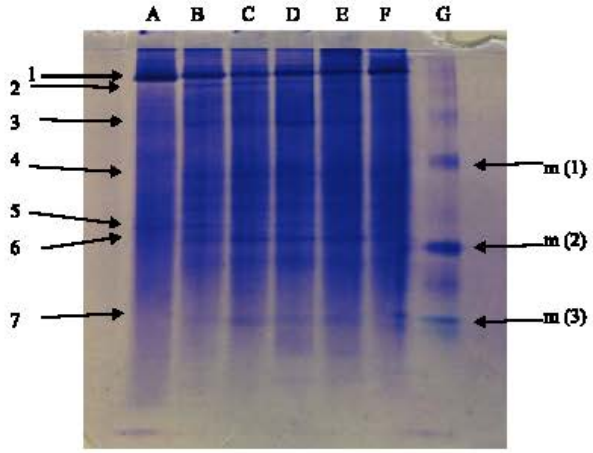

Fig. 4: (Isolation of proteins (PAGE) from leaf (A), callogenic leaf (B), callus (C), embryogenic callus (D), early embryo-holding callus (E), late embryo-holding callus (F) and standard proteins (G). No. of proteins: 1-7. markers with. 60, 45 and 20 KD, respectively: m (1-3)

Table 1: Protein amounts (mg protein/1 g tissue) in different stages of embryo formation

Tissue	Protein concentration mg protein g^{-1} F.W
Leaf	1.9530
Callogenic leaf	1.2460
Callus	0.7630
Embryogenesis callus	0.8263
Early embryo holding callus	0.9270
Late embryo holding callus	1.1100

differentiation. Hence according to the Table 2, proteins 2, 6 and 7 are the main proteins involved in differentiation and proteins 1, 3, 4 and 5 are conservative proteins involved in general activities of differentiation. Also regarding to Table 3, peptides 1, 4, 5, 7, 8, 9, 10, 11 and 14 are main peptides involved in differentiation and peptides 2, 3, 6, 12, 13 and 15 are conservative peptides playing their role in general activities of differentiation.

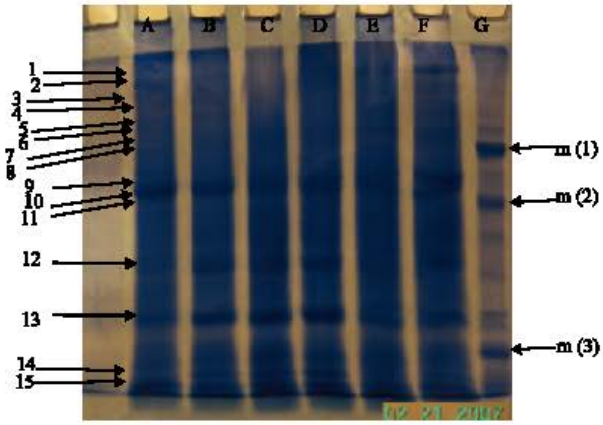

Fig. 5: Isolation of peptides (SDS-PAGE) of leaf (A), callogenic leaf (B), callus (C), embryogenic callus (D), early embryo-holding callus (E), late embryo-holding callus (F) and standard proteins (G) No. of peptides: 1-5 markers with 60, 45 and 20 KD, respectively: m (1-3)

Table 2: Analysis of proteins' electrophoresis in various stages of embryo formation, using PAGE method

No.	R_m	Molecular weight	Leaf	Callogenic leaf	Callus	Embryogenetic callus	Early embryo holding callus	Late embryo holding callus
1	0.088	114.4	+++++	++++	++++	++++	++++	+++
2	0.110	108.7		++	++	++	++	++
3	0.220	84.8	+	++	++	+++	++++	+
4	0.370	60.4	+	++	+++	+++	++++	+
5	0.530	42.0	++	+	+	+	+	+
6	0.570	38.4		+	+++	+++	+++	+
7	0.800	22.8			++	++	+	

+ +++++: Relative quantities

Table 3: Analysis of peptides' electrophoresis in different stages of embryo formation with SDS-PAGE method

No.	R_m	Molecular weight	Leaf	Callogenic leaf	Callus	Embryogenetic callus	Early embryo holding callus	Late embryo holding callus
1	0.080	111.30	++++	+++			+++	++++
2	0.100	107.10	++++	+++	+	+	+++	++++
3	0.200	88.00	+++	+++	+	+	+++	++
4	0.210	86.25	++	++			+++	+++
5	0.230	82.70	++		+	+	+++	+++
6	0.260	78.30	+++	+++	++	++	+++	+++
7	0.270	76.80			++	+++	+++	+++
8	0.280	75.30	++					
9	0.400	59.50				+++++	+++++	+++++
10	0.411	58.30	++++++	++++++	+++++++			
11	0.416	57.70				+++++	+++++	+++++
12	0.630	38.00	+++++	++++++	+++++++	+++++++	+++++	+++++
13	0.770	28.80	+++++	++++++	++++++	++++++	+++++	+++++
14	0.920	21.50		++++	++++	++++		
15	0.940	20.70	++	+++	++++	++++	+	+

+ +++++++: Relative quantities

DISCUSSION

Assaying protein changes during different stages of embryo growth is a way to get knowledge about embryogenesis mechanism. Roja Rani *et al.* (2005) reported the increase of 22 and 65 KD proteins concurrent with the formation of somatic embryos and during later stages of embryonic axes maturation. Synthesis of embryogenic-specific polypeptides concurrent with embryogenic callus development has also been shown (Kairong *et al.*, 1999). Besides, biosynthesis increase and storage of some proteins concurrent with embryogenesis increase (Novozhilova *et al.*, 2004) and embryogenesis decrease (Dahmer *et al.*, 1992; Robert *et al.*, 1989) have been shown. In the present research protein changes during different stages of embryo formation were evident. Quantitative evaluation of total protein amount in different stages showed that protein content is very high in leaves (1.953 mg protein g^{-1} F.W.) and decreases gradually as it reaches to its lowest level in callus (0.763 mg protein/1 g tissue) and again increases gradually to late embryo-holding callus (1.11 mg protein g^{-1} F.W.). Protein electrophoresis (PAGE) shows that during embryogenesis: Proteins of 114.4 and 42 KD decrease (down regulation of this proteins are noticeable). Proteins of 108.7 KD increase and proteins of 84.8, 60.4, 38.4 and 22.8 KD increase at first and then decrease (up regulation of this proteins are noticeable).

According to the results from SDS-PAGE electrophoresis, during embryogenesis: Peptides with molecular weights of 111.3, 107.1, 88, 82.7 and 78.3 KD decrease at first and then increase, peptides with molecular weights of 75.3 and 58.3 KD decrease (down regulation of this peptides are noticeable). Peptides with molecular weights of 86.25, 76.8, 59.5 and 57.7 KD increase gradually and peptides with molecular weights of 38, 28.8, 21.5 and 20.7 KD increase at first and then decrease (up regulation of this peptides are noticeable).

The roles of AGPs in the development of somatic embryo (Majewsla-Sawla and Nothnagel, 2000; Vroemen *et al.*, 1999) and increasing the number of embryos (Van Hengel *et al.*, 2001) and the role of LCOs as the signal molecules in somatic embryogenesis which are involved in the regulation of somatic embryo development (Arnold *et al.*, 2002) have previously been examined.

It has been evidenced that some proteins which are increased under the effect of different stresses such as drought stress (Liang *et al.*, 2004; Tan and Kamada, 2000), osmotic and cold stresses (Liang *et al.*, 2004), water stress (Campalans *et al.*, 1999) and heat stress (Lubaretz and Nieden, 2002), are also involved in embryogenesis. Hence it can be concluded that just like stress, which through induction of some genes (Reinbothe *et al.*, 1994) causes the formation of some specific-protective-proteins, in this differential process, also, some genes are induced leading to the formation and increase of some proteins and some genes go silent leading to decrease or vanishing of their corresponding proteins. In this study, decrease, increase or changes in proteins and peptides which are the outcome of changes taken place in the corresponding genes, shows their roles in the different stages of embryo formation.

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