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Somatic embryogenesis in *Solanum tuberosum* from cell suspension cultures: histological analysis and extracellular protein patterns

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Cell suspension;
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Summary

An embryogenic cell suspension, continuously grown in Murashige and Skoog (MS) medium with 0.5 mg/L of 2,4-dichlorophenoxyacetic acid, was established from friable callus of *Solanum tuberosum* internode sections. The cell suspension was predominantly composed of cell masses and free embryogenic cells. When transferred to an auxin-free medium with zeatin, somatic embryos (SEs) developed and converted to complete plants when cultured on solid MS medium without growth regulators. The system produced approximately 600 SEs per 50 mL of medium. In this investigation, accumulation of extracellular proteins (EPs) of different molecular weights were found associated to different phases of the embryogenic process. At the initiation of the cell suspension, cell clusters and free cells present in the culture (phase "A") secreted a 78 kDa EP, unique to this phase. In phase "B", which is related to embryonic cell determination process, proteins (7–14 kDa) were secreted mainly by embryogenic cells. In phase "C", SEs in different developmental stages secreted protein of 32 kDa, which appeared as a particular feature of the phase. EPs of phase "D", secreted by torpedo and mature embryos, had molecular weights between 20 and 50 kDa. Further studies will be necessary to identify these proteins and link them to previously identified somatic embryogenesis-related proteins. Histological analysis of the potato embryogenesis in liquid media showed unicellular origin of the SE.

Abbreviations: 2, 4-D, 2, 4-dichlorophenoxyacetic acid; BA, benzyladenine; GA_3, gibberellic acid; MS, Murashige and Skoog salt and vitamin mixture; SDS PAGE, sodium dodecyl sulfate polyacrilamide gel electrophoresis; SEs, somatic embryos

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Introduction

During the past two decades, there has been great scientific effort to obtain somatic embryos (SEs) from a range of potato genotypes and from different potato tissues, including anthers (Sopory et al., 1978), immature zygotic embryos (Petrova and Dedicova, 1992), stem (De García and Martınez, 1995; Seabrook and Douglass, 2001; Seabrook et al., 2001), tuber sections and leaves (JayaSree et al., 2001; Seabrook and Douglass, 2001), and roots (Seabrook and Douglass, 2001). The large-scale production of potato plants using bioreactors could be more efficient when SEs are used as propagules (Schafer-Menuhr et al., 2003), or for rapid multiplication of plants via synthetic seeds, where SEs can be encapsulated for direct seeding (Smith, 1995; Fieger et al., 2002; Nyende et al., 2002). For mass propagation, establishment of somatic embryogenesis from cell suspension cultures is needed to assure massive production of SEs.

To induce somatic embryogenesis in chicory, the existing gene expression pattern in the starting material must be modified and replaced with an embryogenic gene expression program in the cells, which become competent to give rise to SEs (Randoux et al., 2002).

The establishment of a highly productive system of somatic embryogenesis from cell suspension cultures of potato (*Solanum tuberosum*) is described. The protocol reported here produced a large number of SEs developmentally competent to regenerate complete plants. Some features of the ontogeny of the SEs, and its unicellular origin are demonstrated by histological analysis. Extracellular protein (EP) patterns associated with different phases of somatic embryogenesis were analyzed.

Material and methods

Plant material

Internode sections were obtained from in vitro propagated plantlets of potato (*S. tuberosum* L. cv. Désirée).

Induction and establishment of cell suspension cultures

To induce callus tissue, nodal sections (1–1.5 cm long) were cultured on Murashige and Skoog (MS1) medium (Table 1), based on MS salts (Murashige and Skoog, 1962), and incubated in the dark at $25 \pm 1\,^{\circ}C$ for 2 months, following the protocol established by De García and Martínez (1995). One gram of callus tissue was inoculated into 100 mL of liquid medium MS2 (Table 1), placed on an orbital shaker (160 rpm), and incubated in darkness at $25 \pm 1\,^{\circ}C$ for 2 weeks. All media renewals were performed by decanting the suspension every 15 days. Tissues were fractionated, after 2 weeks of growing, in pieces of different sizes and filtered through sterile 100 μm mesh, transferred to fresh MS2 medium and placed in the same environmental conditions. Two weeks later, conditioned MS2 medium was collected for protein extraction (EPs Phase "A"), cells were transferred to MS3 medium (Table 1), and maintained under continuous light conditions ($50\,\mu mol/m^2\,s$), at $25 \pm 1\,^{\circ}C$. After 15 days, conditioned MS3 medium was collected for EP analysis (Phase "B"), and cells were recultured in fresh medium MS2. Cells remained in this medium until pro-embryogenic groups and globular embryos were observed (30 days). At this time, the conditioned medium was collected to obtain EPs (Phase "C"), and was substituted by MS4 medium. After 30 days, conditioned MS4 medium was collected for protein extraction (Phase "D"). Embryos at this time had reached maturity and were transferred to solid medium MS8 (Table 1), where the embryos developed into complete plantlets.

At the end of the process (3 months), 10 flasks were randomly selected, and the number of embryos was counted to determine the efficiency of embryo production from the cell suspension cultures.

Table 1. Culture media used for the establishment of somatic embryogenesis in *Solanum tuberosum* cv Désirée from cell suspension cultures. Concentrations in mg/L.

Component:	MS1	MS2	MS3	MS4	MS8
Salts	MS[a]	MS	MS	MS/2	MS
Myo-inositol	100	100	100	100	100
Thiamine	0.1	0.1	0.1	0.1	0.1
Pyridoxine	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5
Glycine	2	2	2	2	2
Yeast extract	—	1000	—	—	
Sucrose	25000	25000	25000	25000	25000
Coconut milk	—	—	—	100 mL	
Citric acid		50	50	50	
Ascorbic acid		50	50	50	
Kinetin	—	0.5	—	—	
2,4-D[b]	4	0.5	—	—	
Zeatin	—	—	1	0.5	
Gelrite	2000	—	—	—	2000
pH	5.6	5.6	5.6	5.6	

[a] Murashige and Skoog (1962).
[b] 2,4-Dichlorophenoxyacetic acid.

Transfer of plants to soil

Forty plantlets, 8 cm long, were potted in a mixture of soil and river sand 3:1, and placed under high humidity (80–93% relative humidity), and low-light conditions (10 μmol/m^2/s). After 10 days, plantlets were transferred to a greenhouse.

Histological methods

Samples from cultures were fixed in 40% formaldehyde, 10% glacial acetic acid, 50% ethanol, dehydrated in a graded alcohol series containing 50%, 70%, 85%, 95% and 100% alcohol by volume, and then transferred to tertiary butyl alcohol; the time of immersion in each concentration was 1 day. The samples were then embedded in paraplast. Serial sections from 10 to 12 μm were stained with safranin and fast green.

Cell suspension culture growth kinetics

Cell suspension culture growth kinetics was established from a suspension culture that was initiated by inoculating 1 g of callus tissue in 50 mL MS2 liquid medium. Fresh and dry weight, and cell number, was determined every day for 15 days. To get fresh weight values, 1 mL of the suspension culture was transferred to a preweighed Eppendorf tube, centrifuged (10,946*g*) for 5 min, the supernatant was discarded, and the Eppendorf tube with the cells was weighted. To get the dry weight, the Eppendorf tube with the cells was placed in an oven at 60 °C for 48 h, and then weighted. Cell number was determined from 20 μL cell suspension culture, using a hemacytometer.

Extracellular protein extraction

Conditioned medium from Phases "A–D" (200 mL) was passed through a Millipore 0.22 μm filter and the proteins were precipitated by addition of 1/10 vol 100% trichloroacetic acid, 4 °C for 2 h as described by Colmenares et al. (1998).

SDS-PAGE electrophoresis

The EPs were collected by centrifugation at 6700*g* for 20 min, 4 °C and resuspended in Laemmli buffer. Electrophoresis was performed on sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) 14% gels according to Laemmli (1970) and the gels were stained with Coomassie brilliant blue.

Results

Establishment of cell suspension cultures

Callus formation was observed 15 days after the initiation of the cultures on MS1 medium. Sealing callus was formed on both sides of the internodes. Two months later the callus appeared to be friable and surrounded all the explants. Cell suspension cultures were established from 1 g of callus tissue, which was mechanically desegregated in cellular groups and free cells. Tissues were cultured for 15 days in liquid MS2 medium. The suspension cultures were then filtered through 100 μm mesh to get a heterogeneous cell suspension culture mainly composed of free embryogenic and non-embryogenic cells and small cell groups (Fig. 1). These cultures were placed for 15 more days on MS2 medium (under dark conditions). This was called Phase "A". Histological observations of this suspension culture showed that embryogenic cells were iso-diametric, with thick walls, dense cytoplasm, prominent nuclei, and contained a high number of starch grains (Fig. 2). Non-embryogenic cells were larger than embryogenic ones, vacuolated and with few starch grains. At the end of this period the amount of embryogenic cells and cell groups increased. Suspension cultures were then transferred to MS3 medium (without 2,4-D) and placed in light conditions in order to start Phase "B". The conditioned MS2 medium was collected to get EPs of Phase "A". After 15 days the number of embryogenic cells increased (Fig. 3), and conditioned MS3 was collected to obtain EPs of Phase B; cells were recultured in MS3 medium to start Phase "C". Fifteen days later, globular stage embryos began to develop from embryogenic cells. Light microscopy analysis demonstrated that before initiating differentiation of the SE, the embryogenic cell developed a thick cell wall (Fig. 2) and the globular embryos were separated from the cell groups by a thin layer of protodermic cells (Fig. 4). At this time, the conditioned medium MS3 was collected and substituted by MS4 medium, to begin Phase "D". The number of globular embryos increased during the first 15 days, and heart and torpedo states started to appear. In Fig. 5, a cross-section of a globular embryo can be observed. Heart-shaped embryos (Fig. 6) developed on MS4 medium, but rapidly changed to a torpedo embryos stage. A cross-section of a torpedo embryo is shown in Fig. 7. Thirty days later, many embryos reached cotyledonary and mature stages and some others germinated (Fig. 8). Conditioned MS4 medium was collected to determine EPs of Phase "D". Mature embryos and small plantlets were

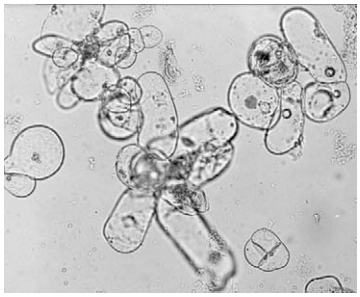

Figure 1. Clusters of embryogenic and non-embryogenic cells in a *S. tuberosum* suspension culture ($\times$ 10).

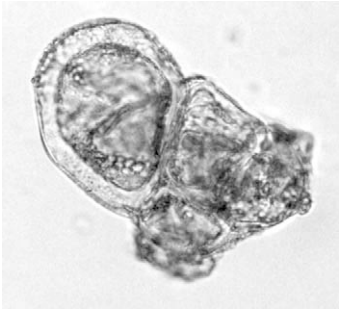

Figure 2. Embryogenic cell showing thick cell wall ($\times$ 40).

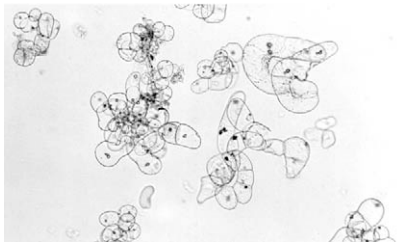

Figure 3. Clusters of embryogenic cells growing in suspension culture (Phase "B") ($\times$ 20).

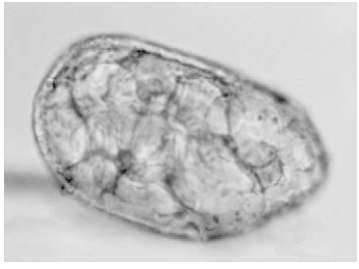

Figure 4. Globular embryo showing a thin layer of protodermis ($\times$ 36).

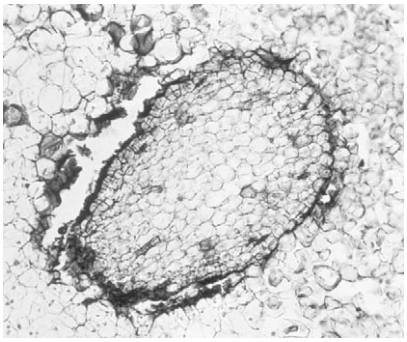

Figure 5. Cross-section of a globular embryo ($\times$ 20).

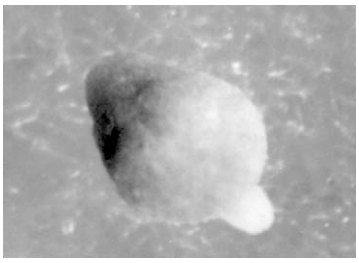

Figure 6. Heart-shaped embryo from cell suspension culture (Phase "D") ($\times$ 10).

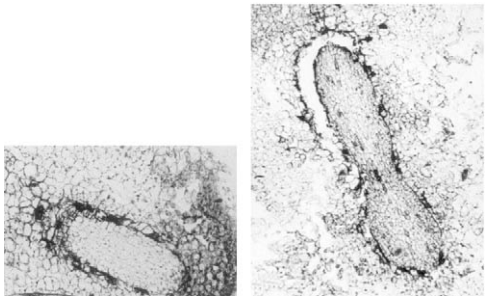

Figure 7. Cross-section of a torpedo embryos ($\times$ 10).

Figure 8. Fully matured SE as observed under stereomicroscope.

transferred to solid MS8 medium to complete their development.

The growth kinetics of *S. tuberosum* cell suspension culture (Fig. 9) showed a smooth increase in the growth of the cells, to the fifth day. Then cells grew very fast to reach maximum values on day 13, attainment values of 93.0 mg/mL of fresh weight, 2.5 mg/mL of dry weight, and 6.87×10^5 viable cells/mL.

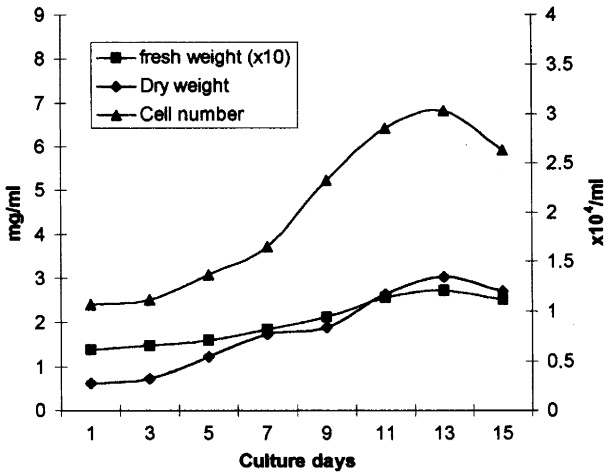

Figure 9. Growth kinetic of cell suspension culture of *S. tuberosum* cv. Désirée, growing in MS2 medium.

The efficiency of the system was evaluated by quantifying the number of embryos per 50 mL liquid medium, in 10 flasks selected randomly at the end of the process (3 months). We found an average of 78.4 ± 17.8 cell clusters, with 7.4 ± 1.5 embryos per cluster; 37.6 ± 9, four free embryos floating in the medium with a total of 599.9 ± 129.3 SEs.

Extracellular protein patterns from media in which embryos developed

EPs were precipitated from the conditioned media obtained at the end of each of the four phases ("A–D") that were established to study the potato somatic embryogenesis. The SDS-PAGE analysis revealed different protein patterns of expression on each of these somatic embryogenesis phases. Conditioned medium from Phase "A", which was in contact with cell clusters and free cells in culture, presented protein bands that ranged 12–78 kDa. The 78 kDa band was unique for this phase. Also, protein bands of 12, 18, 20, 25, 38 and 50 kDa were present (Fig. 10).

Protein patterns from conditioned medium of Phase "B", which was secreted mainly from embryogenic cells, revealed the existence of low-molecular-weight protein bands (7–14 kDa). Also, protein bands of 29 kDa and a group of bands between 35 and 40 were present. Protein bands of 25 and 50 that were visible at phase A, were also present in this phase; on the other hand, 78 kDa protein disappeared (Fig. 11). Phase "C" corresponds to proteins secreted from embryogenic cell clusters and globular embryos in suspension cultures. In this case, we could not see that the low-molecular 17, 26, 30, 35, 42 and 44 kDa were also

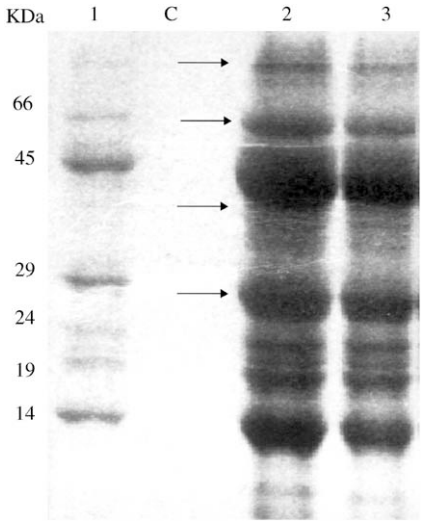

Figure 10. SDS-PAGE of EP from conditioned medium of Phase "A". Lane 1: molecular weight standards, lane "C": control (proteins from culture medium without cells in suspension), lane 2: 0.6 μg of EP, and line 3: 1.2 μg of EP. Arrows (→) indicate 25, 38,50,78 kDa proteins.

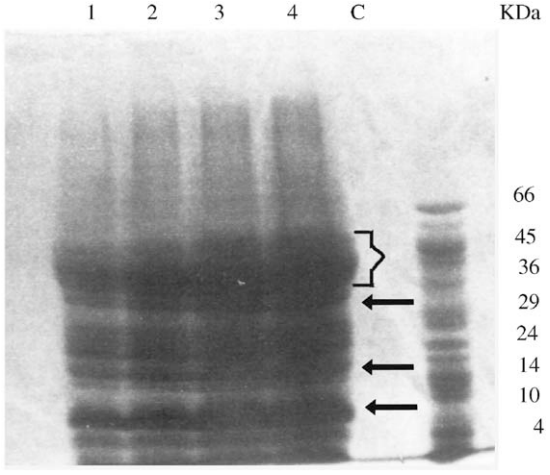

Figure 11. SDS-PAGE of EP from conditioned medium of Phase "B". Lane 1: 0.23 μg of EP, lane 2: 0.46 μg of EP, lane 3: 0.7 μg of EP, lane 4: 1.15 μg of EP, lane C: control (proteins from culture medium without cells in suspension), and lane 5: molecular weight standards. Arrows (→) indicate 7,14, and 29 kDa proteins. Key ({) indicates a group of 35–40 kDa proteins.

observed (Fig. 12). In Phase "D", where conditioned medium was in contact with embryos in different development stages and germinated embryos, high-molecular-weight proteins were not observed, but protein bands of 30, 35 and 44 kDa that were present in Phase "C" appeared also in this phase. The 38 kDa band that was present in

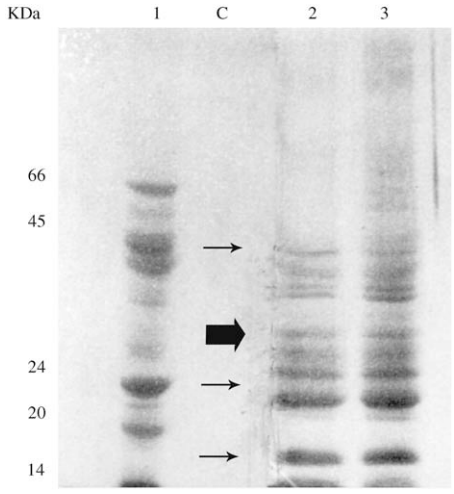

Figure 12. SDS-PAGE of EP from conditioned medium of Phase "C". Lane 1: molecular weight standards, lane "C": control (proteins from culture medium without cells in suspension), lane 2: 0.6 μg of EP, and lane 3: 1.2 μg of EP. Arrows (→) indicate 17, 26 and 44 kDa proteins. Double arrow (➡) indicates 32 kDa protein.

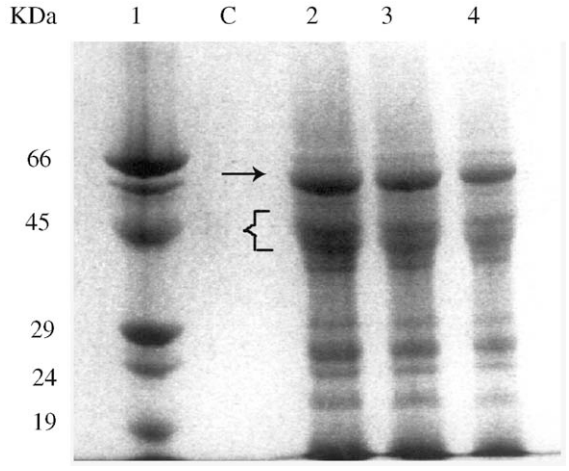

Figure 13. SDS-PAGE of EP from conditioned medium of Phase "D". Lane 1: molecular weight standards, lane C: control (proteins from culture medium without cells in suspension), lane 2: 4.8 μg of EP, lane 3: 3.5 μg of EP, and lane 4: 2.4 μg of EP. Arrow (→) indicates 50 kDa protein. Key ({) indicates a group of 30–44 kDa proteins.

Phase "A", reappeared in this phase. Also, the 50 kDa band that was seen in Phases "A" and "B" was also visible in Phase "D" (Fig. 13).

Discussion

Potato somatic embryogenesis has been reported using solid media from different explants (Sopory et al., 1978; Petrova and Dedicova, 1992; De García and Martínez, 1995; JayaSree et al., 2001), and from a range of genotypes and ploidy levels (Seabrook and Douglass, 2001). The genetic segregation for the process has been analyzed (Seabrook et al., 2001). More recently, the induction of SEs from potato cell suspension cultures and the encapsulation of SE to produce artificial seed were also reported (Fieger et al., 2002).

In this investigation, we report the establishment of a protocol which yielded a considerable amount of SEs in liquid media. This system was initiated from cell suspension cultures of stem internode sections of *S. tuberosum*. The system produced 600 ± 129 SEs per 50 mL of media, after 60 days of the embryogenesis cell suspension setup. This value is significantly higher than the number of SEs produced by other embryogenic liquid systems established for other plant species, like carrot (De Vries et al., 1988a, b), grapevine (Goebel-Tourand et al., 1993) and *Acantopanax koreanum* (Choi et al., 1997).

It has been reported that the rejuvenation of potato tissues associated with in vitro culture influences the amount of callus and shoot regeneration (Quaraishi and John, 1985; Martel and De García, 1992). We found that this is also true in the potato cell suspension cultures, the frequent reculturing of the cell suspension appeared to influence both the time required to develop SEs and the number of SEs formed. In this work, we analyzed the growth kinetics of the cell suspension and determined that the cells should be transferred every 15 days to preserve competent cells with rapid response to somatic embryogenesis stimulus.

The development of the embryos from cell suspension culture is independent of the gibberellic acid (GA_3), which was not added to the media. It has been reported that the addition of GA_3 to the culture media was required for shoot development and elongation in potato callus culture. In its absence, only nodule-like structures were formed on the callus surface (Roest and Bokelman, 1976; Jarret et al., 1981; Martel and De García, 1992).

De García and Martínez (1995) observed that when 2,4-dichlorophenoxyacetic acid (2,4-D) is used to induce embryogenic callus, differentiation of embryos can be reached in media with or without GA_3. This observation is confirmed in the present work. JayaSree et al. (2001), working with leaf explants, demonstrated that callus initiated with 2,4-D and benzyladenine (BA) is nodular and embryogenic giving rise to SEs in media with, or without GA_3, in the presence of BA and/or zeatin. Callus initiated without 2,4-D but with naphthalene acetic acid and BA, mainly produced shoots via

organogenesis, despite the growth factor used to induce differentiation (JayaSree et al., 2001).

The histological characterization of the potato embryogenesis process in liquid media showed the unicellular origin of the SE. The initiation of SE was observed when one cell developed a thick wall, providing the conditions for a fine temporal and spatial regulation of cell division (Ammirato 1987). Vroemen et al. (1996) and Van Hengel et al. (2001) analyzed the role of some compounds related to cell wall formation on somatic embryogenesis process and demonstrated that arabinogalactan proteins restore somatic embryogenesis in carrot protoplast cultures, and this effect was enhanced by EP3 endochitinase.

Different stages of SE development (globular, heart and torpedo) were observed, but heart stage embryos were less frequent than the others. This could be due to the fact that the embryogenesis of potato in liquid media proceeds quickly through this stage, so the heart stage embryos are seldom observed.

Embryo-specific proteins or genes have been recently described (Coutos-Thevenot et al., 1992; Dupire et al., 1999; Hecht et al., 2001; Shah et al., 2002). Few proteins associated with the somatic embryogenesis process have been identified and protein patterns were relatively stable during the culture (Hahne et al.,1988; Ramagopal, 1989; Boyer et al., 1993; Oropeza et al., 2001). In this research, we found that there are several changes in protein patterns during the development of SEs. In Phase "A" we found a 78 kDa protein that was unique to this phase. Proteins of 25, 38 and 50 kDa were also present in this phase. Hvoslef-Eide and Corke (1997) reported a protein of 50 kDa in birch embryogenic cells, 24 h after they were transferred to embryo induction medium (with auxin, cytokinin and casein hydrolyzate). Proteins of 25 and 38 kDa have been identified by Helleboid et al. (2000) as pathogenesis-related proteins that accumulate in the embryogenic culture medium of *Chicorium* hybrid clone "474". The 38 kDa protein is a β-1,3-glucanase and the 25 kDa protein is an osmotin-like protein. The authors suggested that the accumulation of these proteins could be partially correlated with the somatic embryogenesis process.

In Phase "C", the proteins in the medium were secreted by SEs in different developmental stages. Protein of 32 kDa appeared only in this phase. De Vries et al. (1988b) reported a secreted 32 kDa protein with peroxidase activity in carrot somatic embryogenic culture medium. They assumed that at least part of the effects of the secreted glycoproteins on embryogenesis may be explained in terms of a mechanism to restrict cell expansion. EPs phase "D" that were secreted in the present study by torpedo and mature embryos had molecular weights between 20 and 50 kDa. These proteins could be correlated with the proteins extracted from lyophilized embryogenic cell lines of birch by Hvoslef-Eide and Corke (1997). They claimed that these "embryo-specific proteins" are also present in SEs at the torpedo stage.

In conclusion, in this work we described culture conditions for inducing somatic embryogenesis from internode tissue and a regeneration protocol for *S. tuberosum* plants in liquid and solid medium. It is a highly efficient protocol which is expected to be useful for the genetic improvement of potato through genetic engineering. Also, we have related specific protein patterns to each of the different somatic embryogenesis phases observed during this process. Further studies will be necessary to identify these proteins and associate them with previously identified somatic embryogenesis-related proteins.

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