African Journal of Biotechnology Vol. 11(47), pp. 10761-10768, 12 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI:10.5897/AJB11.3702
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Changes in the protein profile of Habanero pepper (*Capsicum chinense* J.) somatic embryos during development

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Accepted 1 February, 2012

Protein profile was studied during the development of *Capsicum chinense* somatic embryos. The total protein content and profile of polypeptides (by sodium dodecyl sulfate polyacrylamide gel electrophoresis) of somatic embryos at different developmental stages (globular, heart-shaped, torpedo and cotyledonary stages) were analyzed. The protein profile of zygotic embryos included nine exclusive bands with molecular weights of 4.0, 5.2, 8.1, 13.7, 20.9, 23.7, 41, 50 and 69.3 kDa; these bands were not observed in the protein profile of somatic embryos. Coincidently, five of these bands possessed similar molecular weights to those reported for storage proteins in other plant species. Protein content showed a clear decreasing tendency with increasing somatic embryo development. The lowest protein content was detected in somatic embryos at the cotyledonary stage (0.436 µg/mg fresh weight), and the highest content was found in somatic embryos at the globular stage (2.98 µg/mg fresh weight). Total proteins two-dimensional electrophoresis (2-DE) analysis of mature zygotic embryo (prior to the desiccation) and cotyledonal somatic embryo, showed significant differences in the protein profile of both types of embryos. Zygotic embryo showed the proteins expression of isoelectric point between 4 to 7 and 7 to 10, and molecular weights between 25 to 36 KDa, which were not expressed in the cotyledonal somatic embryo. The low protein content during the development of the somatic embryos, particularly at the cotyledonary stage, is a factor that could be related with the low rate of conversion to plantlets and the high frequency of deformed somatic embryos of *C. chinense*.

Key words: Recalcitrance, maturation, germination, conversion.

INTRODUCTION

Somatic embryogenesis, which is based on cellular totipotency, refers to the process in which somatic or non-sexual cells are induced to form bipolar embryos through a series of developmental steps similar to those occurring during *in vivo* embryogenesis. Since the early descriptions of the process in carrot (Steward et al., 1958; Reinert, 1958), somatic embryo formation has been achieved for a variety of plant species, including angiosperms and gymnosperms (Brown et al., 1995; Dunstan et al., 1995; KrishnaRaj and Vasil, 1995; Thorpe and Stasolla, 2001). However, some species are re-calcitrant to *in vitro* conditions. Although *in vitro* recalcitrance is a major problem in plant biotechnology programs it is rarely considered in any detail (Benson, 2000). The factors that cause recalcitrance in plants are still unclear. Some evidences indicate that recalcitrance is closely related to genotype, *in vitro* manipulation, and the stress to which cells are subjected during *in vitro* culture (Stasolla et al., 2002; Benson, 2000).

Recalcitrance can manifest in any stage of *in vitro* plant regeneration. *Capsicum* is considered an *in vitro* recalcitrant genus because of the low efficiency and reproducibility of its protocols, the low rate of conversion

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to plantlets and the high frequency of deformed somatic embryos (Buyucalaka and Mavituna, 1996; Binzel et al., 1996; López-Puc et al., 2006; Zapata-Castillo et al., 2007). Recent studies have reported a substantial increase in the efficiency of somatic embryos obtained, either directly or indirectly from explants, working with the *Capsicum chinense* species (López-Puc et al., 2006; Zapata-Castillo et al., 2007). However, these regeneration systems are limited by a low germination rate and a high frequency of deformed embryos, which hinders the formation of complete plants. Meanwhile, the causes of this response in some plant species are not clearly known, although it is known that a number of factors, of diverse nature (physiological, genetic, biochemical, etc.) may be involved in the response of plants during *in vitro* manipulation.

In several plant species, including *Medicago truncatula* (Imin et al., 2004, 2005), *Picea glauca* (Lippert et al., 2005), *Cyclamen persicum* (Winkelmann et al., 2006) and *Vitis vinifera* (Marsoni et al., 2008), proteomics has been used to study somatic embryogenesis. Proteins directly influence cellular biochemistry and provide a more accurate analysis of change during growth and development cellular (Chen and Harmon, 2006). Storage proteins were the first compounds used as markers in comparing the developmental programs of somatic and zygotic embryogenesis (Hakman et al., 1990; Hakman, 1993). Numerous reports exist on proteins associated with somatic embryogenesis (SE) in plants, based on their similarity with their zygotic counterparts. Most knowledge so far has come from studies on alfalfa (Stuart et al., 1988; Krochko et al., 1992, 1994; Lai et al., 1992; Lecouteux et al., 1993; Lai and Mckersie, 1994) and soybean (Christou and Yan, 1989; Slawinska and Obendorf, 1991; Komatsuda et al., 1992; Dahmer et al., 1992; Stejskal and Griga, 1995; Chanprame et al., 1998; Griga et al., 2007), which represent traditional experimental models of SE.

However, there is a lack of information on biochemical aspects involved in somatic embryogenesis of *Capsicum* genus. Given the inability of somatic embryo germination of Habanero pepper and considering the importance of proteins during development and conversion of embryos into plantlets, the objective of this study was to determine the content and profile of the proteins during the development of somatic embryos of *C. chinense*.

MATERIALS AND METHODS

Preparation of plant material

Seeds of Habanero pepper *cv*. Rux-02 were surface-sterilized with a solution of ethanol at 70% for 5 min, rinsed three times in sterile distilled water, soaked in a solution of commercial sodium hypochlorite at 13% for 15 min, and rinsed again three times (1 min) in sterile distilled water. The sterile seeds were cultured in glass jars with 20 ml germination medium composed of mineral salts recommended by Murashige and Skoog (MS, 1962), supplemented with 1.156 µM gibberellic acid (GA_3), 3% sucrose and 0.2% gelrite. The pH was adjusted to 5.7 before sterilization in autoclave. The cultures were incubated in darkness for seven days to accelerate germination. After the seeds had germinated, they were transferred to a photoperiod of 16 h lights (40 to 50 µmol $m^{-2}s^{-1}$) at 25 ± 2°C. The hypocotyls were extracted from the dissection of plantlets at 20 to 25 days of germination.

Somatic embryogenesis induction (SE)

Under aseptic conditions, segments from *in vitro* plantlet hypocotyls were isolated by cutting with a razor blade. These isolated segments were used as explants. Induction of SE and somatic embryo production were performed as reported by López-Puc et al. (2006). Excised hypocotyls were placed in 250 ml Erlenmeyer flasks containing 100 ml MS medium supplemented with 3% sucrose, 9 µM 2,4-dichlorophenoxiacetic (2,4-D) acid, 29 µM thiamine-HCl, 42 µM cysteine-HCl, 55 µM myoinositol and 0.2% gelrite. Explants were maintained at 25 ± 2°C in photoperiod (16 h light, 40 to 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ / 8 h dark). A total of five explants were placed in each flask, and five replicas were used. All reagents for plant growth culture were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of zygotic embryo

Green and fully developed fruits of Habanero pepper *cv*. Rux-02 were harvested from plants grown under greenhouse conditions. To obtain zygotic embryos, seed were taken immediately after collecting the fruits, to prevent desiccation of the embryo. The extraction of the seeds was carried out to longitudinal cutting to the fruit. The zygotic embryo contained in each seed was isolated using forceps and scalpel, and with the aid of a stereo microscope (Nikon, MilesCo Scientific, USA).

Protein extraction for electrophoresis and quantitation

Somatic embryos were separated by stage of development (globular, heart-shaped, torpedo and cotyledonary stages). Three independent samples (replicates) of 100 mg each from protein extraction were performed from each developed stage, including the samples of the control (zygotic embryos). Samples of somatic embryos and mature zygotic embryos were homogenized with 11 mM Tris-HCl buffer (pH 7.5) containing 10 mM magnesium chloride, 1 mM ethylenediaminetetraacetic acid, 5% 2-mercaptoethanol, 2% polyvinylpyrrolidone, 10% glycerol, and centrifuged at 16000 × *g* for 10 min. Electrophoresis was carried out according to Laemmli (1970). Soluble proteins in supernatant were separated using a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking gel [pH 6.8], 15% running gel [pH 8.8]) at 4°C). Tris-glycine buffer (pH 8.3) containing 0.1% SDS was used as the electrode solution, while 10 µg of protein were loaded in each lane of the gel. Electrophoretic separations were performed at a constant current of 90 mA for 12 h. After electrophoresis, the gels were silver stained. Soluble protein quantification was determined using the Bradford (1976) method by measuring the absorbance (A_{595}) using a spectrophotometer (Genesys 5 UV-SENSE). All experiments were repeated twice and were conducted using a random design with at least three replicates.

Two-dimensional isoelectrofocusing (IEF)/SDS-PAGE

Samples of somatic embryos in the cotyledonary stage and mature

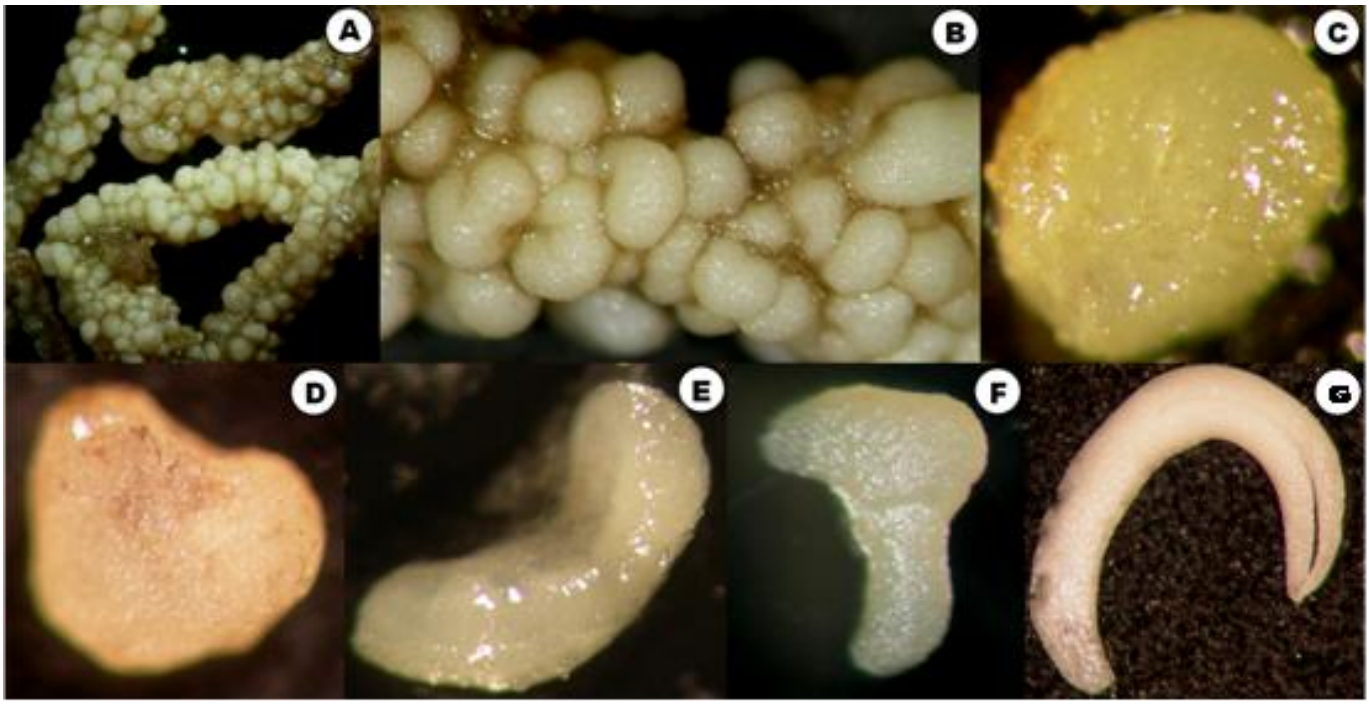

Figure 1. Somatic embryogenesis and developmental stages in *Capsicum chinense*. Somatic embryos obtained directly from explants after A) 2 weeks of culture and B) 5 weeks of culture. Developmental stages correspond to C) globular, D) heart-shaped, E) torpedo, and F) cotyledonary; G) zygotic embryo isolated from *C. chinense*.

zygotic embryos were quickly frozen with liquid nitrogen and homogenized in buffer A (7 M urea, 2 M thiourea, 4% NP-40, 1% dithiothreitol (DDT), 1% ampholytes [pH 3–10], 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM ethylenediaminetetraacetic acid, 40 mM Tris-HCl, and 0.05% polyvinylpolypyrrolidone (PVPP)). Extracts were centrifuged at 15000 × *g* for 15 min at 4°C. Supernatants were precipitated with cooled 10% trichloroacetic acid (TCA) in acetone that contained 0.07% β-mercaptoethanol. Samples were incubated at –20°C for 2 h to complete precipitation. Precipitates were centrifuged at 3000 × g for 15 min, and pellets were washed three times with acetone containing 0.07% β-mercaptoethanol. Subsequently, pellets were resuspended in buffer B (7 M urea, 2 M thiourea, 4% NP-40, 1% DTT, and 2% ampholytes [pH 3 to 10]). This resuspension was continually mixed and then centrifuged at 16000 × g for 15 min at 4°C. Supernatants were recovered and stored at –80°C until use.

The determination of soluble protein content was performed after the samples were thawed gradually according to Bradford (1976) method, using bovine serum albumin as the standard IEF was carried out with 20 µg of total protein extract using an immobilized linear 3 to 10 pH gradient (7 cm × 3.3 mm, dry strip; Invitrogen, CA, USA). The strips were rehydrated for 24 h at room temperature with protein supernatant in buffer that contained 9.5 M urea, 2% NP-40, 1% DDT, 2% ampholytes, and 0.05% bromophenol blue. IEF was carried out using the ZOOM® IPGRunner system (Invitrogen) at 200 V for 20 min, 450 V for 20 min, 750 V for 20 min, and 1000 V for 60 min. Focused strips were equilibrated for 15 min in NuPAGE® LDS buffer (Invitrogen). Equilibrated strips were placed on top of vertical 15% polyacrylamide gel. Electrophoresis was carried out at 4°C according to Laemmli (1970) for 8 h at 160 V. Gels were silver stained. Three independent protein extractions were performed from both samples (somatic embryos in the cotyledonary stage and mature zygotic embryos). Each extraction was analyzed by two gel replicates.

Data analysis

Results were analyzed using analysis of variance and means compared using Tukey's test ($P \leq 0.05$). SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was used.

RESULTS AND DISCUSSION

Direct somatic embryogenesis was induced from hypocotyl segments cultured on MS medium supplemented with 9 µM 2,4-D. Somatic embryos in the globular stage became visible after after weeks of culture (Figure 1A). After five weeks, the transferred embryogenic mass to liquid medium showed an abundant proliferation of somatic embryos (Figures 1A and B) which distinguished all of the developmental stages (Figures 1C to F). This process was highly efficient and reproducible, and corroborated as reported by López-Puc et al. (2007).

Soluble protein content of developing somatic embryos

Results of protein content show significant differences in all of the evaluated development stages (Figure 2). Contrary to that reported for other species, it was observed that the total protein content decreased as the somatic embryos of *C. chinense* were more advanced in their development. The lowest protein content in the

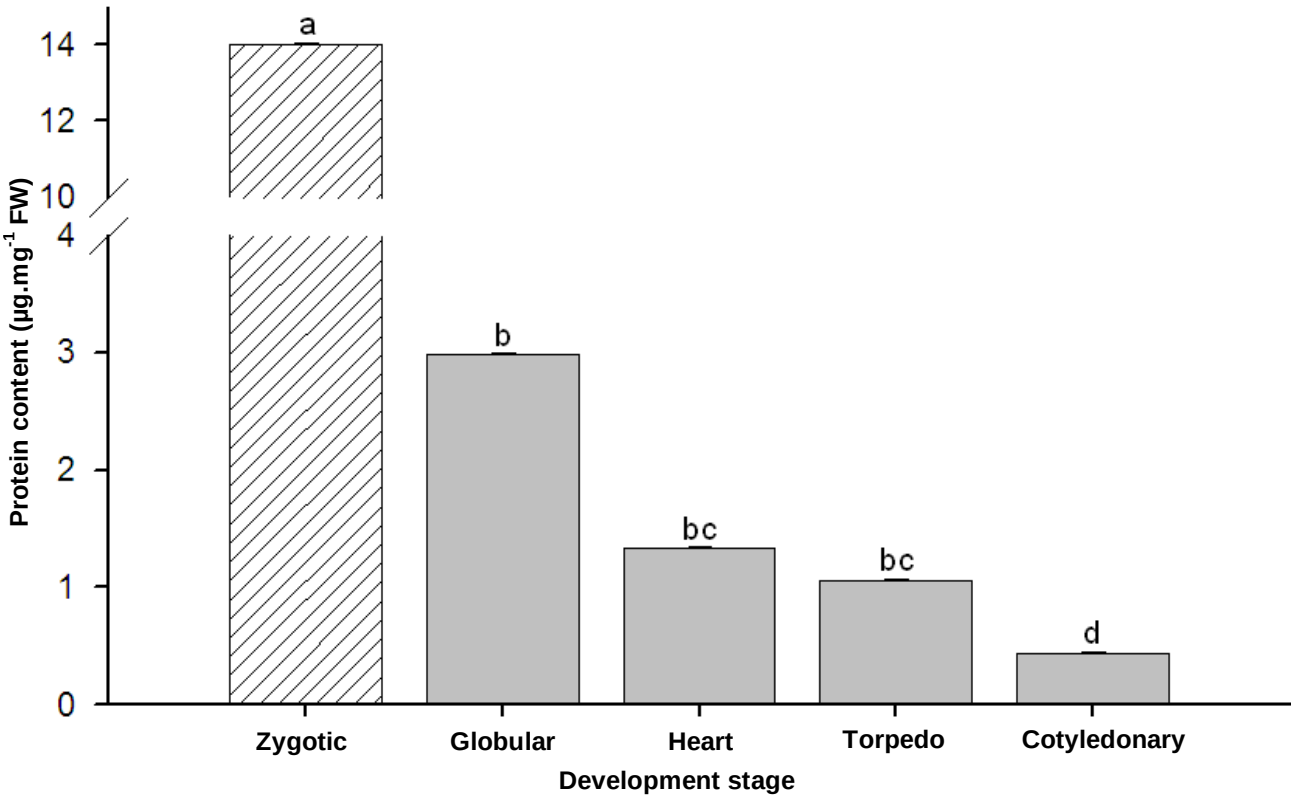

Figure 2. Total protein content of somatic embryos of *Capsicum chinense* at different developmental stages. Data are the means of at least 3 independent replicates; FW, fresh weight. Error bars represent standard deviation. Different letters indicate statistically significant differences according to Tukey's test ($P \leq 0.05$).

somatic embryos was registered in the cotyledonary stage (0.436 µg/mg FW), whereas the highest protein content was observed in the globular stage (2.98 µg/mg FW). Protein content of cotyledonary somatic embryos was dramatically lower than the mature zygotic embryo (0.436 vs. 14 µg/mg FW). Similar studies have been conducted on *Hyoscyamus niger* L. (Ebrahimzadeh et al., 2007), *Pisum sativum* (Griga et al., 2007), *Pinus taeda* (Brownfield et al., 2007), *Vitis vinifera* (Marsoni et al., 2008), *Cyclamen persicum* Mill. (Winkelmann et al., 2006), *Arachis hypogaea* L. (Roja et al., 2005), *Picea glauca* (Lippert et al., 2005), *Hevea brasiliensis* (Lardet et al., 1999), and *Cupressus sempervirens* L. (Sallandrouze et al., 1999). These reports have evidenced a clear tendency of increasing protein content with increasing somatic embryo development, although the range varied significantly by species (range = 2 to 120 µg/mg).

Composition of total proteins

SDS-PAGE revealed nine distinctive bands in the zygotic embryos corresponding to 4.0, 5.2, 8.1, 13.7, 20.9, 23.7, 41, 50 and 69.7 kDa (Figure 3); seven bands (whose molecular weights were 19.4, 20.2, 31.0, 38.3, 45.5, 53.6 and 73.2 kDa) were detected not only in the somatic embryos (at all developmental stages), but in the zygotic embryos too. Three bands (4.5, 4.8 and 49.1 kDa) were observed in the somatic embryos at the globular stage but were not present in the most advanced developmental stages. Somatic embryos in the heart-shaped and torpedo stages showed great similarity in terms of protein profile; and cotyledonary embryos expressed two proteins of 20.5 and 51.8 kDa, which were specific to this developmental stage. Comparing protein profiles of seeds and somatic embryos from *P. taeda*, Brownfield et al. (2007) detected in both biological structures three proteins whose molecular weights were 22.5, 37.5 and 47 kDa that corresponded to storage proteins. They also reported the presence of two proteins (14 and 14.5 kDa) in seeds that were likely related to the desiccation of zygotic embryos. In contrast, in a study that included seeds of four varieties of *Capsicum annuum* and two varieties of *Capsicum frutescens*, Odeigan et al. (1999), obtained 12 bands that could be distinguished in a range of molecular weight between 22 and 98 kDa. Moreover, in a similar study, but working with seeds of 72 accessions of *Solanum melongena*, Karihaloo et al. (2002) found 35 bands ranging from 14.5 to 52.5 kDa. Results evidenced a protein profile of nine bands exclusive to zygotic embryos. Coincidently five of these bands possessed similar molecular weights to those

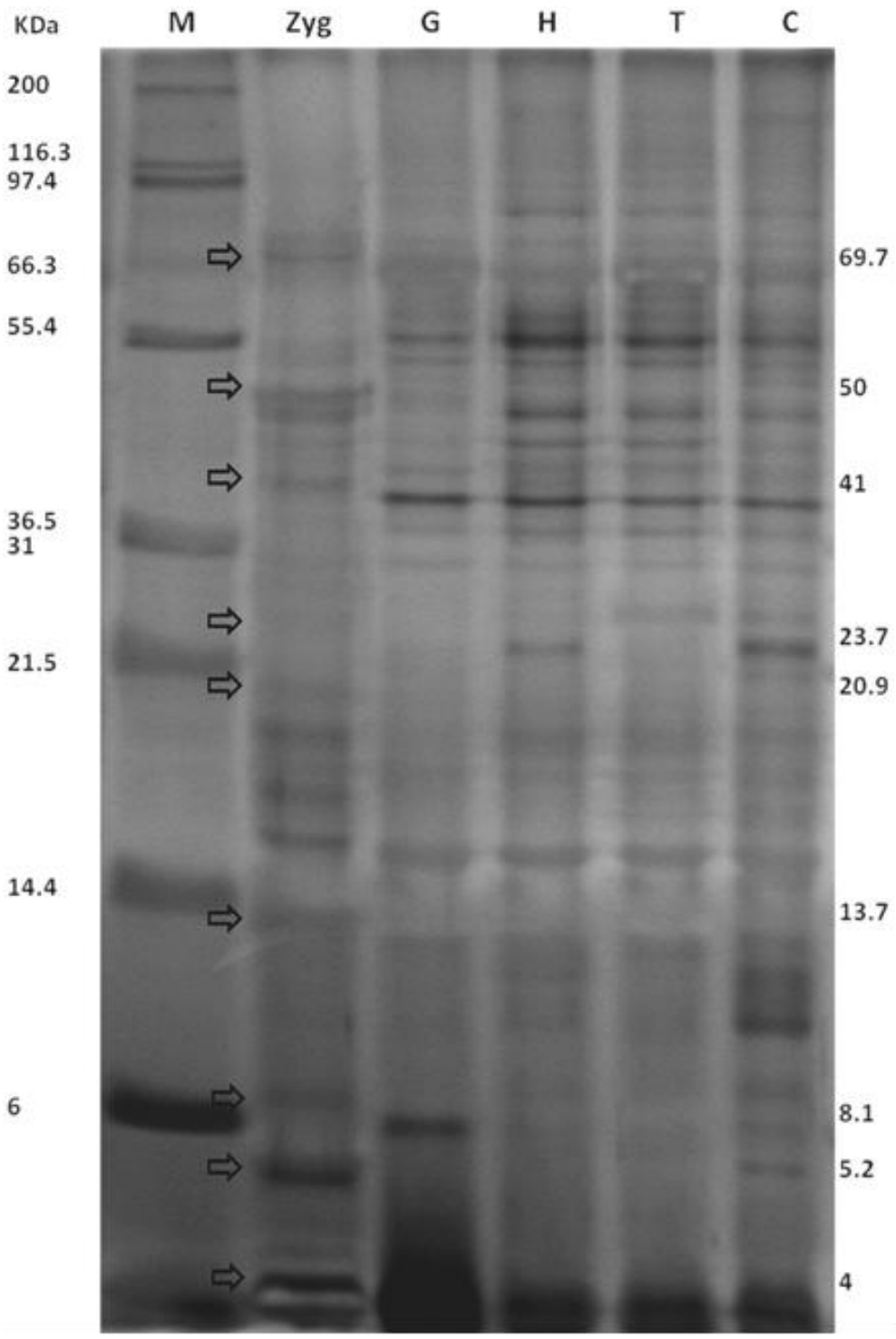

Figure 3. Protein pattern (12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis) of somatic embryos from *Capsicum chinense* jacq. M, Molecular weight marker; Zyg, zygotic embryo; G, globular somatic embryo; H, heart-shaped somatic embryo; T, torpedo-shaped somatic embryo; C, cotyledonary somatic embryo.

reported for storage proteins in other species (Derbyshire et al., 1976; Higgins, 1984, Shewry et al., 1995; Griga et al., 2007; Brownfield et al., 2007; Vladova et al., 2004).

Two-dimensional gel electrophoresis

Comparing the protein patterns of the somatic and

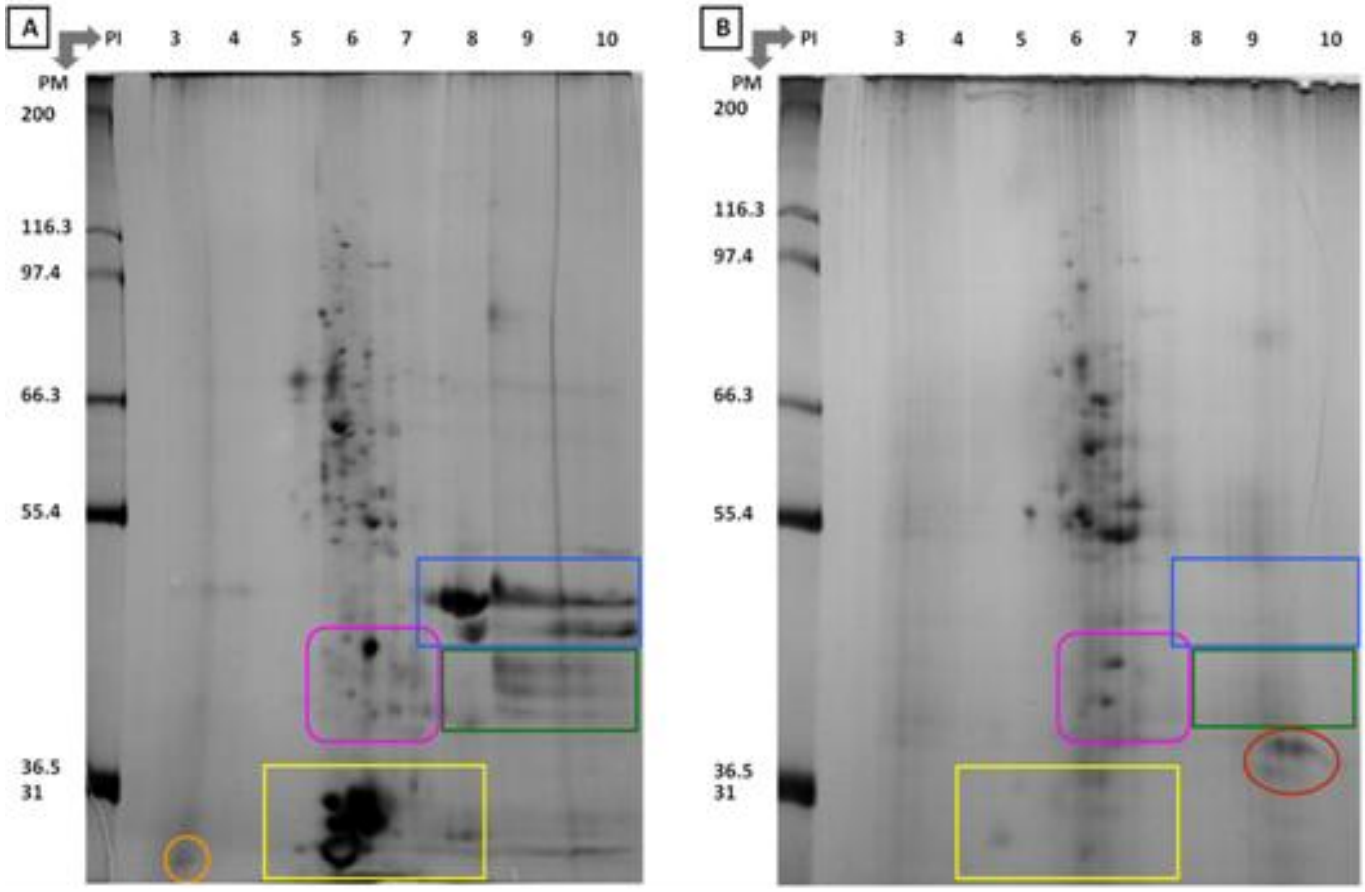

Figure 4. Two-dimensional isoelectrofocusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis (IEF/SDS-PAGE) of A) zygotic embryo and B) cotyledonary somatic embryo from *Capsicum chinense*. A total of 25 µg protein was separated on the first dimension in a lineal gradient (p*I* 3–10); the second dimension was by 15% SDS-PAGE; gels were silver stained.

zygotic embryos, the 2-dimensional analysis (Figure 4) shows proteins that ranged from 20 to 130 kDa and an isoelectric point (p*I*) from 3 to 10. However, in both types of embryos, the majority of proteins had a molecular weight between 33 and 120 kDa and a p*I* between 4 and 7. Specifically, a group of proteins was found in the zygotic embryos that were not expressed in the somatic embryos. These proteins had a molecular weight between 40 and 45 kDa and a p*I* range of 7 to 10. Based on their molecular weight and p*I*, these proteins could be storage proteins (likely corresponding to globulins); however, the protein sequences are required for identification. In addition, protein patterns were evidenced in other groups of proteins that were present in the zygotic embryos but not in the somatic embryos, and vice versa. Proteins of 45 and 50 kDa and p*I* 3 were observed only in the zygotic embryos, whereas proteins between 36 and 38.1 kDa and between p*I* 8.8 and 9.8 were expressed only in the somatic embryos. It is likely that the proteins were associated with embryo origin, even sexual or somatic origin, respectively. It was also observed that small group of proteins was present in both types of embryos, but their expression was much lower in the somatic embryos than in the zygotic embryos.

In a similar study, the presence of 200 proteins with high similarity between zygotic and somatic embryos of *Cyclamen persicum* Mill. (Winkelmann et al., 2006) was reported. These researchers identified a protein of approximately 27 kDa and 5 to 6 p*I*, similar to 11S globulin. They also observed proteins of 10 to 15 kDa and p*I* 4.5 to 5.5; these were highly expressed, but their expression decreased notably during germination. One protein was very similar to vicilin, suggesting that these proteins could belong to the 7S group of globulins. In contrast, Lippert et al. (2005) reported the expression of 1250 proteins. A maturation treatment with abscisic acid (ABA) revealed the higher expression of a protein of 45.2 kDa and p*I* 8.09 corresponding to vicilin, a storage protein. This finding contrasts notably with our results, because unlike in the zygotic embryos, expression of storage proteins in somatic embryos decreases from a low level to none at all. Low protein expression could contribute to the inability of somatic embryos to germinate. The majority of proteins in dicotyledonous seeds correspond to globulins of the 7 to 8S and 11 to 14S groups (Derbyshire et al., 1976; Higgins, 1984). 7S

globulins are from 150 to 180 kDa, with three subunits bound by weak interactions (Derbyshire et al., 1976; Higgins, 1984; Shewry et al., 1995). In contrast, 11S globulins are hexamers (350 to 460 kDa) with intermediate subunits bound by disulphide bridges that create monomers of 40 and 20 kDa when denaturalized. Vladova et al. (2004) found two 11S globulin subunits on two varieties of *C. annuum*, and Griga et al. (2007) reported on the expression of storage protein–type globulins (legumin, vicilin, convicilin) as well as their subunits. The highest expression corresponded to convicilin and vicilin proteins at 47 to 50 kDa and 70 to 75 kDa, respectively.

Biochemical and molecular changes associated with SE have been studied in such species as rapeseed (Crouch, 1982), carrot (Choi and Sung, 1984; Dodeman and Ducreux, 1996), ruffle (Chen and Luthe, 1987), break-wind (Stirn and Jacobsen, 1987), cotton (Shoemaker et al., 1987), trifolium (McGee et al., 1989), *Dactylis glomerata* (Hahne et al., 1988), coffee (Yuffa et al., 1994), *Camellia japonica* (Pedroso et al., 1995), soybean (Stejskal and Griga, 1995), barley (Stirn et al., 1995), sugarcane (Blanco et al., 1997), and birch (Hvoslef-Eide and Corke, 1997). However, there have been no reports related to biochemical changes during SE in *Capsicum*. This is the first report in this aspect, and contrary to expectations, these results reveal a decrease in protein content with increasing development of somatic embryos of *C. chinense,* as well as low protein expression (ranged = 45 to 50 kDa and p*I* 5 to 10).

In conclusion, the protein content behavior of somatic embryos of *C. chinense* throughout successive stages of its development differs from what has been reported for other species. The low protein content during the development of the somatic embryos, particularly at the cotyledonary stage, is a factor that could be related to the *in vitro* behavior of *C. chinense* during the germination and plant conversion. On the other hand, our results show notable differences between protein profiles of zygotic and somatic embryos of *C. chinense*, as observed through SDS-PAGE and the two-dimensional electrophoresis (2-DE) analyses. These results will make it possible to design new strategies to achieve complete plants from somatic embryos of Habanero pepper.

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