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M.A. Fontes · W.C. Otoni · S.M.B. Carolino
S.H. Brommonschenkel · E.P.B. Fontes · M. Fári
R.P. Louro

Hyperhydricity in pepper plants regenerated in vitro: involvement of BiP (Binding Protein) and ultrastructural aspects

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Abstract Hyperhydricity in regenerated pepper plants was monitored by the induction of the ER-luminal resident protein, as observed by immunoblotting. Immunoblotting of total protein using an anti-soybean BiP serum indicated that the induction and accumulation of an 80-kDa protein was related to BiP (Binding protein), a 78-kDa ER-resident molecular chaperone. The anti-BiP serum cross-reacted with an 80-kDa protein which was significantly induced by hyperhydricity. Based on similar molecular weight and immunological reactivity we concluded that the 80-kDa protein induced in hyperhydric plants is a BiP homologue. The ultrastructural organisation of leaves in non-hyperhydric and hyperhydric pepper (*Capsicum annuum* L.) plants was investigated with the aim of identifying the subcellular changes associated with this phenomenon. In non-hyperhydric leaves the chloroplasts of the palisade cells had normally developed thylakoids and grana and a low accumulation or absence of starch grains and plastoglobules. In the hyperhydric plants, however, the chloroplasts exhibited thylakoid disorganisation, low grana number, an accumulation of large starch grains and a low accumulation or absence of plastoglobules. Although the structure of mitochondria and peroxisomes did not change in hyperhydric plants, the number of peroxisomes did increase.

Key words *Capsicum annuum* L. · Hyperhydricity · Ultrastucture · Proteins

Abbreviations *BA:* N^6-benzylaminopurine · *BCPIP:* 5-Bromo-4-chloro-indolylphosphate · *IAA:* Indole-3-acetic acid · *MES:* 2N-morpholino ethane sulfonate · *NAA:* α-Naphthaleneacetic acid · *NBT:* Nitroblue tetrazolium chloride · *PMSF:* Phenylmethylsulfonyl fluoride · *Pipes:* 1,4-Piperazynediethane sulfonic acid · *SEM:* Scanning electron microscopy · *SDS-PAGE:* SDS polyacrylamide gel electrophoresis · *TEM:* Transmission electron microscopy

Introduction

Hyperhydricity involves multiple factors that, depending on the specific physiological response to culture conditions and the species studied, are expressed in various degrees of abnormal morphogenesis. It is characterised by gradations of morphological and physiological disorders including a glassy, waterlogged tissue appearance and distorted growth, and has been well documented in several current reviews on the subject (Gaspar 1991; Ziv 1991; Debergh et al. 1992; Ziv and Ariel 1994). The phenomenon influences photosynthesis, transpiration and CO_2 and O_2 gas exchange, all dominant processes the inhibition of which could be detrimental to plant quality and survival. Also, hyperhydric conditions have been shown to induce changes in protein synthesis as a consequence of various disorders, thus affecting interrelated metabolic pathways (Ziv 1991). These changes in protein

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M.A. Fontes · W.C. Otoni (✉)
Departamento de Biologia Vegetal, Av. P.H. Rolphs, s/n, 36571–000, Viçosa, MG, Brazil (9)), and Núcleo de Biotecnologia Aplicada à Agropecuária (BIOAGRO), Universidade Federal de Viçosa, Viçosa, MG, Brazil
e-mail: wotoni@mail.ufv.br

S.M.B. Carolino · S.H. Brommonschenkel · E.P.B. Fontes
Departamento de Biologia Vegetal, Av. P.H. Rolphs, s/n
36571–000, Viçosa, MG, Brazil

M. Fári
Agricultural Biotechnology Center (ABC), Gödöllo, Szent-Gyorgyi A. u. 4, H-2100 Hungary/Embrapa Semi-Árido (CPATSA), Br 428, km 152, Zona Rural, Petrolina – PE

R.P. Louro
Laboratório de Ultraestrutura Vegetal, Departamento de Botânica, IB/CCS, Universidade Federal do Rio de Janeiro, 21941–590, Rio de Janeiro, Brazil

synthesis are related to photosynthesis, cellulose and lignin synthesis or processes associated with ethylene production (Kevers et al. 1984; Van Huystee 1987).

Chilli pepper (*Capsicum annuum* L.) is a unique example within the cultivated Solanaceae family that, unlike other Solanaceous species, has been a recalcitrant species with respect to its capacity for in vitro plant regeneration (Liu et al. 1990). The very low morphogenetic capacity of its vegetative tissues, cells and protoplasts as well as low micropropagation rate have been reported by several authors (Fári and Andrásfalvy 1994; Szász et al. 1995). One of the explanations for such recalcitrance may relate to in vitro stressful-related factors, whose physiological and molecular bases are poorly understood. Therefore, pepper seems to be a promising experimental model by which to better understand hyperhydricity-associated processes.

In the study presented here, quantitative and qualitative physiological analyses were carried out on normal and hyperhydric in vitro pepper plants based on differences in total protein patterns as determined by SDS-PAGE and immunoblotting with stress-induced Hsp70 family proteins. Also, using scanning and transmission electron microscopy we studied the ultrastructural pattern of leaves in order to identify the subcellular changes promoted by hyperhydricity.

Materials and methods

Plant material and establishment of aseptic plants

Seeds of pepper (*Capsicum annuum* L. cv 'Agronômico G10') were purchased from commercial establishments in Viçosa, Brazil. Surface sterilisation was performed by immersing of the seeds first in 70% (v/v) ethanol for 1 min, followed by 15 min in a 2.5% (v/v) sodium hypochlorite solution containing 1% (v/v) Tween 80; this was followed by four rinses in sterile distilled water. Thereafter, the seeds were soaked for 24 h at 26°±2 °C in sterile distilled water on a rotatory shaker (100 rpm) and then germinated on MS-based medium (Murashige and Skoog 1962) lacking growth regulators, pH 5.7, solidified with 0.3% (w/v) Phytagel (Sigma). Cultures were maintained under a 16/8-h light/dark regime, with 36 μmol m^{-2} s^{-1} light radiation being provided by one Gro-Lux lamp (Sylvania, 20 W, Brazil) and two fluorescent tubes (Luz do Dia Especial, 20 W, Osram, Brazil). The culture room was kept at 26°±2 °C. In vitro-grown seedlings (14–18 days after germination) were used as the source of explants; the roots were freed from agar and trimmed, and the hypocotyls were cut 1–2 mm below the cotyledonary node. Rooted hypocotyls were placed inverted into 50 ml of a shoot induction medium (Valera-Montero and Ochoa-Alejo 1992) for 14–16 days under the same conditions as for germinated seeds. The shoot induction medium consisted of MS salts supplemented with B5 vitamins (Gamborg et al. 1968), 100 mg l^{-1} myo-inositol, 3% (w/v) sucrose, 1.95 g l^{-1} MES, 10 μ*M* $AgNO_3$, 5.0 mg l^{-1} BA and 0.3 mg l^{-1} IAA, semi-solidified with 0.3% (w/v) Phytagel (pH 5.7). After 14 days of culture, hypocotyls with adventitious buds were placed in a normal orientation into a shoot elongation medium lacking growth regulators [MS medium, B5 vitamins, 100 mg l^{-1} myo-inositol, 3% (w/v) sucrose, 0.3% (w/v) Phytagel, pH 5.7]. The medium was dispensed as 15-ml aliquots into the test tubes (25×150 mm). Elongated shoots were individualised and transferred to a rooting medium [half-strength MS salts, B5 vitamins, 0.2 mg l^{-1} NAA, 100 mg l^{-1} myo-inositol, 3% (w/v) sucrose and 0.3% (w/v) Phytagel, pH 5.7]. The medium was poured into Magenta boxes (Sigma Chemical) and sealed with either PVC film (Resinite, Alba Quimica Argentina) or hypoallergenic tape (Micropore 3 M, Brazil). The experiment was performed twice using a completely randomised design, with 30 replicates per treatment.

Ultrastructural analysis: transmission and scanning electron microscopy

Leaf samples were collected from in vitro-grown (60-day-old) normal and hyperhydric plants obtained from independent regeneration events. Ten leaf samples were taken from fully expanded leaves below the terminal bud fixed with 4% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 1.25% (v/v) Pipes buffer (Salema and Brandão 1973) at pH 7.2 for 2 h under low vacuum and then washed three times (10 min each) in the same buffer. The material was post-fixed with 1% (v/v) osmium tetroxide in 1.25% (v/v) Pipes buffer for 1 h and subsequently dehydrated in graded acetone. For TEM analysis, the specimens were embedded in Spurr resin and polymerised for 15 h at 70 °C. Five samples from each of the hyperhydric and non-hyperhydric plants, obtained from different regeneration events were fixed and processed as described above. For SEM, leaf samples were critical-pointed-dried with CO_2, and fragments were positioned on a stub prior to gold sputtering in a Balzer's Sputter Coater SCD050. Following coating with a thin layer of gold (about 20 nm) the specimens were observed and micrographed using a Zeiss DSEM 960 (Zeiss, Germany) scanning electron microscope. For TEM, ultrathin sections (about 70 nm in thickness) were cut on a Reichert Ultracuts (Leica, Germany) using diamond knives (Diatome), collected on copper grids and stained for 20 min in a 1% alcoholic solution of uranyl acetate, followed by 5 min in lead citrate. Sections were examined in a Zeiss EM 900 (Zeiss, Germany) electron microscope.

Protein extraction and quantification, SDS-PAGE electrophoresis and immunoblot analysis

Normal and hyperhydric frozen leaf samples were ground in liquid nitrogen with a pestle and mortar and homogenised at 25 °C with extraction buffer [100 m*M* Tris-HCl, 50 m*M* NaCl and 1 m*M* PMSF, pH 7.5] at a ratio of 500 mg of tissue per 5 ml of extraction buffer. Cell debris was removed by centrifugation (14,000 rpm, 15 min) and the protein-containing supernatants retained. Protein quantification and gel electrophoresis were performed according to Bradford (1976) and Laemmli (1970), respectively. Protein extracts were incubated at 100 °C, for 3 min, with the sample buffer [10% (v/v) glycerol, 2.3% (v/v) SDS, 0.25% bromophenol blue, 5% 2-mercaptoethanol, 0.0625 *M* Tris-HCl, pH 6.8], before loading the gels. Aliquots of protein extract (30 μl) were loaded onto 5% SDS-PAGE, electrophoresed for 16 h at 37 V in a running buffer [0.0025 *M* Tris-HCl, 0.2 *M* glycine, 1 m*M* EDTA and 3.5 m*M* SDS]. The gel was stained with 40% (v/v) methanol, 7.5% (v/v) acetic acid and 0.01% Comassie Brilliant Blue R-250 for 8 h and then de-stained in 10% (v/v) methanol and 7.5% (v/v) acetic acid. After electrophoresis, the proteins were transferred to a nitrocellulose membrane using a blot apparatus (BioRad, USA) according to the manufacturer's instructions. Following transference (about 2 h; 700 mA) the membrane was incubated in a blocking solution (Blotting Grade Blocker Non-fat Dry Milk, BioRad USA) for 1 h at room temperature. The membrane was washed (three times, 15 min each) using TBS-T [0.01 *M* Tris-HCl, pH 7.6, 1.5 m*M* NaCl and 0.1% (v/v) Tween 20] and incubated (2–4 h) with an anti-soybean BiP rabbit polyclonal antibody (Figueiredo et al. 1997) using a 1:2000 dilution ratio. The membrane was repeatedly washed in TBS-T (three times, 25 min each) and incubated (2 h) with IgG alkaline phosphatase conjugate (Sigma) at a 1:500 dilution. Following

incubation, the membrane was washed intensively with TBS-T and then incubated (about 5 min) in enzyme buffer (0.1 M Tris-HCl, pH 9.8, 0.1 *M* NaCl, 0.5 *M* $MgCl_2$). Alkaline phosphatase activity was detected with NBT and BCPIP BRL substrates (Gibco BRL).

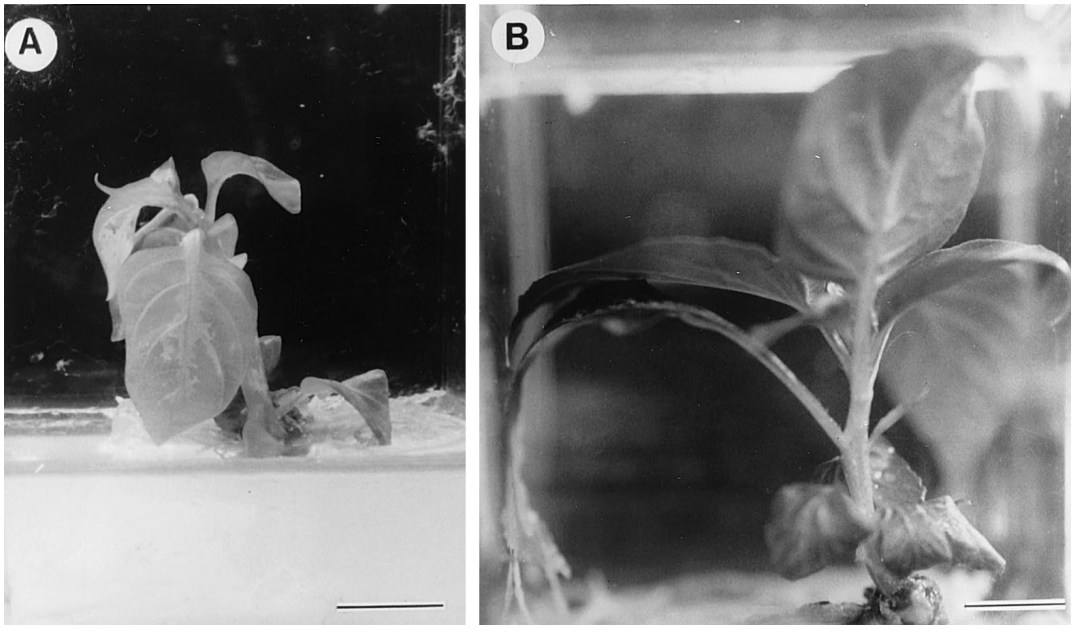
Results and discussion

The aerial part of hyperhydric regenerants had a vitreous appearance consistent with characteristics attributed to this phenomenon, including translucent, turgid and brittle leaves with a smaller leaf area expansion than those of non-hyperhydric plants. During the phases of bud elongation and rooting, up to 44% of the plants exhibited symptoms of hyperhydricity. The shoots were stunted, with an average plant size of 3 cm as compared to 6.5 cm for non-hyperhydric plants (Fig. 1A, B). This high frequency of hyperhydric shoots may also be related to the type of flask sealing, which may have led to the accumulation of ethylene and/or to an increase in relative humidity inside the flasks and, consequently, to the symptoms of hyperhydricity as reported by Han et al. (1995, 1996) and Lakshmanan et al. (1997). When the flasks were sealed with hypoallergenic Micropore tape instead of transparent PVC film, the symptoms of hyperhydricity were not observed. This was partly due to the fact that the hypoallergenic tape is porous and therefore increases gas exchange between the flask and the outer environment and partly to a reduced relative humidity within the culture vessels. Indeed, the type and tightness of culture vessel enclosures determines the concentration of CO_2, O_2, ethylene and water vapour in the culture gaseous atmosphere (Ziv 1991).

The SDS-PAGE analyses of protein accumulation in hyperhydric leaves of in vitro-regenerated pepper shoots revealed the reduced accumulation of a 55- to 60-kDa protein (Fig. 2, compare lanes N and H) and the distinct conduction of a 80-kDa protein. Several lines of evidence suggest that the down-accumulated protein may be the enzyme 1,5-ribulose bisphosphate carboxylase/oxygenase (Rubisco). First, its electrophoretic migration is consistent with the molecular weight of the large subunit of Rubisco. Second, in normal in vitro-regenerated shoots the 55- to 60-kDa protein accumulates as a major leaf protein. Finally, a down-regulation of Rubisco synthesis has been often associated with low photosynthetic rates, the latter having been described as resulting from hyperhydricity (Ziv and Ariel 1992; Jones et al. 1993). Indeed, protein levels were lower in hyperhydric leaves and a 30-kDa protein was found to be present in hyperhydric plants but not in normal leaves (Ziv 1991; Ziv and Ariel 1992). Peroxidases associated with lignin synthesis, which have been shown to be related to lignification, were detected in larger amounts in hyperhydric plants and found to have a molecular weight of 30–32 kDa (Van Huystee 1987). A significant reduction in total protein was also reported in hyperhydric leaves of *Gypsophila paniculata* as compared to glaucous leaves (Paek et al. 1991).

The induction of an 80-kDa protein accumulation prompted us to examine whether the induced protein was related to BiP (Binding protein), a 78-kDa endoplasmic reticulum (ER)-resident molecular chaperone. Total protein was immunoblotted using an anti-soybean BiP serum (Fig. 3). The anti-BiP serum cross-reacted with an 80-kDa protein (lane N), which was

Fig. 1A, B Hyperhydric and normal pepper shoots (*Capsicum annuum* L. cv 'Agronômico G10') regenerated in vitro from inverted hypocotyls. **A** Plant with symptoms of hyperhydricity, **B** normal plant. *Bar*: 1.0 cm



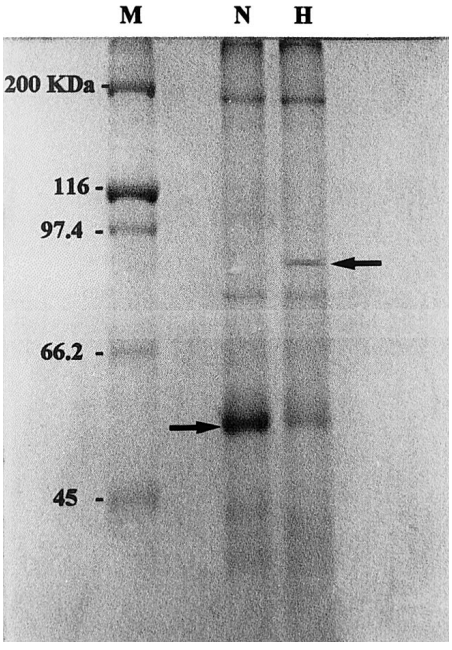


Fig. 2 Electrophoretic pattern of total proteins of normal and hyperhydric plants. Total protein was extracted from leaves of normal (*N*) and hyperhydric (*H*) pepper (*Capsicum annuum* L. cv 'Agronômico G10') plants. *M* corresponds to the electrophoretic pattern of the molecular marker. ← Protein of about 80 kDa, → protein of about 55–60 kDa

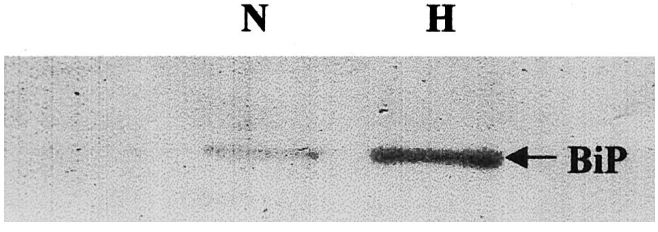


Fig. 3 BiP synthesis in a normal (*N*) and hyperhydric (*H*) pepper (*Capsicum annuum* L. cv 'Agronômico G10') plants. Leaf-soluble proteins were fractionated by SDS-PAGE, and the BiP protein was identified by immunoblotting using a polyclonal soybean BiP antibody. Approximately 30 μg total protein was applied to each column

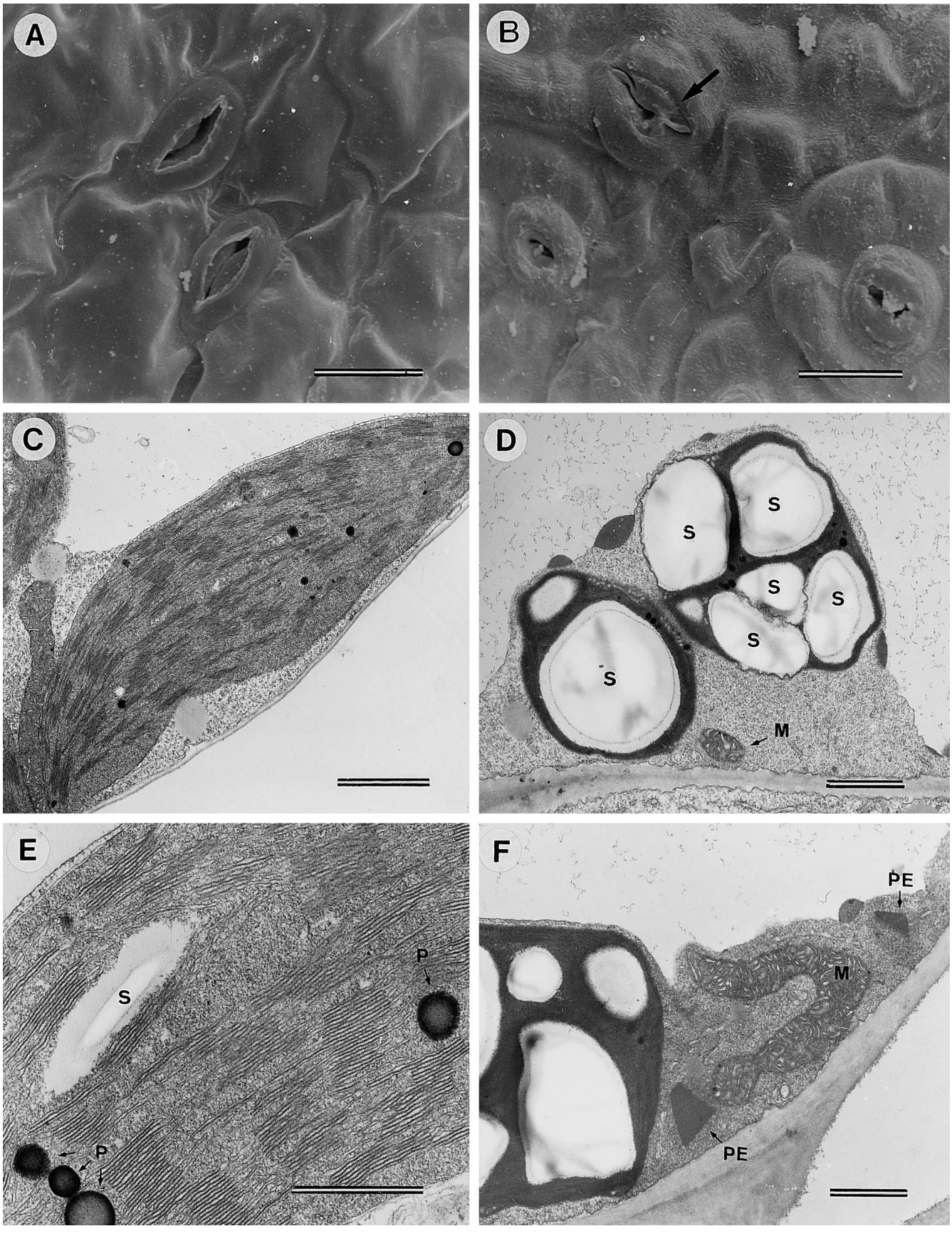
significantly induced by hyperhydricity (lane H). Based on the similar molecular weight and immunological reactivity we concluded that the 80-kDa protein induced in hyperhydric plants is a BiP homologue. As a member of the Hsp70 family, the synthesis of BiP is regulated by conditions of physiological stress that promote an increase in cell secretory activity or in the accumulation of misfolded proteins in the ER (Fontes et al. 1991). The up-regulation of BiP synthesis under stress has been shown to be an efficient approach by which to monitor intracellular stress in plants.

Using SEM, we observed the epidermal abaxial surface of leaves. In general, the leaf surfaces of the two types of plant were quite regular, with stomata at the same level as the remaining epidermal cells. With respect to morphology, the stomata of non-hyperhydric plants (Fig. 4A), displayed normal morphology, whereas the guard cells of hyperhydric plants were larger in size than those of normal plants due to a greater water absorption leading to turgidity and probably to changes in cell-wall structure (Fig. 4B). The cell wall bordering the stomata pore in guard cells from hyperhydric leaves protruded and appeared to be torn in several places, resulting in some cases in guard-cell deformation (Fig. 4B). These deformations may result from structural changes in the guard cells followed by changes in cell-wall composition. Similar results were reported by Miguens et al. (1993) for hyperhydric *Datura insignis* plants. Changes of this nature were also accompanied by deformation of the guard-cell walls due to a loss of elasticity or to modifications in the pattern of deposition of cellulose microfibrils (Ziv and Ariel 1992, 1994).

Under TEM the palisade chloroplasts of normal plants showed well-developed thylakoids organized into numerous grana. Plastoglobules and a few starch grains were located in stroma, usually between the thylakoid membranes (Fig. 4C, E). The accumulation of these structures has been attributed to chloroplast senescence, nutritional deficit, plant treatment with herbicides and other stress conditions (Dixit 1988). The presence of plastoglobules in normal plants growing in elongation and rooting medium may be explained by the concentration of nutrients in the culture medium (MS medium with half-strength salts) and/or to ethylene accumulation caused by the type of flask sealing, both of which lead to senescence, as reported by Han et al. (1995). Louro (l994) working with eucalyptus hybrid (*Eucalyptus grandis* × *E. urophylla*) also observed an increase in plastoglobule density in plants cultivated in elongation rooting medium. In his case, however, this phenomenon was attributed to nutritional deficiency probably caused by the addition of activated charcoal to the culture medium.

The chloroplasts of the palisade parenchyma of the hyperhydric plants stored voluminous starch grains, had few plastoglobules and their inner membrane were poorly developed, with thylakoid disorganisation and

Fig. 4 **A** Scanning electron microscopy showing the adaxial surface of a non-hyperhydric pepper leaf. *Bar*: 100 μm. **B** Scanning electron microscopy showing the adaxial surface of a hyperhydric pepper leaf. Note the cell wall bordering the stomata pore in guard cells (*arrow*) appears to protrude and be torn in several places. *Bar*: 100 μm. **C** Transmission electron microscopy showing the plastoglobules in chloroplasts of normal palisade leaf cells. *Bar*: 1 μm. **D** Detailled palisade cell in hyperhydric leaf showing cytoplasm with mitochondria (*M*), chloroplast accumulating large starch grains (*S*) and small plastoglobules. *Bar*: 1 μm. **E** Detailled chloroplast in a normal leaf showing thylakoids, grana, plastoglobules (*P*) and small starch grains (*S*). *Bar*: 1 μm. **F** Detailled cytoplasm in a hyperhydric leaf mesophyll with peroxisomes (*PE*), mitochondria (*M*) and part of a chloroplast containing starch grains. *Bar*: 1 μm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low granum density (Fig. 4D). Starch accumulation in chloroplasts of hyperhydric plantlets was significantly higher than in those of non-hyperhydric plants. This accumulation may reflect a delay in sugar mobilisation caused by changes in membrane permeability, and in our culture system the partition of photoassimilated substances was probably impaired so that starch grains accumulated at their site of synthesis. The accumulation of these voluminous grains has been reported in plants growing under stressful conditions both in vivo (Dixit 1988; Stoyanova and Velikova 1997/1998) and in vitro (Wetzstein and Sommer 1982; Han et al. 1992). The mitochondria and microbodies in cells of palisade parenchyma in normal and hyperhydric plants commonly occurred in close spatial association with chloroplasts. The mitochondria were rounded to elongated and presented well-developed cristae (Fig. 4C, D, F). The peroxisomes had an outer limiting membrane which enclosed a matrix and large crystalline inclusions (Fig. 4F), and these organelles were observed more often in hyperhydric plants.

In fact, the hyperhydric pepper leaves presented abnormal chloroplasts as judged by the thylakoid organisation (Fig. 4D). These morphological changes have been related to the low photosynthetic capacity of the leaves. The low photosynthesis rate, as determined by the reduction in palisade parenchyma and by the presence of chloroplasts with abnormal granum and stroma organisation with reduced amounts of chlorophyll, is one of the major factors responsible for the translucent aspect of hyperhydric plants (Kevers et al. 1984; Paek et al. 1991; Ziv and Ariel 1992; Delarue et al. 1997). In addition to the abnormal chloroplasts observed in the palisade parenchyma of the hyperhydric leaves, the vacuoles were also altered in size (data not shown). The expanded vacuoles may be a result of an increased secretory activity of the cells or might reflect alterations in the overall morphology of the secretory system. In both cases, an induction of the ER-resident molecular chaperone synthesis is expected to occur (Vitale et al. 1993). The induction of BiP synthesis in hyperhydric pepper leaves may be a result of a permanently induced stress condition as observed by the abnormal organelle morphology under hyperhydricity.

The present results confirm previous reports regarding the negative effects of hyperhydricity on morphogenesis in vitro. Also, the findings of this research support other investigations already in progress in our laboratorywith respect to the optimization of cultural and physical conditions that may lead to an effective reduction of pepper hyperhydricity. On the basis of our observations, we suggest that additional efforts should be made to evaluate the reliability of using BiP as a marker for monitoring its accumulation due to in vitro stress conditions, mainly for those species where hyperhydricity is also a limiting factor.

The present results taken together constitute, to the best of our knowledge, the first report on the involvement of BiP in hyperhydricity in regenerated palisade plants.

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