

Comparative investigations on protoplast culture of some Brazilian and Hungarian sweet pepper cultivars and hybrids

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Summary: Cotyledon protoplasts were isolated from 16–18-day-old in vitro grown seedlings of 9 Brazilian and 3 Hungarian pepper varieties and hybrids. Large numbers (average 9.59×10^6 protoplasts g^{-1} fresh weight) of highly viable (average 87.0%) protoplasts were released using a pectocellulolytic enzyme mixture. Protoplasts were cultured in K8p medium using an alginate disc embedding method. The osmotic pressure of the medium surrounding the alginate-embedded protoplasts was reduced by replenishing the liquid medium at K8p:K8 ratios of 1:0, 2:1, 1:1 in the first, second, and third week, respectively. Initial plating efficiency (IPE) average was 38.5% and after 21 days protoplasts reached microcolonies (15–20 cells) stages. Microcolonies were transferred after 3–4 weeks to a MS-based medium supplemented with 1.0 mg l^{-1} zeatin, 3.0% (w/v) sucrose, 0.24% (w/v) phytigel and pH 5.8, whereupon they formed callus. Final plating efficiency (FPE) average was 0.29% at a plating density of 1.0×10^5 protoplasts ml^{-1} . Protoplast-derived calli were cultured on a range of MS-based media supplemented with either BAP, IAA, TDZ, and zeatin. No morphogenic response was observed in any genotype investigated.

Introduction

Isolated plant protoplasts are cells which have had their cell wall removed by mechanical action or enzymatic digestion. Theoretically, all plant cells are totipotent and, therefore, protoplasts may be able to re-synthesise the cell wall, divide, form colonies and calli, and, eventually, regenerate a new plant. This capacity enables protoplasts to be used as important techniques in plant breeding (Menczel et al., 1981; Barsby et al., 1984; Power and Chapman, 1985; Sundberg and Glimelius, 1986; Ochatt and Power, 1992), or physiological studies (Galun, 1981; Bengochea and Dodds, 1986). The development of reproducible and efficient systems of isolation, cultivation and plant regeneration from protoplasts is a fundamental prerequisite for using these techniques. One of the most important breeding characteristics of the *Capsicum* genus is its resistance to diseases, especially those caused by virus and bacteria (Boukema et al., 1980; Peter et

al., 1984; Boiteux et al., 1993). According to these authors, there are several wild species of the genus which have been recognised as sources of resistance. Interspecific incompatibility and/or hybrid sterility, however, has hindered the transfer of desirable characteristics by conventional hybridisation methods, thus protoplasts are an important tool in solving this problem. Unfortunately, the in vitro cultures of the *Capsicum* genus are usually unable to provide satisfactory morphogenic responses. Chilli pepper (*Capsicum annuum* L.) is a unique example within the Solanaceae family that, unlike other Solanaceous species, has been a recalcitrant species with regard to its capacity for in vitro plant regeneration (Fári, 1986; Liu et al. 1990). Its very low morphogenetic capacity of vegetative tissues, cells and protoplasts as well as micropropagation was reported by several authors (Fári and Andrásfalvy 1994; Szász et al. 1995). One of the explanations of the failure may be that its cause is some in vitro stress-related factor whose physio-

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logical and molecular bases are poorly understood (Fontes et al. 1999). The study of the host-pathogen interaction at the cell and molecular level using infection of isolated protoplasts by electroporation with viral DNA or RNA is another important aspect of this work. It has contributed to studies of virus replication and to explain the mechanisms by which the pepper plants become resistant to infection (Gera et al., 1994; Murphy and Kyle, 1994; Deom et al., 1997). There are some papers on the protoplast isolation technique of *Capsicum* genus (Hsia et al., 1983; Niedz et al., 1987; De Donato et al., 1989; Gera et al., 1994; Murphy and Kyle, 1994; Szász et al., 1995b; Deom et al., 1997) and only three reports on successful plant regeneration (Saxena et al., 1981; Díaz et al., 1988; Prakash et al., 1997). These works are conflicting in terms of methodology and in presentation of results, so that this important genus still does not have a defined protocol of plant regeneration from the protoplast system.

The final objective of our investigations is to elaborate an appropriate gene transfer system for the sweet pepper (*Capsicum annuum* L.) by means of protoplast electroporation method involving broad spectra of genotypes cultivated under different tropical and temperate climate conditions. The goal of the present study was to define suitable protocols for isolation, cultivation and plant regeneration of some Brazilian and Hungarian pepper standard cultivars and hybrids started from cotyledon-derived protoplasts.

Materials and methods

In vitro culture establishment

Pepper standard cultivar and hybrid seeds were obtained from Top Seed Company (Itaipava, Brazil) and from commercial shops. The Brazilian cultivars Agronômico G10, Casca Dura Ikeda, Hércules, Magda, Quadrado Verde, Quadrado Vermelho, Yolo Wonder (All Big) and hybrids Magali and Tango and the Hungarian cultivars Greigo, Cecei and Szentesi (Research Institute for Vegetable Crops, Station Budatétény, Hungary) were studied. Surface-sterilisation was performed by immersion of the seeds 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, followed by four rinses in sterile distilled water. Thereafter seeds were soaked for 24 h, at $26 \pm 2^\circ\text{C}$, in sterile distilled water on a rotatory shaker (100 rpm), and germinated on MS-based medium (Murashige and Skoog, 1962) lacking growth regulators, pH 5.7, solidified with 0.3% (w/v) Phytigel (Sigma). Cultures were maintained under 16/8 h light/dark regime, $36 \mu\text{mol m}^{-2} \text{s}^{-1}$ light radiation, provided by one Gro-Lux lamp (Sylvania, 20 W, Brazil) and two fluorescent tubes (Luz do Dia Especial, 20 W, Osram, Brazil). Temperature of the culture room was kept at $26 \pm 2^\circ\text{C}$. *In vitro* grown seedlings (14–18 days after germination) were used as the source of explants. The medium was poured into 250 ml glass flasks containing approximately 50 ml medium and sealed with polypropylene lids.

Protoplast isolation, embedding and culture

Sixteen to eighteen-day-old axenic seedlings (counted from the radicle emergence) were used as a source of explants for protoplast isolation. Approximately 1.0 to 1.5 g plant material were excised and transversally sectioned in 1 to 1.5 mm wide strips, and pre-plasmolized in 15 to 20 ml of TVL (Glimelius 1984; Sundeborg and Glimelius, 1986) solution for one hour. The TVL solution was then discarded and 25 ml of one the following enzymatic solutions were added:

E1: (Díaz et al., 1988): 1% (p/v) Cellulase Onozuka R-10 (Yakult Honsha Company Ltd., Japan), 0.25% (p/v) Macerozyme R-10 (Yakult Honsha Company Ltd., Japan) diluted in CPW 13M medium (Power and Chapman, 1985) and pH 5.8;

E2: (Szász et al., 1995; Szász, 1996): 1% (p/v) Cellulase Onozuka R-10, 0.6% (p/v) Macerozyme R-10, diluted in K3 (Nagy and Maliga, 1976) medium with 0.4 M sucrose and pH 5.6;

E3: 1% (p/v) Cellulase Onozuka R-10, 0.25% (p/v) Macerozyme R-10, 0.5% (p/v) Driselase (Sigma Chemical Company, USA), diluted in K3 medium and pH 5.6;

E4: 1% (p/v) Cellulase Onozuka R-10, 0.5% (p/v) Macerozyme R-10, 0.25% (p/v) Driselase diluted in K3 medium and pH 5.6.

Incubation was performed during 12 to 13 hours, in the dark, in a stationary system at $26^\circ \pm 2^\circ\text{C}$. After the incubation phase the suspension obtained was filtered in a sterile 64 (m plastic sieve (Wilson Sieves, Nottingham, UK) and the filtrate was collected by pipetting with Pasteur pipettes, and transferred to 15 ml centrifuge tubes. The filtrate was then diluted by the addition of 2.0 to 2.5 ml of CPW16 medium (Banks and Evans, 1976) and centrifuged (110g; 5 minutes). Floating protoplasts formed a band in the upper part of the tube was then collected and transferred to a new centrifuge tube, the volume completed with W5 solution (Menczel et al., 1981) and then centrifuged (75 g, 5 minutes). The pellet was resuspended in W5 solution and centrifuged again (75g; 5 minutes). After rinsing twice, the yield and viability of the protoplast isolates were assessed, and a minimum of 250 protoplasts were counted. The density of the suspended protoplasts was measured with a haemocytometer, adjusted to 1.0×10^5 protoplasts per ml, and their viability assessed using fluorescein diacetate (Larkin, 1976). Protoplast size was determined by using a micrometric ocular under optical microscopy. The protoplast plating density was 1×10^5 protoplasts ml^{-1} , in K8p medium (Kao and Michayluk, 1975) twice concentrated, except for glucose (Szász, 1996). Protoplasts were embedded in sodium alginate as described by (Szász et al., 1995; Szász, 1996). The dishes were sealed with transparent PVC film and kept in the dark for a week at $26^\circ (\pm 2^\circ\text{C})$. The dishes were then transferred to the light with irradiance about $12 \mu\text{moles m}^{-2} \text{s}^{-1}$, with a 16 hour

photoperiod at 26 ± 2 °C. The osmotic pressure of the medium was reduced by the weekly substitution of the medium (Szász et al., 1995; Szász, 1996). The initial plating efficiency (IPE) was obtained by counting the proportion of the originally plated protoplasts which underwent first mitotic division, after 10 days, within a random population of at least 200 protoplasts per dish. The final plating efficiency (FPE), percentage of plated protoplasts, which had divided to form colonies of at least 1.0–2.0 mm in diameter, was assessed after 28 days. All cultures were maintained at 27 °C in darkness until microcalli were formed. After the fourth week, the calli obtained were removed from the gel using a 6 mm diameter cork opener, transferred to MSZ medium (Díaz et al., 1988) and kept at 26 ± 2 °C, 16 hour photoperiod and irradiance around $24 \mu\text{moles m}^{-2} \text{s}^{-1}$. After two weeks, the calli were transferred to different regeneration media containing the basic MS salts, supplemented by the B5 vitamin complex, 100 mg l⁻¹ mio-inositol, 3% (w/v) sucrose, 0.24% (w/v) phytigel, and different combinations of growth regulators (Table 1). The R medium were also used, and supplemented with 4.0 mg l⁻¹ IAA and 2.56 mg l⁻¹ kinetin (Saxena et al., 1981) and S, supplemented with 2.0 mg l⁻¹ BAP (Díaz et al., 1988) with the addition of 1.5% activated charcoal. There were four replications for each culture medium containing 12 explants per dish, in a total of 48 calli for each cultivar and hybrid. The culture media were poured in Petri dishes (90 x 15 mm) and the cultures were maintained at 26 ± 2 °C, 16 hour photoperiod regime, under a irradiance around $24 \mu\text{moles m}^{-2} \text{s}^{-1}$ during the first two weeks and $36 \mu\text{moles m}^{-2} \text{s}^{-1}$ for the following weeks.

Results and discussion

Under the same incubation conditions, volume of enzymatic mixture, digested tissue mass, the enzymatic

Table 1 Culture media used in the subculture of protoplast-derived calli from different Brazilian and Hungarian pepper cultivars and hybrids (*Capsicum annuum* L.) after six weeks of culture

Media	Growth regulators (mg l ⁻¹)			
	BAP	Zeatin	NAA	IAA
A**	2.0	—	—	—
B	2.0	—	0.01	—
C	2.0	—	0.1	—
D	2.0	0.25	0.01	—
E	2.0	0.25	0.1	—
F	2.0	0.5	0.01	—
G	2.0	0.5	0.1	—
H	2.0	—	—	0.01
I	2.0	—	—	0.1
J	2.0	0.25	—	0.01
K	2.0	0.25	—	0.1
L	2.0	0.5	—	0.01
M	2.0	0.5	—	0.1
N*	2.0	—	—	—
O*	2.0	0.25	—	0.01
P*	2.0	0.25	—	0.1
Q*	2.0	0.25	—	—

*Culture media containing half the concentration of salts

** According to Díaz et al. (1988).

solution E4 enabled higher protoplast yields and viability when compared with the other solutions (Table 2). With the enzyme mixture used, protoplasts were isolated from all the genotypes at a proper yield (Table 4). The enzymatic solution E1, originally described by Díaz et al. (1988), was used successfully in isolating protoplasts from the Dulce Italiano cultivar. However, in this study, it was not efficient in isolating the protoplasts when compared with the other enzymatic solutions (Table 2). When the E1 enzymatic solution was used, a suspension of protoplasts of inferior quality (less pure protoplasts in the filtrates) was obtained at the end of the purification process, because the cell walls were not effectively digested, resulting in a large quantity of cell debris. Likewise, the enzymatic solution E₂ was not ideal for the four analysed cultivars because it did not give yields comparable to the E₃ and E₄ solutions, in spite of being the best in terms of protoplast viability (Table 3). This enzyme mixture, originally described by Szász et al. (1995) and Szász (1996), was used successfully in isolating protoplasts from cotyledons, hypocotyls and leaves from 31 pepper cultivars (*Capsicum* sp). The enzymatic solutions, E3 and E4, enabled higher protoplast yields, with an average of 5.62 ± 1.53 and $9.97 \pm 1.72 \times 10^6$ protoplast g⁻¹ of fresh material, respectively. Both enzymes allowed higher viabilities for isolated protoplasts, with a mean of $80.06 \pm 2.59\%$ and $85.96 \pm 2.27\%$, respectively. The enzyme combination in the E4 solution was suitable for obtaining protoplasts with high purity degrees, yield and viability (Tables 2 and 3), and it was chosen thereafter for protoplast isolation of other cultivars and hybrids (Table 4). This

Table 2 Yield means and standard deviation of protoplasts isolated from 16 to 18-day old cotyledons from four Brazilian pepper (*Capsicum annuum* L.) cultivars in different enzymatic solutions (E₁, E₂, E₃, E₄)

Cultivar	Yield*			
	E ₁	E ₂	E ₃	E ₄
Hércules	1.50 ± 1.41	3.25 ± 0.58	4.25 ± 1.73	8.52 ± 1.26
Ikeda	1.15 ± 1.00	3.10 ± 1.82	5.78 ± 1.50	10.50 ± 1.63
Q. vermelho	1.95 ± 0.58	3.55 ± 1.00	6.90 ± 0.81	10.43 ± 1.82
Yolo Wonder	1.25 ± 1.73	2.75 ± 1.31	5.55 ± 2.08	10.42 ± 2.16
Mean	1.46 ± 1.18	3.16 ± 1.17	5.62 ± 1.53	9.97 ± 1.72

* Yields expressed in 1×10^6 protoplasts g⁻¹ of fresh material. Each value represents the mean of eight countings on the hemacytometer; \pm standard deviations for the mean.

Table 3 Viability means and standard variation of four pepper cultivars (*Capsicum annuum* L.) submitted to different enzymatic solutions (E₁, E₂, E₃ and E₄)

Cultivar	Viability (%)**			
	E ₁	E ₂	E ₃	E ₄
Hércules	82.96 ± 1.89	88.97 ± 2.04	77.61 ± 2.44	89.08 ± 2.17
Ikeda	71.17 ± 1.69	90.37 ± 1.16	82.86 ± 2.23	89.65 ± 1.42
Q. Vermelho	82.57 ± 2.2	80.78 ± 3.2	77.10 ± 2.74	77.35 ± 2.62
Yolo Wonder	78.30 ± 2.67	88.13 ± 1.90	82.69 ± 2.98	87.93 ± 2.57
Mean	78.75 ± 2.11	87.06 ± 2.09	80.06 ± 2.59	85.96 ± 2.27

** Each value represents the mean of a counting of 250 protoplasts, \pm standard deviation from the mean.

enzymatic solution enabled higher protoplast yields (Figure 1A) with a mean of $9.59 \pm 1.53 \times 10^6$ protoplasts g^{-1} of fresh weight (Table 4). Among tested cultivars, Casca Dura Ikeda showed the best result for protoplast yield (10.5×10^6 protoplasts g^{-1} of fresh material). For the hybrid Tango the obtained yield ($11.48 \pm 0.50 \times 10^6$ protoplasts g^{-1} of fresh material) was superior to the other cultivars. Except for the Quadrado Vermelho cultivar and the hybrid 'Magali' hybrid, the protoplasts viabilities were higher than 85% (Table 4). The best result was recorded for the Agronomico G10 cultivar ($91.14 \pm 1.34\%$). Niedz et al. (1987) reported a mean viability of 73% whereas De Donato et al. (1989) reported a mean viability of 95% and Prakash et al. (1997) 70 to 75% viability. High protoplast viability is crucial immediately after isolation, and also during subsequent culture. According to Ochatt and Power (1992), in general, the viability of isolated protoplasts should not be lower than 60%.

In spite of the different genotypes used, the average size of the protoplast obtained ($33.24 \pm 1.0 \mu\text{m}$ diameter) from all cultivars and hybrids were very similar (Table 4). Protoplast isolation is influenced by several factors, including the choice of medium, plant species and/or cultivar, together with the source of tissue (e.g. leaves, cell suspensions, cotyledons, roots, pollen tetrads) and the physiological status of the donor plant. In the present study cotyledon explants were used with success in obtaining reproducible yields and viabilities. Different sources of explant were used for protoplast isolation in the genus *Capsicum*, for instance, leaves from plants grown *in vitro* (Saxena et al., 1981; Murphy and Kyle, 1994; Szász et al., 1995; Prakash et al., 1997), or in shoot apex (Díaz et al., 1988), cotyledon (De Donato et al., 1989; Szász et al., 1995; Szász, 1996), and hypocotyl (Szász et al., 1995). The type of osmoticum used was one the main difference amongst reports on the isolation and cultivation of protoplasts of *Capsicum* genus. Saxena et al. (1988) used 0.5 M mannitol, diluted in the CPW salts (Power and Chapman 1985) used 0.7 M mannitol, diluted in the same salt in some

C. annuum and *C. chinense* cultivars. De Donato et al. (1989) compared the osmotic effects of sorbitol, mannitol, glucose and sucrose and found that 0.4 M mannitol was the best for the different cultivars analysed. Murphy and Kyle (1994) observed that different concentrations of mannitol (0.5–0.7 M) were necessary for the isolation of protoplasts from different *C. annuum* cultivars. Szász et al. (1995b), however, used 0.4 M sucrose as an osmotic solution to isolate protoplasts of 31 *Capsicum* ssp. genotypes. Prakash et al. (1997) used mannitol at 9% (p/v) diluted in CPW salts in the isolation of protoplasts in the California Wonder cultivar. These variations may be only genotype-specific, but also reflect the demands as to the type of tissue from which the protoplasts are isolated (Szász et al., 1995). The protoplasts isolated from the enzymatic solution E4 performed well under cultivation, except for the Yolo Wonder and Magda cultivars and the Tango hybrid, which had no cell division (Table 5). The cultivation method proposed by Díaz et al. (1988) with some modifications, mainly the use of K8p medium in place of the KM8p and sodium alginate instead of agarose, as suggested by Szász (1996) and Szász et al. (1995). Protoplast cultivation in alginate gel was adopted in the present study considering positive effects compared to other cultivation methods. Cells in suspension (Ochoa-Alejo and Salgado-Garciglia, 1992; Hoshino et al., 1994; Sanot-Díaz et al., 1994) and pepper protoplasts (Szász et al., 1995; Szász 1996) under cultivation produced and released phenolic compounds in the culture medium, decreasing the pH value of the medium as well as intoxicating the cells, preventing their division. The gel, which imbibes the cells, is formed at room temperature by the polymerisation of the alginate with calcium ions (Sundenberg and Glimelius, 1986; Szász et al., 1995) so the risk of possible damages by temperature stress, commonly observed when cultivation is undertaken in semi-solid medium with agar or agarose, is reduced. In some culture systems, cell division and plant regeneration were obtained and optimised when protoplasts of *Arabidopsis thaliana* (Damm and Willmitzer, 1988), *Helianthus annuus* (Schmitz and Schnabl, 1989; Fisher and Hahne, 1992), *Citrus sinensis* (L.) Osbeck cv Hamlin (Niedz, 1993), *Hordeum vulgare* (Golds et al., 1994), *Allium ampeloprasum* (Buíteveld and Creemers-Molenaar, 1994) were also cultivated in alginate. The liberation of phenol compounds in the surrounding liquid culture medium and

Table 4 Mean and standard deviation of yield, viability and size of protoplasts of Brazilian and Hungarian pepper (*Capsicum annuum* L.) cultivars and hybrids isolated from the E₄ enzymatic solution

Cultivar	Yield*	Viability (%)	Size (μm)
Agronomico G10	8.98 ± 0.95	91.14 ± 1.34	33.62 ± 1.01
Casca Dura Ikeda	10.50 ± 1.63	89.65 ± 1.42	34.61 ± 0.98
Hércules	8.55 ± 1.26	89.08 ± 2.17	33.74 ± 0.91
Magda	9.45 ± 2.08	89.57 ± 1.51	32.87 ± 1.57
Quadrado Verde	8.80 ± 1.41	88.96 ± 3.37	31.85 ± 1.05
Quadrado Verm.	10.43 ± 1.82	77.20 ± 2.93	33.36 ± 1.28
Yolo Wonder	10.42 ± 2.16	87.93 ± 2.57	30.57 ± 0.88
Híbrido Magali	8.75 ± 1.73	77.80 ± 2.46	33.98 ± 1.18
Híbrido Tango	11.48 ± 0.50	85.22 ± 3.78	31.68 ± 1.03
Greigo	9.85 ± 1.73	90.84 ± 2.14	34.11 ± 0.64
Cecei	9.50 ± 1.15	87.18 ± 2.79	33.73 ± 0.71
Szentesi	8.45 ± 1.91	89.48 ± 2.53	32.24 ± 0.84
Mean	9.59 ± 1.53	87.00 ± 2.41	33.11 ± 1.00

*Yield expressed in 1×10^6 protoplasts g^{-1} of fresh material. The yield values represent the mean of eight counting on the hemacytometer; each viability and size value represent the mean of 250 protoplast countings, standard deviations from the mean.

Table 5 Means and standard deviations of the initial (IPE) and final (EFP) plating efficiencies of protoplasts of pepper (*Capsicum annuum* L.) cultivars and hybrids after 30 days cultivation

Cultivars/Hybrids	IPE (%)	FPE (%)
Casca Dura Ikeda	38.20 ± 1.50	0.32 ± 0.01
Yolo Wonder	—	—
Tango	—	—
Magali	40.70 ± 0.8	80.28 ± 0.02
Greigo	36.73 ± 1.24	0.27 ± 0.01
Magda	—	—
Mean*	38.55 ± 1.20	0.29 ± 0.01

* Mean among the cultivars with response; — no division; \pm standard deviations from the mean.

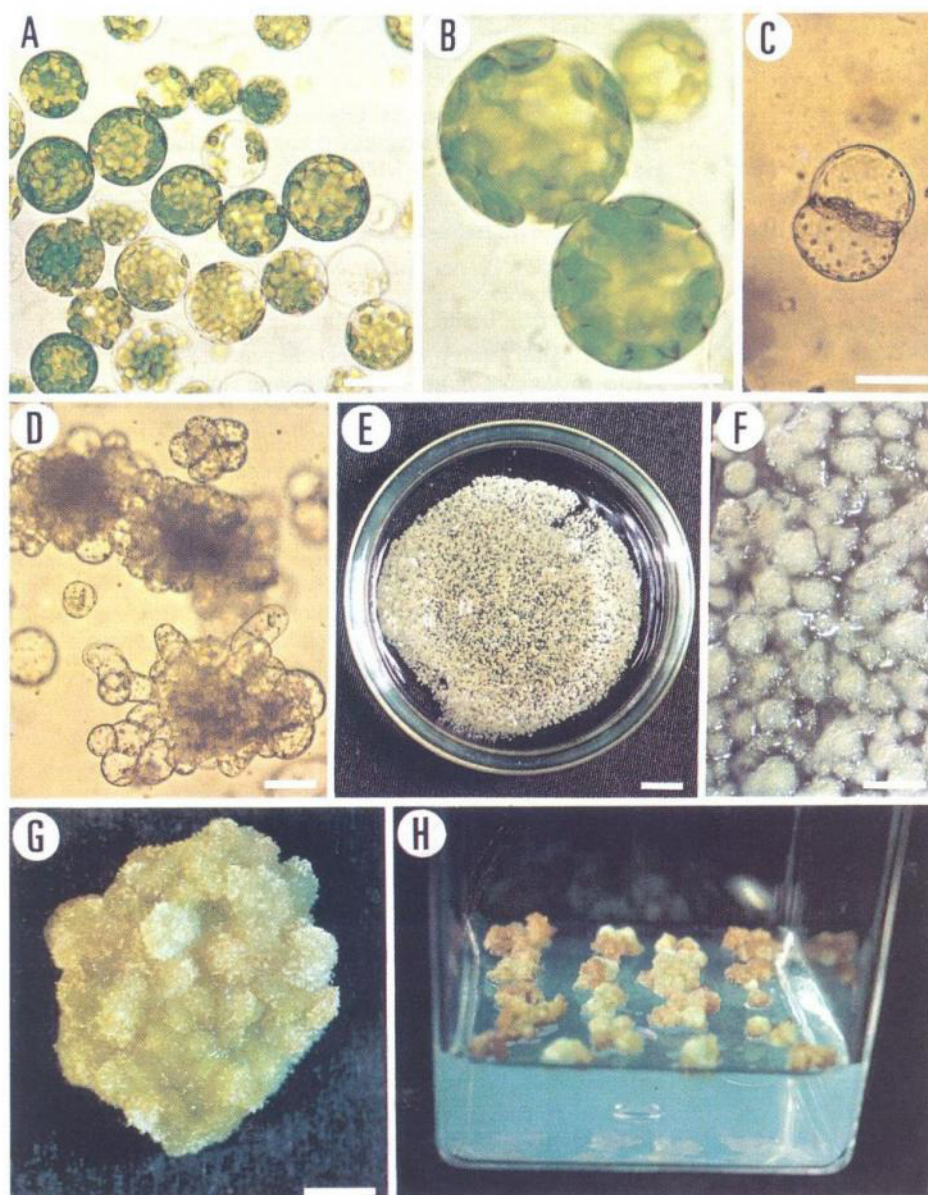


Figure 1 Isolation and culture of cotyledon-derived protoplasts from the Brazilian pepper variety, cv Casca Dura Ikeda. **A** – Fresh isolated protoplasts (Bar = 30 μ m); **B** – Detail of recently isolated protoplasts showing the uniform distribution of chloroplasts (Bar = 15 μ m); **C** – First mitotic division after seven days in culture in sodium alginate (Bar = 15 μ m); **D** – Protoplast-derived microcolonies after 21 days of culture (Bar = 15 μ m); **E** – Detail of protoplast calli cultured in sodium alginate gel (Bar = 1 cm); **F** – Protoplast-derived calli after 32 days of cultivation (Bar = 1.5 mm); **G** – Protoplast-derived calli in greening phase, after three weeks of cultivation in MSZ culture medium (Bar = 0.5 cm); **H** – Protoplast-derived calli in regeneration medium, showing phenol oxidation (Bar = 1 cm).

the transport of nutrients and growth regulators in the alginate gel were increased by this technique. A greater uniformity of protoplast dispersion in the matrix was also observed and it could also be used for other recalcitrant protoplast systems (Szász et al., 1995). The first divisions were observed between sixth to seventh days of cultivation, on the average (Figure 1C). Similar results were obtained by Saxena et al. (1981), Díaz et al. (1988) and Prakash et al. (1997). Niedz et al. (1987) observed the first division after five-day cultivation, and De Donato et al. (1989) after 48 hours. The subsequent mitotic divisions were observed

shortly afterwards, leading to the formation of microcolonies. The microcolonies containing 15 to 20 cells were, on the average, observed at the 21st day of cultivation (Figure 1D). After 30 days, the first calli were formed (Figure 1F, the EFP determined and the callus cultivated in greening medium (Figure 1G). The absence of cell division of the Yolo Wonder and Magda cultivars and of the Tango hybrid, however, may have been caused by the unsuitability of the culture medium and/or the osmotic solution. Out of the seven reports on the isolation and cultivation of protoplasts of the *Capsicum* genus, reports by Saxena et al. (1981) Niedz et al. (1987) and Murphy and Kyle (1994) did not provide data for EIP, hindering the comparison of the results with the present study. The EIP means, obtained by Díaz et al. (1988) were observed at the 14th day of cultivation, and a mean of 22.75% was obtained. The exception was *Capsicum annum* cv. Nigrum, where no response for cell division was found. At the 10th day of cultivation, De Donato et al. (1989) and Szász et al. (1995b) observed 9.5% and 29.6% EIP, respectively, while Prakash et al. (1997) obtained 20–25% at the 12th day.

The data obtained in the present experiment together with those of the available literature, showed that the cultivation system in alginate was superior, because the EIP means were 38.54% except for the Yolo Wonder and Magda cultivars and the Tango hybrid, which did not respond in cultivation. The Magda cultivar and Tango hybrid protoplasts did not divide, although they remained intact. However, the

Yolo Wonder cultivar protoplasts burst in culture; conversely to those in the De Donato et al. (1989) study had an EIP around 5%. Contamination by bacteria in other cultivars and hybrids analysed in this study did not allow cultivation, thus their response still remains an open question.

The Casca Dura Ikeda and Greigo cultivars and the Magali hybrid callus formed in alginate gel (Figure 1E), were transferred to MSZ medium (Figure 1G) after the fourth week; in all cases, they increased significantly in diameter, and began to turn green after the first week of

cultivation. The calli obtained in MSZ medium were transferred to the regeneration (Table 1) and the R and S media and kept under the same conditions of cultivation. The callus began to oxidize (Figure 1G) in all the culture media after the second week in cultivation, including those cultivated in medium containing activated carbon. The lowest oxidation rates for the Magali and Casca Dura Ikeda cultivars were observed in the C and R media and varied between 45.83 (2.12% and 37.50 (3.34%, respectively. The R culture medium was the most suitable for callus subculture (Saxena et al. 1981) as the callus maintained a greenish colour and had lower phenol oxidation rates. The calli were subcultured in this medium every two weeks but no morphogenic response was observed during four months in cultivation, whereas phenol oxidation was the major problem in protoplast cultivation.

Despite the lack of successful organogenesis, several important results were obtained on cultural performance of pepper protoplasts. They may serve as a basis for future studies of plant regeneration from protoplast-derived calli, as well as for studies of DNA or RNA replication involved with the resistance mechanisms of pepper plants to diseases.

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