In Vitro Cell.Dev.Biol.-Plant (2009) 45:650–658
DOI 10.1007/s11627-009-9193-y

DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

In vitro propagation and agronomic performance of regenerated chili pepper (C*apsicum* spp.) plants from commercially important genotypes

Ma. Guadalupe Valadez-Bustos · Gerardo Armando Aguado-Santacruz · Guillermo Carrillo-Castañeda · Víctor Heber Aguilar-Rincón · Eduardo Espitia-Rangel · Salvador Montes-Hernández · Alejandrina Robledo-Paz

Received: 8 January 2008 / Accepted: 22 January 2009 / Published online: 5 March 2009 / Editor: N. Ochoa-Alejo

Abstract The application of modern biotechnology for improvement of chili pepper productivity requires an efficient *in vitro* plant regeneration protocol. In this study, a reliable protocol was developed for the *in vitro* regeneration of four types of chili, *Capsicum annuum* var. *annuum* (Jalapeño and Serrano), *C. annuum* var. *glabriusculum/aviculare* (Piquin), and *C. chinense* (Habanero) by direct organogenesis using three different explants (cotyledon, hypocotyls, and embryo) and three induction media. All evaluated culture media promoted the formation of adventitious shoots. When embryos or hypocotyls were used as explants, morphologically normal adventitious shoots developed, while culturing cotyledons resulted in non-elongating rosette-shaped shoots. The highest *in vitro* regeneration efficiency (14.6 shoots per explant) was achieved when Habanero chili hypocotyls were grown on Murashige and Skoog medium containing 1.7 μM indole-3-acetic acid and 22.2 μM N^6-benzyladenine. This regeneration rate is higher than that obtained in previous reports. Regenerated plants were ready to be transferred to the greenhouse 13 wk after the explant culture. An evaluation carried out under greenhouse conditions showed differences in agronomic performance between *in vitro* regenerated plants and plants developed from seeds with the magnitude of the differences depending on the genotype being studied.

Keywords *Capsicum* · Chili pepper · Plant regeneration · Tissue culture · Organogenesis · Seed production

M. G. Valadez-Bustos · G. Carrillo-Castañeda · V. H. Aguilar-Rincón · A. Robledo-Paz (✉)
Colegio de Postgraduados, Campus Montecillo,
km 36.5 Carretera México-Texcoco,
Montecillo, Estado de México 56230, México
e-mail: arobledo@colpos.mx

G. A. Aguado-Santacruz
Unidad de Biotecnología, Campo Experimental Bajío,
Instituto Nacional de Investigaciones,
Forestales Agrícolas y Pecuarias,
km 6.5 Carretera Celaya-San Miguel Allende,
Celaya, Guanajuato 38110, México

E. Espitia-Rangel · S. Montes-Hernández
Campo Experimental Bajío, Instituto Nacional de Investigaciones,
Forestales Agrícolas y Pecuarias,
km 6.5 Carretera Celaya-San Miguel Allende,
Celaya, Guanajuato 38110, México

Introduction

Chili pepper is an indispensable ingredient employed for food preparation in the world and an important product utilized in the pharmaceutical, food, cosmetic, and poultry industries. The main producers of chili are China, Mexico, Turkey, and USA. The export value of this species represents US $2,811,590,000 worldwide, while in Mexico its production represents US $576,690,000 (FAO 2006).

As with other crops, the production of chili pepper is affected by biotic and abiotic factors that reduce its crop quality and yield (Nuez et al. 1996). For this reason, the search for biotechnological alternatives that may increase the productivity of this Solanaceae member is necessary. The development of efficient protocols for *in vitro* tissue culture and plant regeneration is required for the application of biotechnological tools, such as asexual reproduction of elite stocks, recovery of useful somaclonal variants, germplasm preservation as well as the production of transgenic plants with improved agronomic traits, interspecific hybrids, and haploid plants (Ezura 1997; Aguado-Santacruz et al. 2004).

Although *in vitro* regeneration of chili peppers has been achieved through different protocols and morphogenic ways (Prakash et al. 1997; Kintzios et al. 2001; Valera-Montero and Phillips 2005; López-Puc et al. 2006; Joshi and Kothari 2007), it has been difficult to establish efficient, reliable, and broad-spectrum protocols for members of this genus. This obstacle is related to the high diversity of the existing genotypes, which results in a great variety of responses to *in vitro* tissue culture. Therefore, it is necessary to establish reliable regeneration systems for chili peppers, especially for genotypes developed for commercial purposes. Moreover, the comparative agronomic performance in terms of valuable traits, such as fruit or seed production, between regenerated and seed-derived plants has not been properly addressed, with only one study reporting the genetic and morphological variation of R_0 and R_1 chili pepper plants regenerated from *in vitro* tissue culture (Amzad et al. 2003). Such information is vital to reliably determine the advantages and disadvantages of this technology. Therefore, the objectives of this study were (1) to evaluate different media formulations, explants, and chili germplasm sources for induction of *in vitro* morphogenic responses and regeneration of complete chili plants and (2) to compare the agronomic performances of the regenerated plants to that of plants developed from seeds under greenhouse conditions.

Materials and Methods

Plant materials. Seeds from two species of *Capsicum* obtained from the National Institute for Forestry, Agriculture and Livestock Research (INIFAP), Veracruz, México were used in this study. The evaluated materials included the following: *Capsicum annuum* var. *annuum* (Jalapeño cv. Don Pancho and Serrano cv. Tampiqueño-74); *C. annuum* var. *glabriusculum/aviculare* (Piquin cv. Güemez); *C. chinense* (Habanero cv. Naranja).

Explant sterilization and isolation. The *in vitro* morphogenic responses of three sources of explant (cotyledons, hypocotyls, and zygotic embryos) were evaluated. Seeds of the different species of chili pepper were surface sterilized by soaking them for 5 min in a solution containing 1 g l^{-1} Daconil (Chlorothalonil tetrachloroisophthalonitrile; Quimica Industrial Agricola, Mexico, Mexico). Subsequently, the seeds were transferred for 20 min to 30% (*v*/*v*) NaClO solution (Cloralex®, Tlalnepantla, Mexico) amended with 1.0 ml l^{-1} of Tween 20 (Sigma–Aldrich, St. Louis, MO) and then rinsed four times with sterilized distilled water.

Disinfected seeds were placed on one half Murashige and Skoog (MS) medium (Murashige and Skoog 1962), 1% (*w*/*v*) sucrose and solidified with 0.6% agar–agar (Merck, Darmstadt, Germany). Cotyledons and hypocotyls were isolated from 7-d-old seedlings (Fig. 1*a*), while the zygotic embryos were isolated directly from the chili pepper seeds. Cotyledons were placed with the abaxial side in contact with the medium, while the proximal (nearest to the apical meristem), 0.7 cm long hypocotyl sections were cultured vertically and inverted to their original position on the plant. In order to obtain the embryo explants, seeds were cut into two sections before germination; one portion contained the cotyledons and part of the hypocotyls, while the other section comprised the proximal section of the hypocotyl and the radicle.

Media for induction of morphogenic responses. Cotyledons, hypocotyls, and embryos were placed on three Petri dishes (ten explants per Petri dish) with 30 ml of induction media. There were three variations of the induction media: Medium A, Medium B, and Medium C. The induction media contained the basic salts of the MS medium, 3% sucrose, 11.8 μM $AgNO_3$, 0.7% (*w*/*v*) agar–agar, and the following respective concentrations of N^6-benzyladenine (BA) and indole-3-acetic acid (IAA): medium A, 13.3 and 1.1 μM; medium B, 17.8 and 1.7 μM; medium C, 22.2 and 1.7 μM. The pH of the media was adjusted to 5.7±0.1. The media were sterilized by autoclaving at 1.05 kg cm^{-2} for 15 min. The percentage of explants that formed shoots was evaluated 4 wk after the initial culture.

Media for elongation of adventitious shoots. After 4 wk of growth on induction media A, B, and C, the explants were transferred to glass jars containing 30 ml of the elongation medium, which included the salts of the MS medium, 3% sucrose, 2.9 μM gibberellic acid (GA_3), 23.5 μM $AgNO_3$, and 0.7% agar–agar. When hypocotyls were cultured on elongation medium, they were inverted in relation to their initial culture position. After 3 mo, the number of differentiated shoots (longer than 5 mm) per explant was determined.

Medium for rooting of the shoots. Once the adventitious shoots were 2 cm long, they were transferred into 250 ml glass jars containing 30 ml of rooting media that included the salts of the MS medium, 3% sucrose, and 0.7% agar–agar.

Growth conditions. Environmental conditions during seed germination, *in vitro* culture of explants and shoot regeneration and rooting were 26±2°C and a 16 h photoperiod (photon flux=25 μmol m^{-2} s^{-1}).

Cultivation of seed-derived plants. In order to characterize and compare the performance of plants developed from seeds to that of *in vitro* regenerated plants, seeds from the four types of chili peppers under study were germinated *in vitro* on one-half-MS medium with 1% sucrose and 0.6% agar–agar.

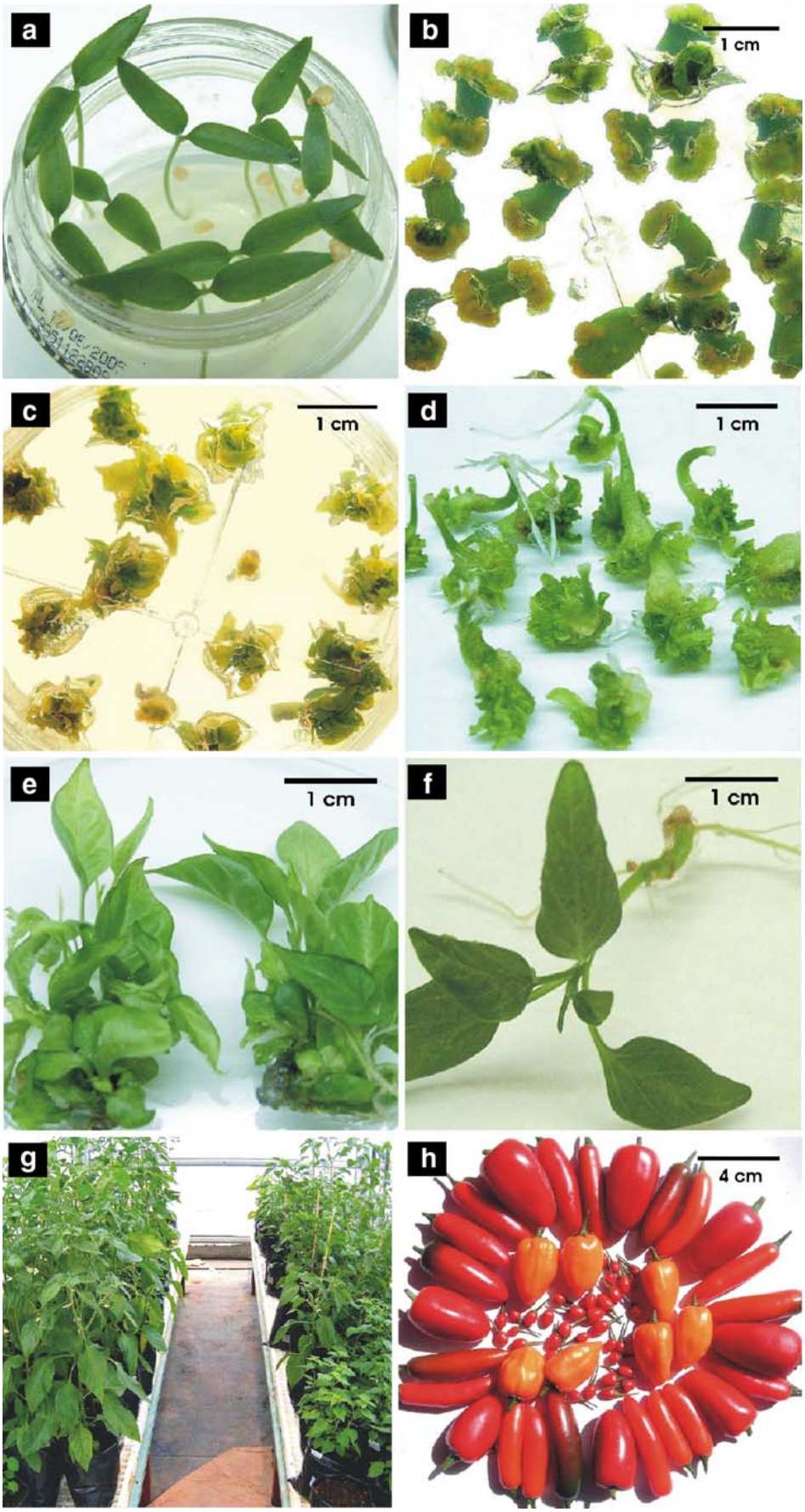

Figure 1. *In vitro* plant regeneration of *Capsicum* spp. (*a*) Seedlings after 7 d following radicle emergence, (*b*) Formation of shoot primordia from cotyledons after 12 d of growth on induction media, (*c*) Shoot primordia 12-d-old generated from hypocotyls, (*d*) Adventitious shoot 30-d-old formed from hypocotyls, (*e*) Adventitious shoots 3-mo-old formed from embryos, (*f*) A complete *in vitro* regenerated plant ready for greenhouse acclimation, (*g*) Regenerated chili plants after establishment in the greenhouse, (*h*) Fruits obtained from the different types of chili peppers regenerated *in vitro*.

Acclimation and hardening of regenerated and seed-derived plants in the greenhouse. The plantlets regenerated *in vitro* and seedlings developed from seeds that reached lengths of 3–4 cm were extracted from the culture media. Roots were rinsed with running water to eliminate residue from the culture media and then soaked in a fungicidal solution (Daconil 1 g l^{-1}) for 3 min. Subsequently, the plantlets and seedlings were transferred into 6-kg pots containing peat moss (Sunshine®), covered with perforated transparent polyethylene bags, and placed in a greenhouse under a black mesh cover (photon flux=345 μmol m^{-2} s^{-1}) which was removed after 3 wk. The plantlets and seedlings were fertilized twice a week with 600 ml of a solution of 1 g l^{-1} Miracle Grow® (Scotts Co., Marysville, OH) and 0.5 g l^{-1} phosphonitrate. The conditions in the greenhouse during acclimation and hardening were photon flux = 600 μmol m^{-2} s^{-1}, 35°C and 23% of relative humidity.

The following traits in the adult plants derived either from *in vitro* culture or from seeds were evaluated: survival (determined 3 wk after transference to soil), number of days to flowering and fructification, number of days to the first harvest, length, width, and weight of the fruits, fruits per plant, seeds per fruit, plant height, yield per plant, and thousand-seed weight.

Statistical analysis. One-way analysis of variance was used to test significant differences in the evaluated variables. Means were then compared according to the LSD test ($p<$ 0.05; Steel and Torrie 1992).

Results and Discussion

Effect of genotype on the in vitro tissue culture response of chili. Adventitious shoots were differentiated in all chili materials tested (Table 1). Jalapeño and Piquin types showed the highest and lowest percentages of explants with shoots, 66.1% and 50.2%, respectively. These results differ from those reported by Ochoa-Alejo and Ireta-Moreno (1990), who observed only 6% of explants with shoots in Jalapeño chili. On the other hand, the Habanero type produced the highest number of shoots per explant (11.0) and Jalapeño the lowest (5.8; Table 1).

The low percentage of explants with shoots obtained in Piquin chili had also been observed by Ramírez-Malagón and Ochoa-Alejo (1996), and it can be attributed to the high genetic variability present in this wild chili. There are only a few reports that focus on *in vitro* plant regeneration of Piquin chili (Ramírez Malagón and Ochoa-Alejo 1996; Cárdenas-Avila et al. 1997), which is a valuable genetic resource because it carries genes potentially useful for genetic improvement of *Capsicum* spp. (Hernández-Verdugo et al. 1998), such as those involved in tolerance to pests, diseases, and different abiotic stresses that significantly reduce the yield of this crop.

Capsicum chinense is another economically important species for which information about *in vitro* response is limited. Some authors consider it as recalcitrant for *in vitro* culture (López-Puc et al. 2006; Pinzón 2007), but our results with Habanero chili indicate that *in vitro* culture of this species is feasible when an adequate variety, culture medium, explant, and growth conditions are used.

Effect of induction media on the in vitro tissue culture response of chili. All of the media evaluated in our investigation favored the differentiation of adventitious shoots. This response was dependent on the explant and cultivar under study. Media A and B resulted in the highest percentages of explants that formed shoots (59.8% and 62.5%, respectively; $p<0.05$). In contrast, medium C was the least efficient in terms of the number of explants that formed shoots (Table 1). These results contrast with those reported by Valera-Montero and Ochoa-Alejo (1992) who obtained the highest adventitious shoot differentiation efficiencies using medium C in cultivars of Tampiqueño-74, Salvatierra, and Chile de Agua. With respect to the number of

Table 1. Effect of genotype, culture medium, and explant source on the differentiation of adventitious shoots

Variable	Chili type				Induction media			Explant		
	Hab	Piq	Ser	Jal	A	B	C	Emb	Hyp	Cot
Explants with shoots (%)[a]	62.1b[b]	50.2c	59.4b	66.1a	59.8a	62.5a	56.0b	89.7a	73.1b	15.6c
No. of shoots per explants[c]	11.0a	8.1b	6.8c	5.8d	7.9ab	8.1a	7.8b	7.5b	8.3a	–

Experiments were performed three times

Hab Habanero, *Piq* Piquin, *Ser* Serrano, *Jal* Jalapeño, *Emb* Embryo, *Hyp* Hypocotyl, *Cot* Cotyledon

[a] Values observed after 1 mo of explants culture

[c] Values observed after 3 mo of explants culture (shoots ≥ 5 mm long)

[b] Means followed by different *letters* are significantly different ($P\leq0.05$) within chili type, induction media, and explant source tested

shoots per explant, there were no significant differences among the media tested (Table 1).

Likewise, the results obtained in this paper confirm the synergistic effect of the combination of BA with IAA in the regeneration of adventitious shoots observed previously by other authors (Ramírez-Malagón and Ochoa-Alejo 1996; Kumar et al. 2005; Peddaboina et al. 2005). Conversely, Sanatombi and Sharma (2006) and Sripichit et al. (1987) observed that the use of BA as the only growth regulator was more effective for inducing the formation of adventitious shoots in some cultivars of *C. annuum*.

Effect of explant source on the in vitro tissue culture response of chili. Significant differences were observed among the three sources of explants, with embryos producing the best response in terms of the percentage of explants that formed shoots (89.7%, Table 1) and hypocotyls generating the highest number of shoots per explant (8.3). One advantage of using embryos as the starting material is that they can be grown directly on the induction medium without waiting for seeds to germinate and form seedlings from which the cotyledons and hypocotyls can be isolated. This is especially important in chili types that have irregular and retarded germination, such as Piquin and Habanero chilis. Utilization of these explants has allowed the reduction of the time required to obtain *in vitro* plants, as well as solving a well-known problem related to the elongation of the adventitious shoots (Binzel et al. 1996).

The favorable morphogenic response observed in hypocotyl and embryo explants may be due to the fact that they were obtained from young tissues, which have a higher capacity to respond to *in vitro* culture than older explants. Some authors have indicated that these differences are related to a higher number of totipotent cells, mitotic activities, and endogenous levels of growth regulators, especially cytokinins in young tissues (Dabauza and Peña 2001; Kaparakis and Alderson 2003).

On the other hand, it was not possible to count the shoots formed in cotyledons because they produced leaf rosettes without well-defined stems. Again, the formation of deformed and rosette-grouped shoots that do not elongate is a phenomenon frequently observed during *in vitro* culture of chili (Agrawal et al. 1989; Husain et al. 1999; Dabauza and Peña 2001; Santana-Buzzy et al. 2005; López-Puc et al. 2006; Pinzón 2007).

Interaction among types of chili, induction media, and sources of explant. Although all types of chili were able to develop adventitious shoots under the experimental conditions tested in this study, the magnitude of the response depended on a combination of genotype, induction media, and explant sources. Therefore, each type of chili had its highest regeneration efficiency in a specific culture medium and from a specific source of explant (Table 2).

According to our results, the best explants and media for adventitious shoot production were hypocotyls cultured on media C and B for Habanero and Jalapeño, respectively, while embryos produced a better response in Piquin and Serrano when cultured on media A and B, respectively (Table 2). When the most efficient conditions for shoot induction in every type of chili were compared, the Habanero and Piquin types obtained the highest numbers of shoots per explant (14.6 and 10.5 shoots, respectively; $p<0.05$).

These efficiencies of shoot formation are higher than those previously reported. For example, Ramírez-Malagón and Ochoa-Alejo (1996) reported only 2.5 and 3.1 shoots formed per explant for the Jalapeño and Serrano types, respectively, and Santana-Buzzy et al. (2005) reported eight shoots per explant for Habanero chili.

Variation in the responses presented by the different types of chili tested in this study confirms that *in vitro* chili regeneration requirements depend on the cultivar being tested, as stated by different authors (Husain et al. 1999; Dabauza and Peña 2001; Steinitz et al. 2003; Kumar et al. 2005; Sanatombi and Sharma 2006; Joshi and Kothari 2007).

In vitro shoot development in the different chili types. When the hypocotyls were cultured on the induction media, the zone in contact with the medium developed protuberances from which adventitious shoots formed after 3 wk (Fig. 1*c*). On the other hand, distal ends of the hypocotyls developed roots, thus, conserving their morphogenic polarity despite being inverted from their original position on the plant during the initial culture of these explants (Fig. 1*d*). Once the adventitious shoots were transferred to the elongation medium and placed with the distal root-forming region in contact with the culture medium, shoot development was promoted in a similar way to that described by Ramírez-Malagón and Ochoa-Alejo (1996).

Table 2. Regeneration of adventitious shoots using the most efficient medium and explant in the four types of chili pepper tested

Chili type	Media	Explant	Explants with shoots (%)[a]	No. of shoots per explant[b]
Habanero	C	Hypocotyl	80.0a[c]	14.6a
Jalapeño	B	Hypocotyl	96.7a	7.1d
Piquin	A	Embryo	90.0a	10.5b
Serrano	B	Embryo	96.7a	8.2c

Experiments were performed three times

[a] Values evaluated after 1 mo of explants culture

[b] Values evaluated after 3 mo of explants culture (shoots≥5-mm long)

[c] Means followed by different *letters* are significantly different ($P\leq0.05$) among the chili-type tested

At the second week of the initial culture of the cotyledons, development of adventitious shoots was observed in the areas where they were cut and on the abaxial side of these explants. Pronounced callus areas were observed on the margins of the cotyledons, which occasionally impeded the development of the shoots (Fig. 1*b*).

When the embryos were used as explants, shoot differentiation was observed 3–4 wk after the initial culture. The embryo section containing both the hypocotyl and the radicle showed the best morphogenetic capacity, as previously reported by Binzel et al. (1996).

Periodic subculture and separation of the shoots formed in the explants promoted their development. Likewise, the inclusion of GA_3 and $AgNO_3$ in the elongation medium stimulated shoot elongation and prevented leaf abscission (Fig. 1*e*) in agreement with previous reports (Robledo-Paz and Carrillo-Castañeda 2004; Kumar et al. 2005; Santana-Buzzy et al. 2005, 2006; Joshi and Kothari 2007).

Regenerated shoots formed roots spontaneously (Fig. 1*f*), a response that was genotype-dependent, ranging from 44% of shoots forming roots in Jalapeño to 88% in Habanero (data not shown). All cultivars considered in this study were able to regenerate complete plants within 13–20 wk. After this period, the regenerated plants were ready to be acclimated and grown in the greenhouse.

Evaluation of agronomic performance of the regenerated plants in the greenhouse. In order to use tissue culture as a tool for the propagation of plants, it is of great importance to analyze the plant survival rate and agronomic performance of the *in vitro* regenerated plants in the greenhouse. Despite this fact, none of the chili regeneration protocols published to date have compared the performance of the plants regenerated *in vitro* with those derived from seeds. In this paper, we addressed this issue and compared important agronomic traits of the regenerated plants with those of plants developed from seeds under the same environmental conditions.

Survival. In vitro culture of plants induces genetic, morphological, and physiological changes in the regenerated plants that affect plant survival and agronomic performance (Estrada-Luna et al. 2001). In this study, at least 60% of the plants regenerated *in vitro* survived the acclimation period, with Habanero plants showing the highest survival index (95%) and the Jalapeño plants exhibiting the lowest survival rate (60%). The plants derived from seeds had a survival rate of 100% (data not shown).

Days to flowering, fructification, and the first harvest. All regenerated plants were normal and able to form fruits similar to those produced by the seed-derived plants (Fig. 1*h*). However, some differences were observed in the agronomic variables analyzed for the different chili types. There were significant differences in the days to flowering for all types of chili, except Piquin. *In vitro* regenerated plants from Jalapeño and Serrano flowered 9 and 13 d before seed-derived plants, respectively (Table 3).

Days to fructification and first harvest showed significant differences only for Habanero chili. Plants derived from seeds had fruits and were harvested 1 mo before the micropropagated plants (Table 3).

Table 3. Agronomic performance of regenerated and seed-derived plants of *Capsicum* spp. under greenhouse conditions

Agronomic traits	Chili type							
	Jalapeño		Serrano		Habanero		Piquin	
	Seed	*In vitro*	Seed	*In vitro*	Seed	*In vitro*	Seed	*In vitro*
Days to flowering	61.0a[a]	51.8b	70.0a	56.7b	60.0b	65.7a	74.6a	73.6a
Days to fructification	90.4a	85.3a	111.5a	100.2a	86.8b	115.4a	102.1a	109.2a
Days to the first harvest	138.9a	131.7a	148.6a	141.9a	125.5b	156.6a	147.0a	147.1a
Fruit width (cm)	2.1a	2.1a	1.0b	1.2a	1.8a	1.7a	0.4a	0.4a
Fruit length (cm)	4.7a	4.4a	5.3a	4.9a	3.8a	2.9b	0.5a	0.5a
Fruit weight (g FW)	12.0a	11.1a	3.9b	5.0a	4.6a	3.2b	0.1a	0.1b
Fruits per plant	24.4b	33.6a	42.8b	58.8a	93.2a	89.2a	396.0a	395.8a
Seeds per fruit	37.5a	28.0b	19.3b	30.0a	12.9a	11.0a	5.8a	6.6a
Plant height (cm)	90.8a	58.8b	117.3a	90.0b	72.8a	50.6b	80.2a	78.3a
Yield per plant (g FW)	297.6b	374.5a	177.0b	296.8a	450.8a	277.8b	47.6a	39.6b
Thousand-seed weight (g)	6.1a	5.8a	3.3b	4.3a	3.8a	3.7a	2.9a	2.8a

[a] Means followed by different *letters* are significantly different ($P \leq 0.05$) among regenerated and seed-derived plants in the evaluated agronomic variables

Length, width, and weight of the fruit. As compared to their seed-derived plant counterparts, the width and weight of fruits were significantly higher in the regenerated plants of the Serrano type (Table 3). As a result, heavier fruits with larger diameters were observed in the micropropagated plants of this chili.

In contrast, plants derived from seeds of Habanero formed wider, longer, and heavier fruits than those produced by the regenerated plants, with significant differences only for length and weight. Hence, the seed-derived Habanero fruits were bigger and weighed more than those produced by the regenerated plants (Table 3).

With respect to Piquin chili, the seed-derived plants produced heavier fruits and no significant differences in the fruit length, diameter, or weight in the Jalapeño chili with respect to origin were detected (Table 3).

Number of fruits and seeds. Statistical differences were only observed for the Jalapeño and Serrano types (Table 3) in the number of fruits per plant and seeds per fruit. Jalapeño micropropagated plants produced nine fruits more than plants derived from seeds, but these fruits had fewer seeds. In the Serrano type, the seed-derived plants produced 16 fruits and 11 seeds per fruit less than the *in vitro* regenerated plants (Table 3).

Plant height and yield per plant. Significant differences in plant height were only detected in the Jalapeño, Serrano, and Habanero types, with the seed-derived plants being taller than the *in vitro* regenerated plants (Table 3). On the other hand, yield per plant was statistically higher in the regenerated plants of the Jalapeño and Serrano types (Table 3), which produced, respectively, 77 and 120 g more fruits than the plants derived from seeds. Differences in yield per plant for the Jalapeño chili were related to a higher number of fruits per plant being produced by the regenerated plants. In the Serrano type, yield per plant was not only influenced by a higher number of fruits per plant but by heavier fruits.

In contrast, yield per plant was smaller in the regenerated plants of Habanero (277.8 g FW) and Piquin (39.6 g FW) than in their seed-derived counterparts (450.8 and 47.6 g FW, respectively); these differences were attributed to the fact that the micropropagated plants produced lighter fruits.

The positive relationship observed among yield, fruits per plant, and fruit weight has been previously reported for this crop by other authors (Kumar et al. 2003; Nandadevi and Hosamani 2003; Deepu et al. 2004).

Thousand-seed weight. Seed quality depends on crop management in the field (application of fertilizers and pest control), harvesting method, and seed post-harvest storage conditions. Characteristics such as water content, weight per volume, purity, size, shape, color, thousand-seed weight, and damage by insects and fungi are considered physical quality parameters for seeds (Kelly 1988; Basra 1995). In this study, thousand-seed weight was considered as a variable indicative of physical quality. Our results showed significant differences only for the Serrano chili, with the micropropagated plants obtaining higher weights than seed-derived plants (Table 3). Differences observed in this type chili are explained by the production of thicker and heavier fruits by the micropropagated plants, resulting in a more developed pericarp, a characteristic that has a positive effect on seed size. This relationship was reported previously for five species of *Capsicum* (Deepu et al. 2004).

Tissue culture generates stress conditions that may cause variations of cytoplasmic or nuclear origin that may be stable and hereditary (somaclonal variation) or nontransmissible (epigenetic changes). In this paper, differences in agronomic performance between *in vitro* regenerated plants and those derived from seeds were observed. Extent and type of variation depended on the genotype being tested.

Differences in agronomic performance observed for the Jalapeño and Serrano types favored the regenerated plants. Conversely, seed-derived plants of Habanero displayed better agronomic characteristics. The micropropagated plants of the Piquin chili did not present any differences from those derived from seeds. The less well-adapted nature of Piquin chili may account for the lack of difference between micropropagated Piquin and chilis derived from seeds. This characteristic provides a wider genetic base than that available to the Jalapeño, Serrano, and Habanero types that have been subject to intense selection and breeding programs. This ample genetic variability creates the variation observed for this chili that ultimately may mask the positive effects of the *in vitro* culture on the agronomic performance of the regenerated plants.

Another factor that could have affected the agronomic performance of the regenerated plants was the number of shoots per explant, since Habanero, Serrano, and Jalapeño produced up to 14.6, 8.2, and 7.1 shoots, respectively. Because the Habanero chili had the highest number of shoots per explant, those shoots were exposed to a more intense competition for space and nutrients and more ethylene accumulation in their growing microenvironment. This could result in plants with less vigor when transferred to the greenhouse. A similar response was observed in *Paulonia elongata*, in which acclimated plantlets regenerated from explants with numerous shoots had less leaf area, dry weight, and vigor than those regenerated from explants with only a few shoots (Castillo-Martínez 2007).

On the other hand, a reinvigorating effect has also been observed in plants regenerated from *in vitro* conditions and may explain why the recovered plants of the Jalapeño and Serrano types showed better development than plants

derived from seeds. This effect may be due to changes observed in the structure and function of the chloroplasts during *in vitro* culture that cause a reduction in the synthesis of abscisic acid (ABA). Gibberellins and cytokinins in the culture medium may also generate morphological and functional alterations in vegetative and floral processes. These alterations have not yet been sufficiently studied in relation to *in vitro* rejuvenation (López-Encinas 2007).

The improved agronomic traits displayed in this study by some of the regenerated chili plants may confer advantages to this crop and, therefore, could be useful for genetic-breeding programs. The variation generated during *in vitro* culture has been successfully applied to the improvement of potatoes, sugar cane, maize, tomatoes, celery, rice, bananas, wheat, and alfalfa, where traits such as disease-, drought-, and salt-tolerance and higher production of tryptophan, herbicide resistance, and higher yield have been conferred (Amzad et al. 2003; Cardone et al. 2004).

To date, it is unknown whether the differences observed in the regenerated plants (R_0; Fig. 1*g*) of these cultivars have heritable bases; therefore, it is important to conduct further research focused on determining whether these differences are maintained in the progeny (R_1) or if they are only epigenetic changes.

Conclusions

An efficient and reliable protocol through direct organogenesis was developed for the *in vitro* plant regeneration of different *Capsicum* species. Efficiencies of plant regeneration depended on genotype, culture media, and explant source under study. The regenerated plants were ready to be transferred to the greenhouse within 13–20 wk. Differences were observed in the physiology of *in vitro* regenerated plants and their counterparts derived from seeds, with micropropagated plants of the Serrano and Jalapeño types displaying improved agronomic performance. Incorporation of some of these favorable traits into breeding programs of chili will depend on a future evaluation on the genetic bases of these alterations.

Acknowledgement The authors would like to express their gratitude to The National Council for Science and Technology (CONACYT, Mexico) for the scholarship awarded to M.G.V.B. (number 184879).

References

Agrawal S.; Chandra N.; Kothari S.-L. Plant regeneration in tissue cultures of pepper (*Capsicum annuum* L. cv. Mathania). *Plant Cell Tiss. Org. Cult*. 16: 47–55; 1989. doi:10.1007/BF00044071.

Aguado-Santacruz G. A.; Martínez-Castillo A.; Moreno-Gómez B.; Peña-Mosqueda D. Cultivo de tejidos vegetales. In: Aguado-Santacruz G. A. (ed) XVI Jornada de ingeniería bioquímica. INIFAP, Unidad de Biotecnología-Instituto Tecnológico de Celaya, Celaya, Gto. Mex, p 51 p; 2004.

Amzad H.-M.; Konisho K.; Minami M.; Nemoto K. Somaclonal variation of regenerated plants in chili pepper (*Capsicum annuum* L). *Euphytica* 130: 233–239; 2003. doi:10.1023/A:1022856725794.

Basra A. S. Seed quality; basic mechanisms and agricultural implications. Food Products, New York1995.

Binzel M.-L.; Sankhla N.; Joshi S.; Sankhla D. *In vitro* regeneration in chili pepper (*Capsicum annuum* L.) from 'half-seed explants'. *Plant Growth Regul.* 20: 287–293; 1996. doi:10.1007/BF00043320.

Cárdenas-Avila M.-L.; Verde-Star J.; Villarreal J.; Valadés M.-C.; Maiti R.-K.; Morales M.-R *In vitro* tissue culture of wild chili «chile piquín» (*Capsicum annuum* L. var. *aviculare* (Dierb.) D'Arcy & Eshbaugh): an alternative method for propagation. *Phyton* 60: 99–102; 1997.

Cardone S.; Olmos S.; Echenique V Métodos para generar variabilidad. In: Echenique V.; Rubinstein C.; Mroginski L. (eds) Biotecnología y mejoramiento vegetal. INTA, Argentina, pp 81–96; 2004.

Castillo-Martínez, C.-R. Transformación genética de *Paulonia elongata* mediada por *Agrobacterium tumefaciens* y por biobalistica. Ph. D. thesis, Colegio de Postgraduados, México; 2007.

Dabauza M.; Peña L High efficiency organogenesis in sweet pepper (*Capsicum annuum* L.) tissues from different seedling explants. *Plant Growth Regul.* 33: 221–229; 2001. doi:10.1023/A:1017585407870.

Deepu M.; Doijode S.-D.; Reddy K.-M Correlation and path coefficient analysis in five species of *Capsicum*. *Capsicum Eggplant Newsl.* 23: 57–60; 2004.

Estrada-Luna A.-A.; Davies F.-T.; Egilla J.-N Physiological changes and growth of micropropagated chile ancho pepper plantlets during acclimatization and post-acclimatization. *Plant Cell Tiss. Org. Cult.* 66: 17–24; 2001. doi:10.1023/A:1010606430060.

Ezura H Micropropagation of *Capsicum* species (pepper). In: Bajaj Y. P. S. (ed) Biotechnology in agriculture and forestry high-tech and micropropagation, vol 39. Springer, Heidelberg, pp 48–59; 1997.

FAOSTAT. Food and Agriculture Organization of the United Nations. Database results. http://faostat.fao.org. Cited 18 Jun 2008; 2006.

Hernández-Verdugo S.; Guevara-González R.-G.; Rivera-Bustamante R.-F.; Vázquez-Yanez C.; Oyama K Los parientes silvestres del chile (*Capsicum* spp.) como recursos genéticos. *Bol. Soc. Bot.* 62: 171–181; 1998.

Husain S.; Jain A.; Kothari S.-L. Phenylacetic acid improves bud elongation and *in vitro* plant regeneration efficiency in *Capsicum annuum* L. *Plant Cell Rep.* 19: 64–68; 1999. doi:10.1007/s002990050711.

Joshi A.; Kothari S.-L High copper levels in the medium improves shoot bud differentiation and elongation from the cultured cotyledons of *Capsicum annuum* L. *Plant Cell Tiss. Org. Cult.* 88: 127–133; 2007. doi:10.1007/s11240-006-9171-6.

Kaparakis G.; Alderson P.-G Differential callus type formation by auxins and cytokinin in *in vitro* cultures of pepper (*Capsicum annuum* L.). *Plant BioSyst.* 137: 275–280; 2003. doi:10.1080/11263500312331351521.

Kelly A. F Seed production of agricultural crops. Longman Scientific and Technical. Wiley, New York1988.

Kintzios S.; Drossopoulos J.-B.; Lymperopoulos C.-H Effect of vitamins and inorganic micronutrients on callus growth and somatic embryogenesis from leaves of chilli pepper. *Plant Cell Tiss. Org. Cult.* 67: 55–62; 2001. doi:10.1023/A:1011610413177.

Kumar B.-K.; Munshi A.-D.; Joshi S.; Kaur C.-H Correlation and path coefficient analysis for yield and biochemical characters in chilli (*Capsicum annuum* L.). *Capsicum Eggplant Newsl.* 22: 67–70; 2003.

Kumar V.; Gururaj H.-B.; Narasimha B.-C.; Giridhar P.; Ravishankar G.-A. Direct shoot organogenesis on shoot apex from seedling

explants of *Capsicum annuum* L. *Sci. Hort.* 106: 237–246; 2005. doi:10.1016/j.scienta.2005.02.021.

López-Encinas, C. Juvenilidad y rejuvenecimiento de plantas cultivadas *in vitro.* http://www.encuentros.uma.es/encuentros27/27juvenil.html. Cited 13 Jul 2007; 2007.

López-Puc G.; Canto-Flick A.; Barredo-Pool F.; Zapata-Castillo P.; Montalvo-Peniche M.-C.; Barahona-Pérez F.; Santana-Buzzy N Direct somatic embryogenesis: a highly efficient protocol for *in vitro* regeneration of habanero pepper (*Capsicum chinense* jacq.). *Hort. Sci.* 41: 1645–1650; 2006.

Murashige T.; Skoog F A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15: 473–497; 1962. doi:10.1111/j.1399-3054.1962.tb08052.x.

Nandadevi; Hosamani R.-M Variability, correlation and path analysis in kharif-grown chilli (*Capsicum annuum* L.) genotypes for different characters. *Capsicum Eggplant Newsl.* 22: 43–46; 2003.

Nuez F.; Gil-Ortega R.; Costa J. El cultivo de pimientos, chiles y ajíes. Mundi-Prensa, México D.F1996.

Ochoa-Alejo N.; Ireta-Moreno L Cultivar differences in shoot-forming capacity of hypocotyl tissues of chilli pepper (*Capsicum annuum* L.) cultured *in vitro*. *Sci. Hort.* 42: 21–28; 1990. doi:10.1016/0304-4238(90)90144-4.

Peddaboina V.; Thamidala C.; Subhash K Selection of atrazine-resistant plants by *in vitro* mutagenesis in pepper (*Capsicum annuum*). *Plant Cell Tiss. Org. Cult.* 83: 75–82; 2005. doi:10.1007/s11240-005-2678-4.

Pinzón, L. Aspectos fisiológicos, anatómicos y morfológicos de la recalcitrancia del cultivo *in vitro* del chile habanero (*Capsicum chinense* Jacq.). Ph. D. thesis, Colegio de Postgraduados, México; 2007.

Prakash A.-H.; Sankara K.; Kumar M Plant regeneration from protoplasts of *Capsicum annuum* L. cv. California Wonder. *J. Biosci.* 22: 339–344; 1997. doi:10.1007/BF02703236.

Ramírez-Malagón R.; Ochoa-Alejo N An improved and reliable chili pepper (*Capsicum annuum* L.) plant regeneration method. *Plant Cell Rep.* 16: 226–231; 1996. doi:10.1007/BF01890873.

Robledo-Paz A.; Carrillo-Castañeda G Regeneración *in vitro* de plantas de chile (*Capsicum annuum* L) mediante cultivo de cotiledones e hipocótilos. *Rev. Fitotec. Mex.* 27: 121–126; 2004.

Sanatombi K.; Sharma G.-J *In vitro* regeneration and mass multiplication of *Capsicum annuum* L. *Agri. Environ.* 4: 205–208; 2006.

Santana-Buzzy N.; Canto-Flick A.; Barahona-Pérez F.; Montalvo-Peniche M.-C.; Zapata-Castillo P.-Y.; Solís-Ruíz A.; Zaldívar-Collí A.; Gutiérrez-Alonso O.; Miranda-Ham M.-L Regeneration of habanero pepper (*Capsicum chinense* Jacq.) via organogenesis. *Hort. Sci.* 40: 1829–1831; 2005.

Santana-Buzzy N.; Canto-Flick A.; Iglesias-Andreu L.-G.; Montalvo-Peniche M.-C.; López-Puc G.; Barahona-Pérez F Improvement of *in vitro* culturing of habanero pepper by inhibition of ethylene effects. *Hort. Sci.* 41: 405–409; 2006.

Sripichit P.; Nawata E.; Shigenaga S *In vitro* shoot-forming capacity of cotyledon explants in red pepper (*Capsicum annuum* L. cv. Yatsufusa). *Jap. J. Breed* 37: 133–142; 1987.

Steel R.-D.; Torrie J. H. Bioestadistica: principios y procedimientos. McGraw-Hill, México1992.

Steinitz B.; Küsek M.; Tabib Y.; Paran I.; Zelcer A. Pepper (*Capsicum annuum* L.) regenerants obtained by direct somatic embryogenesis fail to develop a shoot. *In Vitro Cell Dev. Biol. Plant* 39: 296–303; 2003. doi:10.1079/IVP2002405.

Valera-Montero L.; Ochoa-Alejo N A novel approach for chili pepper (*Capsicum annuum* L.) plant regeneration: shoot induction in rooted hypocotyls. *Plant Sci.* 84: 215–219; 1992. doi:10.1016/0168-9452(92)90137-B.

Valera-Montero L.; Phillips G.-C Long-lasting *Capsicum baccatum* 'organogenic callus' formation. *In Vitro Cell Dev. Biol. Plant* 41: 470–476; 2005. doi:10.1079/IVP2005648.