Regeneration of Habanero Pepper (*Capsicum chinense* Jacq.) Via Organogenesis

Nancy Santana-Buzzy,¹ Adriana Canto-Flick, Felipe Barahona-Pérez, María del Carmen Montalvo-Peniche, Patricia Yolanda Zapata-Castillo, Anabel Solís-Ruiz, Amílcar Zaldívar-Collí, Omar Gutiérrez-Alonso, and María de Lourdes Miranda-Ham

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 # 130, Chuburná de Hidalgo, Mérida, Yucatán, 97200 México

Additional index words. *Capsicum chinense* Jacq., chili pepper, ethylene, regeneration, thidiazuron, TDZ

Abstract. To induce multiple shoots from habanero pepper (*Capsicum chinense* Jacq.), nodes and stem segments were cultivated in MS medium supplemented with varying concentrations of kinetin, benzyladenine, and thidiazuron. The effect of the age of the explant in the medium on shoot formation and their latter development into plants was assessed. Ethylene concentration was measured along the experiments. Thidiazuron was the key growth regulator in the process, which at 3.4 μM induced seven to eight shoots that developed into healthy plants per explant. Plantlets in nonventilated vessels, where ethylene concentration was $0.25 \pm 0.1102 \mu\text{L}\cdot\text{L}^{-1}$, showed early defoliation and the formation of calli on the leaves and stems.

All chili peppers belong to the genus *Capsicum* of the Solanaceae family. There are 27 species of *Capsicum*, yet only five have been domesticated and are currently cultivated: *C. annum* Linné, *C. baccatum* Linné, *C. chinense* Jacquin, *C. frutescens* Linné, and *C. pubescens* Ruiz & Pavon (Ochoa-Alejo and Ramírez-Malagón, 2001).

Although excellent progress has been made in obtaining transgenic plants from many species of the Solanaceae family, chili pepper has lagged behind, most likely due to unavailability of an efficient regeneration protocol (Ebida and Hu, 1993; Liu et al., 1990).

In vitro plant regeneration from cells, tissues, and organ cultures is a fundamental process for the application of plant biotechnology to plant propagation, plant breeding and genetic improvement. Despite the economic importance of chili peppers, plant regeneration systems for *Capsicum* species have not progressed as fast as those for some other solanaceous crops. In the case of *Capsicum* species, they have shown to be difficult with regard to regeneration of whole plants from explants and other tissues, which might be a clear indication of severe recalcitrant morphogenic problems (Ochoa-Alejo and Ramírez-Malagón, 2001).

In *Capsicum annum*, several regeneration protocols based on organogenesis have been described (Agrawal et al., 1989; Binzel et al., 1996; Buyukalaca and Mavituna, 1996; Fári and Andrásfalvy, 1994; Ochoa-Alejo

and Ireta-Moreno, 1990; Valera-Montero and Ochoa-Alejo, 1992). Only two reports regarding regeneration through somatic embryogenesis have been published for *C. annum* (Binzel et al., 1996; Buyukalaca and Mavituna, 1996), whereas there are none for the regeneration of habanero pepper (*C. chinense* Jacq.).

The present report describes the first protocol for efficient micropropagation of habanero pepper (*Capsicum chinense* Jacq.). The use of an ideal explant (nodes) to propagate plants, along with the application of thidiazuron for the induction of multiple shoots, and the maintenance of an adequate gas exchange in the culture vessels, are critical factors for the efficiency of the propagation protocol.

Materials and Methods

In vitro germination of habanero pepper seeds. Seeds from habanero pepper (*C. chinense* Jacq.) 'Orange' were obtained from the INIFAP (Uxmal) pepper germplasm bank. Seeds were desinfested in 70% ethanol, followed by three to four rinses with sterile distilled water. Then, they were submerged in diluted 30% (v/v) commercial bleach for 15 minutes and rinsed four to five times with sterile distilled water. They were put in jars with 20 mL of MS medium (Murashige and Skoog, 1962), supplemented with 29.64 μM thiamine, 554.93 μM myo-inositol, 0.22% (w/v) gelrite, 1.3 μM GA₃ and 3% (w/v) sucrose, pH 5.6. The jars were covered with propylene lids and incubated at $25 \pm 2^\circ\text{C}$ under continuous light (40 to 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Upon germination, plantlets were transferred to the same MS medium, but without growth regulators, to grow.

Shoot induction. Nodes from 25 to 30-d-old

plants were used as explants for the induction. They were placed on either complete or half ionic strength MS medium, both supplemented with 29.64 μM thiamine, 554.93 μM myo-inositol, 0.22% (w/v) gelrite and 3% (w/v) sucrose. The effect of the growth regulators benzylaminopurine (BAP): 15.28 to 30.56 μM , kinetin (KIN): 15.89 to 31.78 μM , and thidiazuron (TDZ): 1.1 to 4.5 μM , was evaluated independently.

To evaluate the effect of age on shoot induction, nodes and stem sections (with three to four axillary buds) from 21, 63, and 84-d-old plants were put on the culture media (MS medium, supplemented with 29.64 μM thiamine, 554.93 μM myo-inositol, 3% (w/v) sucrose, 0.22% (w/v) gelrite, and 3.4 μM TDZ, pH 5.6). Ten explants were used per treatment, with three independent repetitions. The jars were maintained at $25 \pm 2^\circ\text{C}$ under continuous light (40 to 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

The percentage of shoot-forming explants and the number of shoots per explant were recorded after 21 d of culture on the induction medium.

Effect of ethylene on the in vitro development of habanero pepper plantlets. To determine the effect of ethylene, plantlets were cultured in Magenta vessels with or without ventilation. In the case of those with ventilation, a hole (1 cm in diameter) was drilled in the propylene closure, which was then covered with a 3 cm filter paper disk (medium pore), held in place with silicone. Plantlets (0.8 to 1 cm tall) were placed in MS medium, supplemented with 29.64 μM thiamine, 554.93 μM myo-inositol, 0.22% (w/v) gelrite and 3% (w/v) sucrose, without growth regulators for 40 d. Ethylene concentration inside the vessels was determined using a HP8690 gas chromatographer (Series II), with a FID detector. The samples were analyzed with a GS-Q FSOT column (300 $\times$ 0.53 mm, Alltech), at 70 $^\circ\text{C}$ and a carrier gas flow of 10 $\text{mL}\cdot\text{min}^{-1}$. For each treatment, six independent vessels (four plantlets per vessel) were used. They were maintained at $25 \pm 2^\circ\text{C}$ in continuous light (40 to 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

Data analysis. Data were analyzed by an analysis of variance (ANOVA), with STATGRAPHICS 4.1 (Windows).

Results

Shoot induction. The number of shoots per explant was very similar when using complete or half ionic strength MS media (Table 1). Nevertheless, the reduction of salts induced shoots with a rosette morphology, that would not fully develop and finally, necrose. Explants in MS medium without regulators formed shoots and calli (Fig. 1a), probably due to the interaction between the explants and the conditions prevailing inside the culture vessels, in particular, the high concentration of ethylene.

Shoot from nodes in the presence of BAP or KIN presented abnormal leaves and could not elongate. There was a diminished response to these growth regulators (Table 1), both in the number of explants forming shoots and shoots per explant (Fig. 1b). Noteworthy was the result with complete MS medium supplemented with 31.78 μM KIN, that resulted in three to four

Received for publication 24 Feb. 2005. Accepted for publication 4 June 2005. The authors wish to thank CONACyT (Fondo Mixto Yucatán) and Fundación Produce-Yucatán for funding our research.

¹To whom reprint requests should be addressed; e-mail buzzy@cicy.mx.

Table 1. Effect of the media ionic strength and the addition of BAP and KIN on shoot induction in habanero pepper (*Capsicum chinense* Jacq.).

Ionic strength	Medium	Concn (μM)	Shoot-forming explants (%)	Shoots/explant
Full	BAP	15.28	39	1
		19.10	35	1–2
		30.56	44	1–2
	Kin	15.89	44	1
		19.86	51	1–2
Half-strength	BAP	31.78	67	3–4
		15.28	25	---
		19.10	42	1–2
	Kin	30.56	25	1–2
		15.89	42	1
		19.86	25	1–2
		31.78	33	1–2

shoots per explant. These shoots grew slowly, could not elongate, and had a pale green color (Fig. 1c and d). In the margins of the explant, where cuttings were made, a whitish, compact calli formed and grew abundantly.

A better response to TDZ was observed for nodes, rather than with stem sections (Table 2). The chosen TDZ concentration for multiple shoot formation was $3.4 \mu\text{M}$, which yielded seven to eight shoots per explant after 2 weeks. These shoots presented dark green leaves, a

uniform and rapid growth rate and enhanced vigor (Fig. 1e). Once shoots were isolated, they developed into normal plants with healthy roots (Fig. 1f). Stem explants were not as successful in forming shoots under any of the TDZ assayed concentrations (Table 2). The results were similar to those of nodes in MS media containing BAP or KIN.

The best response in shoot formation was observed with 21-d-old nodes or stem sections. In older tissues, the response was less marked, both at the frequency of shoot formation and the number of shoots per explant, confirming the fact that the younger the explant is, a better response can be expected, as generally happens in most plant species (Dabauza and Peña, 2001).

Effect of ethylene. In closed containers, where ethylene accumulation was promoted, the emergence of callus tissue could be observed in the upper side of the leaves and along the stems of plantlets grown in closed containers (Fig. 2a and b). Furthermore, they presented etiolated stems and intense leaf chlorosis, that led to an early fall of leaves, showing a clear senescence process (Fig. 2c). When the elongated shoot buds were kept for three months under these conditions, the plantlets flowered inside the culture vessel (Fig. 2d).

In contrast, shoots and plants grown in ventilated containers showed a normal development pattern, they were vigorous and presented dark green leaves (Fig. 2e and f). There was also a marked difference in the average number of leaves (13.0 vs. 6.5), as well as in plantlets' height (4.916 vs. 5.625 cm), between plantlets kept in ventilated vessels and those in the nonventilated ones, respectively. A similar tendency was observed for fresh weight (0.7 vs. 0.27 g) and dry weight (0.2 vs. 0.06 g), in plantlets' under ventilated conditions, compared to those in closed containers. Ethylene concentration inside the closed vessels was $0.25 \pm 0.1102 \mu\text{L}\cdot\text{L}^{-1}$ ($p = 0.0000761$), while it was below the detection limit in the ventilated ones. Ethylene is a plant grow regulator that is known to influence in vitro morphogenesis. The deleterious effects of the presence of ethylene in closed containers has been demonstrated in other species, such as *Tagetes erecta* (Santamaría et al., 1996).

Discussion

TDZ has showed strong cytokinin-like activity in various assays (Mok et al., 1982). Furthermore, TDZ proved to be very efficient

in stimulating cytokinin-dependent shoot regeneration in a wide variety of plants (Dabauza and Peña, 2001; Huettelman and Preece, 1993; Malik and Saxena, 1992). Szász et al. (1995) reported the use of TDZ in the direct regeneration of pepper plants, using different genotypes of *Capsicum annuum* L. A high number of formed shoots was reported by Dabauza and Peña (2001), working with eight sweet pepper varieties (*Capsicum annuum* L.). Nevertheless, our results demonstrate that there are significant differences in the behaviour of other *Capsicum* species, particularly regarding to their sensitivity to in vitro growing conditions.

This is the first report of the in vitro regeneration of habanero pepper (*Capsicum chinense* Jacq.) plants. The addition of TDZ at a concentration of $3.4 \mu\text{M}$ resulted in vigorous shoots, that presented a normal morphology and a dark green coloration. Once isolated, they continued their normal developmental program. This growth regulator has been successfully employed for the regeneration and elongation in other pepper cultivars (Dabauza and Peña, 2001), but it is the first time it has been used for this purpose in *Capsicum chinense*. Neither BAP, nor KIN in the assayed concentrations were suitable for the induction of shoots. Under these conditions, the shoots tended to form rosettes, with chlorotic leaves, that finally necrose. Most published works on *Capsicum annuum* shoot induction have used a combination of IAA and BA (Arroyo and Revilla, 1991; Christopher and Rajam, 1996; Fárri and Czakó, 1981; Ochoa-Alejo and Ireta-Moreno, 1990). Preliminary experiments to evaluate several auxin-cytokinin combinations resulted on the induction of only one shoot per explant, and in the injured edges of the explants, there were the formation of white callus and small buds.

Habanero pepper plants showed a high sensitivity to ethylene present inside the containers, which could be observed as the persistent formation of whitish callus in the plantlets' surface, evident chlorosis and early abscission of the leaves, along with a rapid loss of vigor, even when growth regulators were absent from the media. However, when the explants were kept in ventilated vessels, the regeneration and development of habanero pepper plantlets proceeded normally.

Numerous reports show that the regeneration of pepper (*Capsicum annuum* L.) plants via organogenesis is severely limited, since in many cultivars the shoot buds either do not elongate or produce aberrant, ill-defined, distorted leaves (Arroyo and Revilla, 1991; Ebida and Hu, 1993; Ochoa-Alejo and Ireta-Moreno, 1990; Phillips and Hubstenberger, 1985). According to Binzel et al. (1996), the major problem in regenerating *Capsicum annuum* plants is not shoot bud induction, but shoot bud elongation.

This protocol has been developed for the regeneration of habanero pepper plants, which can be used for the clonal multiplication of the germplasm that has been kept in vitro, and also, to multiply any other genotype that could be promising for the genetic improvement of this species.

Fig. 1. Shoot induction in habanero pepper. Aspect of the shoots in media without growth regulators (a), with $30.56 \mu\text{M}$ BAP (b), $31.78 \mu\text{M}$ kinetin (c and d) or $3.4 \mu\text{M}$ TDZ (e). Aspect of the development of shoots into normal, whole plants (f).

Table 2. Effect of TDZ on shoot induction from stem sections and nodes of *Capsicum chinense* Jacq.

Explant	Concn (μ M)	Shoot-forming explants (%)	Shoots/explant
Stem sections	0	38	1
	1.1	25	1
	2.3	25	1–2
	3.4	25	1–2
	4.5	38	1–2
Node	0	50	1–2
	1.1	50	2–3
	2.3	55	3–4
	3.4	75	7–8
	4.5	65	4–5

Fig. 2. Effect of ethylene on habanero pepper plants. Aspect of the calli formed on the plantlets' leaves (a) and stems (b) in nonventilated vessels. Intense chlorosis and early defoliation in nonventilated vessels (c). In vitro flowering of the habanero pepper plants in a nonventilated vessel (d). In vitro plants from *C. chinense* nodes in ventilated vessels (e and f).

Literature Cited

- Agrawal, S., N. Chandra, and S.L. Kothary. 1989. Plant regeneration in tissue cultures of pepper (*Capsicum annuum* L. cv. Mathania). *Plant Cell Tiss. Org.* 16:47–55.
- Arroyo, R., and M.A. Revilla. 1991. *In vitro* plant regeneration from cotyledon and hypocotyl segments in two bell pepper cultivars. *Plant Cell Rpt.* 10:414–416.
- Binzel, M.L., N. Sankhla, S. Joshi, and D. Sankhla. 1996. *In vitro* regeneration in chili pepper (*Capsicum annuum* L.) from "half-seed explants". *Plant Growth Regulat.* 20:287–293.
- Buyukalaca, S. and F. Mavituna. 1996. Somatic embryogenesis and plant regeneration of pepper in liquid media. *Plant Cell Tiss. Org.* 46:227–235.
- Christopher, T. and M.V. Rajam. 1996. Effect of genotype, explant and medium on *in vitro* regeneration of red pepper. *Plant Cell Tiss. Org.* 46:245–250.
- Dabauza, M. and L. Peña. 2001. High efficiency organogenesis in sweet pepper (*Capsicum annuum* L.) tissues from different seedling explants. *Plant Growth Regulat.* 33:221–229.
- Ebida, A.I.A. and C.Y. Hu. 1993. *In vitro* morphogenetic responses and plant regeneration from pepper (*Capsicum annuum* L. cv. Early California Wonder) seedling explants. *Plant Cell Rpt.* 13:107–110.
- Fári, M. and A. Andrásfalvy. 1994. Regeneration and cloning of pepper (*Capsicum* sp.) *in vitro*: A review. *Hort. Sci. (Hungary)* 26:9–18.
- Fári, M. and M. Czákó. 1981. Relationship between position and morphogenetic response of pepper hypocotyl explant cultured *in vitro*. *Sci. Hort.* 15:207–213.
- Huetteman, C.A. and J.E. Preece. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tiss. Org.* 33:105–119.
- Liu, W., W.A. Parrot, D.F. Hildebrand, G.B. Collins, and E.G. Williams. 1990. *Agrobacterium* induced gall formation in bell pepper (*Capsicum annuum* L.) and formation of shoot-like structures expressing introduced genes. *Plant Cell Rpt.* 9:360–364.
- Malik, A.K. and P.K. Saxena. 1992. Regeneration in *Phaseolus vulgaris* L.: High-frequency induction of direct shoot formation in intact seedlings by N⁶-benzylaminopurine. *Planta* 186:384–389.
- Mok, M.C., D.W.S. Mok, D.J. Armstrong, K. Shudo, Y. Isogai, and T. Okamoto. 1982. Cytokinin activity of N-phenyl-N'-1,2,3-thiazol-5-yl urea (thidiazuron). *Phytochemistry* 21:1509–1511.
- Murashige, T. and F.A. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plantarum* 15:437.
- Ochoa-Alejo, N. and L. Ireta-Moreno. 1990. Cultivar differences in shoot-forming capacity of hypocotyl tissues of chili pepper (*Capsicum annuum* L.) cultured *in vitro*. *Sci. Hort.* 42:21–28.
- Ochoa-Alejo, N. and R. Ramírez-Malagón. 2001. *In vitro* chili pepper biotechnology. *In vitro Cell Dev.-PL* 37:701–729.
- Phillips, G.C. and J.F. Hubstenberger. 1985. Organogenesis in pepper tissue cultures. *Plant Cell Tiss. Org.* 4:261–269.
- Szász, A., G. Nervo, and M. Fari. 1995. Screening for *in vitro* shoot-forming capacity of seedling explants in bell pepper (*Capsicum annuum* L.) genotypes and efficient plant regeneration using thidiazuron. *Plant Cell Rpt.* 14:666–669.
- Santamaria, J.M., J. Coello, and W.J. Davies. 1996. Is the gaseous environment inside culture vessels responsible for the limited capacity of vitroplants to control water loss? *Plant Physiol.* 111(Suppl.):47.
- Valera-Montero, L.L., and N. Ochoa-Alejo. 1992. A novel approach for chili pepper (*Capsicum annuum* L.) plant regeneration: shoot induction in rooted hypocotyls. *Plant Sci.* 84:215–219.