In Vitro Cell.Dev.Biol.—Plant (2014) 50:471–477
DOI 10.1007/s11627-014-9621-5

MICROPROPAGATION

Effect of immersion cycles on growth, phenolics content, and antioxidant properties of *Castilleja tenuiflora* shoots

Raúl Valdez-Tapia · Jacqueline Capataz-Tafur · Alma Rosa López-Laredo · José Luis Trejo-Espino · Gabriela Trejo-Tapia

Received: 15 October 2013 / Accepted: 15 May 2014 / Published online: 19 June 2014 / Editor: Praveen Saxena

Abstract *Castilleja tenuiflora*, a species highly valued for its medicinal properties, is threatened in the wild. We evaluated the effects of six different immersion cycles in a temporary immersion bioreactor on *C. tenuiflora* shoot growth, proliferation rate, phenolics content, flavonoid content, and antioxidant activity. We also evaluated the regeneration capacity of the shoots. The highest proliferation rate (nine shoots per explant) was obtained using an immersion cycle of 5 min every 12 h, and the longest shoots (38.8±1.9 mm) were obtained using an immersion cycle of 5 min every 24 h. Shoots obtained from immersion cycles of 30 min every 24 h or 5 min every 24 h showed 100% rooting efficiency. Shoots obtained from immersion cycles of 30 min every 3 h or 30 min every 12 h accumulated H_2O_2, developed abnormal stomata, and showed symptoms of hyperhydricity. These characteristics were associated with a low survival rate (16–80%) when the plants were transferred to potting mix. The shoots from an immersion cycle of 30 min every 24 h showed the highest total phenolics content, which coincided with the highest antioxidant activity in the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) free-radical scavenging assay (161.74±10.06 μmol Trolox/g dry weight (DW)). The shoots from an immersion cycle of 5 min every 24 h showed the highest activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay, and those from an immersion cycle of 5 min every 3 h showed the strongest reducing power. These results show that temporary immersion culture represents a reliable and efficient method for *in vitro* micropropagation of *C. tenuiflora*.

Keywords Germplasm conservation · Flavonoids · Free-radical scavenging · Reducing power

R. Valdez-Tapia · A. R. López-Laredo · J. L. Trejo-Espino · G. Trejo-Tapia (✉)
Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional (CEPROBI-IPN), Carr. Yautepec-Jojutla Km 6, Calle CEPROBI No. 8, Col. San Isidro, 62730 Yautepec, Morelos, Mexico
e-mail: gttapia@ipn.mx

J. Capataz-Tafur
Universidad del Papaloapan, C. Circuito Central No. 200, Col. Parque Industrial, 68301 Tuxtepec, Oaxaca, Mexico

Introduction

Castilleja tenuiflora Benth. (Orobanchaceae), commonly known as "Indian paintbrush," is a perennial hemiparasitic plant distributed in mountainous areas of the southern USA and Mexico (Holmgren 1976). It has long been used in folk medicine (Béjar *et al.* 2000), and it is still used in both rural and urban regions to treat the symptoms of various cancers (Alonso-Castro *et al.* 2011) and to treat coughs, inflammation, and gastrointestinal disorders (Biblioteca Digital de la Medicina Tradicional Mexicana 2011). The traditional uses of *C. tenuiflora* may be related to the biological activities of its secondary products, including iridoid glycosides, phenylethanoid glycosides (Gómez-Aguirre *et al.* 2012), and flavonoids (López-Laredo *et al.* 2012).

Wild populations of *C. tenuiflora* are vulnerable to the indiscriminate commercial harvesting of this species. Also, it is available only seasonally, and falling trees and fires often affect its habitat. To avoid possible loss of this important plant and to obtain a permanent source of its secondary metabolites, we have established efficient procedures to propagate *C. tenuiflora* in semisolid cultures *in vitro* (Salcedo-Morales *et al.* 2009; Martínez-Bonfil *et al.* 2011) and to acclimatize the plants propagated in culture (Martínez-Bonfil *et al.* 2011). However, despite having a successful micropropagation protocol, commercial-scale implementation of these procedures remains limited by the high production costs. A promising

alternative is the use of temporary immersion culture (Etienne and Berthouly 2002; Ashraf *et al.* 2013). Temporary immersion culture combines the positive effects of aeration with liquid culture medium, thereby stimulating shoot proliferation and growth (Etienne and Berthouly 2002). This method avoids problems such as asphyxia and hyperhydricity, so the plant materials produced are often of better quality than those produced by conventional culture systems, *i.e.*, on semisolid media (Sreedhar *et al.* 2009). This method is also useful for basic research on plant physiology (Michoux *et al.* 2013).

The main factors influencing the performance of a given plant species in temporary immersion cultures are the immersion time and frequency. These factors need to be optimized for different genotypes and applications (Albarran *et al.* 2005; Ivanov *et al.* 2011; Zhao *et al.* 2012; Ashraf *et al.* 2013). The aim of this study was to evaluate the effect of immersion cycles on the growth, proliferation rate, phenolics contents, and antioxidant properties of *C. tenuiflora* shoots cultured in temporary immersion bioreactors, as well as their effects on the ability of the shoots to regenerate into whole plants.

Materials and Methods

Plant material. Shoot cultures of *C. tenuiflora* were initiated and propagated *in vitro* as described previously (Trejo-Tapia *et al.* 2012). Shoots were subcultured in 200-ml jars (Fig. 1*A*), with a 6-cm-diameter mouth that was sealed with a polypropylene closure. Each jar contained 30 ml of B5 culture medium (Gamborg *et al.* 1968) containing 3% (*w*/*v*) sucrose and no plant growth regulators (PGRs). Cultures were maintained under continuous agitation at 110 rpm on an orbital shaker in a growth room at 25±2 °C under a 16-h light/8-h dark photoperiod with illumination of 103 μmol/m^2/s provided by 75-W cool-white fluorescent lamps (SL5938, Osram, Tultitlan, México).

Temporary immersion culture. The 3-wk-old *in vitro* shoots of *C. tenuiflora* were cultured in RITA® (Sigma-Aldrich, St. Louis, MO) temporary immersion bioreactors with 200 ml of B5 culture medium (Gamborg *et al.* 1968) supplemented with 3% sucrose (*w*/*v*) without PGRs. In each bioreactor, the flow rate of the inlet air was 1 l/min. We evaluated the effects of immersion time (30 or 5 min) and immersion frequency (every 3, 12, or 24 h) in six different immersion cycle (IC) treatments as follows: IC-1, 30 min every 3 h; IC-2, 30 min every 12 h; IC-3, 30 min every 24 h; IC-4, 5 min every 3 h; IC-5, 5 min every 12 h; and IC-6, 5 min every 24 h. Each IC treatment consisted of five replicates of 40 shoots (2.5 cm in length; Fig. 1*B*), giving a total of 200 initial shoots per IC treatment. Cultures were maintained in a growth chamber under the conditions described above. At the end of the 3-wk *in vitro* culture period, all of the shoots were counted and the proliferation rate was calculated as follows: (number of shoots and buds at the end of culture period)/(number of shoots inoculated). We also evaluated the following morphological characteristics: shoot length (mm), hyperhydricity (%), fresh mass (g), and rooting rate (%). The experiments were performed three times.

Anatomical analysis and subcellular localization of H_2O_2. To visualize the subcellular location of H_2O_2, we used a histochemical method based on the generation of an insoluble brown precipitate after the reaction of 3,3-diaminobenzidine (DAB) with H_2O_2 (Thordal-Christensen *et al.* 1997). Leaves from normal and hyperhydric shoots were cut and immersed in DAB solution (1 mg/ml DAB dissolved in deionized water, pH 3.8). Samples were incubated for 8 h at 25 °C, cleared in boiling ethanol (96%) for 10 min, and mounted on slides. The presence of H_2O_2 is indicated by a reddish-brown color. Samples were observed (×40) and images were acquired under a light microscope (Eclipse 80I, Nikon, Tokyo, Japan) equipped with a digital camera (DC330, Dage-MTI, Tokyo, Japan).

Rooting and plant acclimatization. Uniform shoots/young plants with 6–8 expanded leaves (3.0 cm long) were selected from each IC tested and transferred to pots (10×7.5 cm; Fig. 1*D*) filled with a sterilized mixture of peat moss, agrolite (agroLITA, State of Mexico, Mexico), and vermiculite (60:20:20 *v*/*v*/*v*) adjusted to pH 5.8±0.3 and moistened with water (40 ml). The pots were covered individually with plastic foil (12.5×20 cm) for 3 wk to maintain a relative humidity of ≥95% and kept under the conditions described by Martínez-Bonfil *et al.* (2011). In all cases, the following data were recorded after 35 d: survival (%), plant height, number of roots per shoot, and length of the longest root. These experiments were performed three times, and 50 shoots from each IC were cultured each time.

Quantification of total phenolics and flavonoids. Plant material was freeze-dried and ground into a fine powder (particle size <250 μm) using a mortar and pestle. Phenolics and flavonoids were extracted from 100-mg lyophilized tissue in 100-ml methanol, as described previously (López-Laredo *et al.* 2012). The total phenolics content was estimated by the Folin–Ciocalteu method, and the flavonoid content was determined by a colorimetric assay as described previously (López-Laredo *et al.* 2009). Standard curves for total phenolics and flavonoids were prepared with gallic acid and catechin, respectively. Total phenolics content is expressed as milligram gallic acid equivalents per gram dry weight (GAE/g DW). Total flavonoid content is expressed as microgram catechin equivalents per gram dry weight (CE/g DW). Three independent samples were analyzed each time.

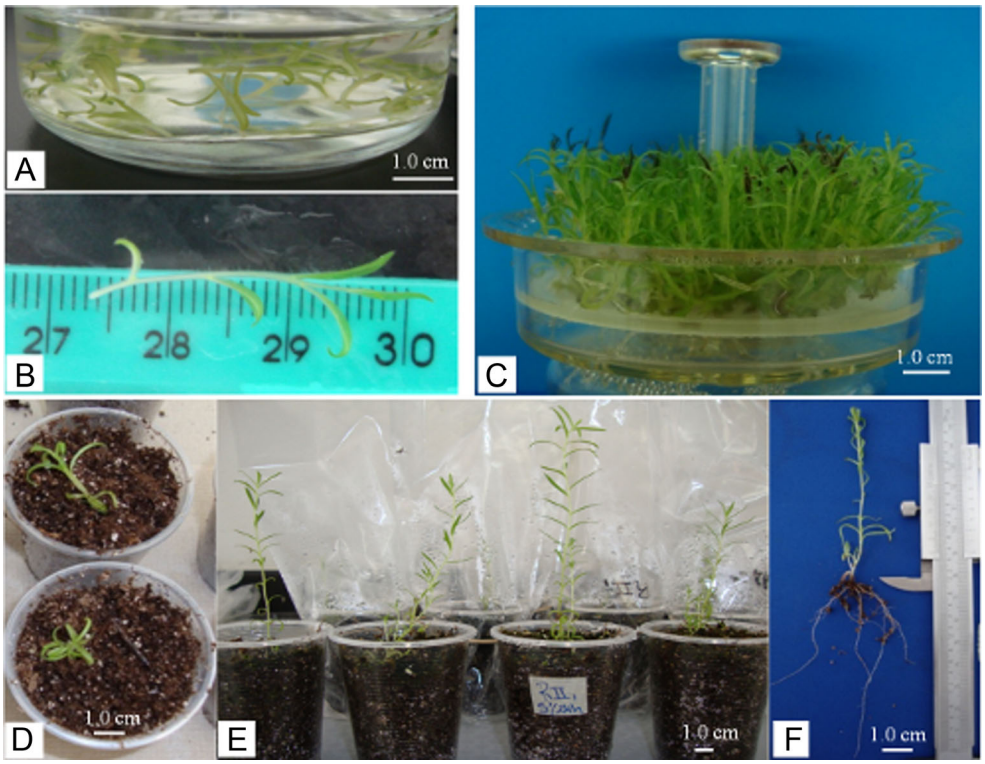

Figure 1. Stages of *C. tenuiflora* plants regenerated in temporary immersion bioreactors (RITA®). (*A*) Shoots cultured in B5 liquid culture medium (Gamborg *et al.* 1968) containing 3% (*w/v*) sucrose and no plant growth regulators. (*B*) Shoot explant. (*C*) Multiple shoot regeneration in RITA® after 3 wk of culture (from treatment IC-5). (*D*) Uniform shoots/young plants transferred to pots filled with a sterilized mixture of peat moss, agrolite, and vermiculite. (*E*) Regenerated plantlets after 3 wk of acclimatization. Third plant from left is from treatment IC-6, which gave the longest shoots. (*F*) Regenerated plant from treatment IC-6, which gave the longest roots.

DPPH and ABTS free-radical scavenging assays. Free-radical scavenging activity was quantified spectrophotometrically using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical scavenging assay as described previously (López-Laredo *et al.* 2009). The results are expressed as micromoles of Trolox per gram DW based on a calibration curve. Three independent samples were analyzed for each assay.

Reducing power assay. Reducing power was quantified by the phosphomolybdenum (PPM) assay as described elsewhere (Prieto *et al.* 1999). The results are expressed as micromoles Trolox per gram DW (Diouf *et al.* 2009) based on a calibration curve. Three independent samples were analyzed.

Statistical analysis. The differences among ICs were tested by two-way ANOVA, and Tukey's all-pairwise multiple comparison procedure was used to determine statistically different values at a significance level of $p<0.05$. Percentage values were arcsine-transformed to obtain normally distributed data. The software SigmaPlot for Windows version 11.0 (Systat Software Inc., San Jose, CA) was used to perform statistical analyses.

Results

Shoot proliferation and morphological characteristics. After 3 wk, the *C. tenuiflora* shoot proliferation rate ranged from six to nine shoots per explant, depending on the IC treatment ($p<0.05$; Table 1). The highest proliferation rate (nine shoots per explant) was in IC-5 (5 min every 12 h; Fig. 1*C*) and the lowest (six shoots per explant) was in IC-3 and IC-6 (30 or 5 min every 24 h, respectively). The longest shoots (38.8± 1.9 mm) were from IC-6, followed by IC-4 and IC-3.

The shoots cultured in immersion bioreactors developed roots from their bases after 2 wk and did not form callus. The highest rooting efficiency (100%) was observed for shoots from IC-3 and IC-6. Shoots from IC-1 and IC-2, which had the longest total daily immersion times (240 and 60 min per day, respectively), showed hyperhydricity at levels ranging from 16 to 40% ($p<0.05$). In hyperhydric shoots, the water content (WC) was 93–96% (Table 1; $p<0.05$). The symptoms of hyperhydricity in shoots were observed after 1 wk of culture, when shoots began to show brittle and translucent leaves with a paler green color than that of normal shoots. Hyperhydric leaves had larger stomata (Fig. 2*B*) than those of normal leaves (Fig. 2*A*). In the histochemical analysis of *C. tenuiflora* leaves, we observed red-brownish polymerization products from DAB reacting with H_2O_2 in hyperhydric leaves (Fig. 2*D*) but not in control leaves (Fig. 2*C*).

Secondary metabolites and antioxidant properties of shoots. The highest contents of total phenolics and flavonoids were in shoots from IC-3 (30-min immersion every 24 h) followed by IC-4 (5-min immersion every 3 h). These shoots also showed among the highest free-radical scavenging and reducing activities (Table 1). Shoots that showed less vigorous

Table 1. Shoot proliferation and length, water content, hyperhydricity, rooting rate, total contents of phenolics, and antioxidant properties of *C. tenuiflora* shoots in a RITA® system with different immersion cycles after 3 wk of culture

Immersion cycle	t_{imm} (min)[z]	F_{imm} (h)[y]	No. shoots/ explant	Shoot length (mm)	WC (%)[x]	H (%)[w]	Rooting rate (%)[v]	Total phenolics content	Total flavonoid content	Free-radical scavenging (μmol Trolox/g DW)		Reducing power (μmol Trolox/g DW)
								(mg GAE/g DW)	(μg CE/g DW)	DPPH	ABTS	
IC-1	30	3	8.0 ± 0.7^{B}	30.0 ± 1.0^{B}	96.0^{A}	40.0^{A}	43.0^{C}	19.01 ± 0.99^{B}	20.64 ± 1.85^{C}	57.79 ± 3.51^{B}	90.58 ± 6.18^{B}	241.58 ± 13.32^{C}
IC-2	30	12	7.6 ± 0.2^{B}	32.4 ± 1.3^{B}	93.8^{A}	16.1^{B}	43.3^{C}	9.61 ± 0.61^{C}	8.81 ± 0.87^{D}	11.86 ± 1.37^{D}	29.58 ± 3.46^{D}	31.25 ± 1.59^{D}
IC-3	30	24	6.0 ± 0.2^{C}	35.0 ± 1.4^{A}	91.5^{B}	0	100^{A}	30.58 ± 2.39^{A}	45.83 ± 4.27^{A}	49.91 ± 1.80^{B}	161.74 ± 10.06^{A}	282.06 ± 17.53^{B}
IC-4	5	3	8.0 ± 0.7^{B}	35.4 ± 1.2^{A}	91.4^{B}	0	50.0^{B}	24.93 ± 1.89^{B}	32.49 ± 4.72^{B}	89.97 ± 9.29^{A}	112.85 ± 7.03^{B}	356.03 ± 24.23^{A}
IC-5	5	12	9.5 ± 0.5^{A}	25.6 ± 0.9^{B}	90.3^{B}	0	36.7^{C}	21.20 ± 1.27^{B}	21.68 ± 1.20^{C}	32.53 ± 14.27^{C}	62.07 ± 17.82^{C}	$42.\ 11\pm8.69^{D}$
IC-6	5	24	6.0 ± 0.4^{C}	38.8 ± 1.9^{A}	90.6^{B}	0	100^{A}	20.21 ± 0.25^{B}	25.43 ± 1.62^{C}	91.62 ± 13.59^{A}	109.23 ± 8.30^{B}	314.14 ± 15.04^{A}

Data represent mean±standard error of five replicates. Values in each *column* followed by different *capital letters* are significantly different at $p<0.05$ by Tukey's multiple range test

ABTS 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, *CE* catechin equivalents, *DPPH* 2,2-diphenyl-1-picrylhydrazyl, *DW* dry weight, *GAE* gallic acid equivalents, *WC* water content, *H* hyperhydricity

[z] Minutes per immersion period

[y] Hours between start times of consecutive immersions

[x] Water content=[(fresh weight−dry weight)/fresh weight]×100

[w] Hyperhydricity=(number of hyperhydric leaves/total number of leaves)×100

[v] Percentage of shoots forming roots

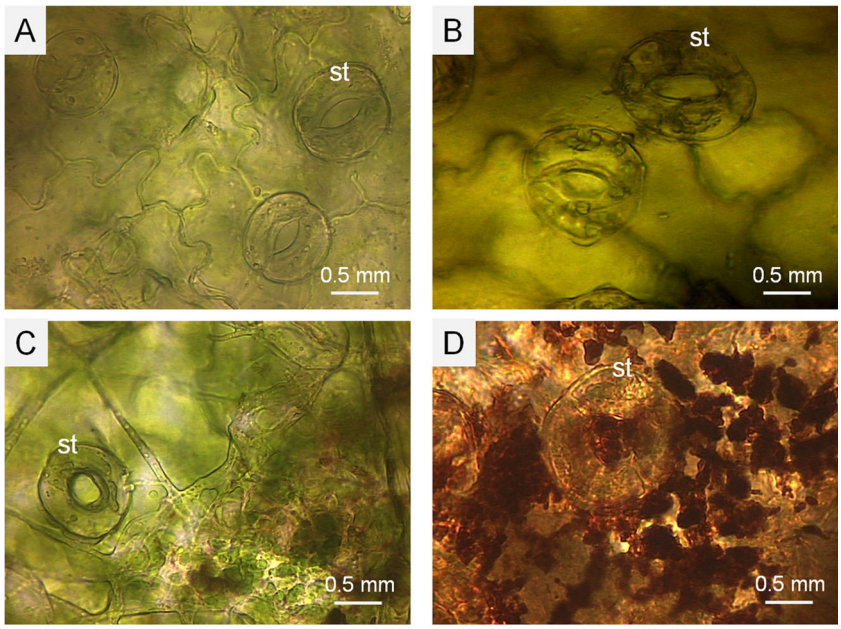

Figure 2. Histochemical analysis of control and hyperhydric leaves of *C. tenuiflora*. (*A*, *B*) Semi-thin sections of a control leaf (*A*) and a hyperhydric leaf showing an altered anatomy (*B*). (*C*, *D*) H_2O_2 accumulation in control leaf (*C*) and hyperhydric leaf (*D*) detected by DAB staining. H_2O_2 is indicated by the reddish-brown color. *st* stoma.

growth and symptoms of hyperhydricity (from IC-1 to IC-2) showed the lowest contents of total phenolics and flavonoids.

Plant characteristics and survival rate. Table 2 shows the characteristics of plants grown in potting mix after different IC treatments. The tallest plants (10.4±0.5 cm; Fig. 1*E*) were those regenerated from shoots from IC-6 (5-min immersion every 24 h). These plants also showed the longest roots (6.0± 0.3 cm; Fig. 1*F*) and were among those with the highest survival rate (100%). Plants regenerated from shoots from ICs 3, 4, and 5 were significantly shorter than those regenerated from shoots from IC-6 ($p<0.05$), ranging from 4.9 to 6.7 cm in height, but they showed high survival rates (100%). As expected, the hyperhydric plants (from IC-1 to IC-2) showed lower survival rates (16–80%).

Discussion

Our results showed that *C. tenuiflora* shoots grown in a temporary immersion bioreactor successfully regenerated into whole plants. A 5-min immersion every 24 h resulted in the production of many vigorous *C. tenuiflora* shoots without symptoms of hyperhydricity. All of the shoots developed under these conditions developed roots, and the regenerated plants showed a 100% survival rate. The temporary immersion bioreactor system improved the morphological characteristics and shoot proliferation rate (nine shoots per explant) of *in vitro C. tenuiflora* shoots, compared with that obtained using the conventional method of propagation on semisolid medium (four shoots per explant; Martínez-Bonfil *et al.* 2011). In the present study, a short immersion time (5 min)

Table 2. Effects of different immersion cycles on characteristics of whole plants regenerated from shoots

Immersion cycle	t_{imm} (min)[z]	F_{imm} (h)[y]	Plant height (cm)	Longest root length (cm)	Survival (%)
IC-1	30	3	4.7 ± 0.3^{C}	3.0 ± 0.3^{C}	16^{C}
IC-2	30	12	5.3 ± 0.5^{C}	4.4 ± 0.4^{C}	80^{B}
IC-3	30	24	4.9 ± 0.2^{C}	3.6 ± 0.3^{C}	100^{A}
IC-4	5	3	6.7 ± 0.4^{B}	4.0 ± 0.4^{C}	100^{A}
IC-5	5	12	6.4 ± 0.6^{B}	5.0 ± 0.4^{B}	100^{A}
IC-6	5	24	10.4 ± 0.5^{A}	6.0 ± 0.3^{A}	100^{A}

Data were collected 5 wk after transplanting shoots/young plants to pots and represent mean±standard error of five replicates (10 plants each). Values in each *column* followed by different *capital letters* are significantly different at $p<0.05$ by Tukey's multiple range test

[z] Minutes per immersion period

[y] Hours between start times of consecutive immersions

every 24 h gave better results than more frequent immersions (every 3 and 12 h) or longer immersions (30 min). The same trend of shorter immersions producing better results has been observed for shoot proliferation of *Saccharum* spp. (Lorenzo *et al.* 1998) and *Ananas comosus* (Escalona *et al.* 1999), while in other cases, longer and more frequent immersions produced better results. For instance, for microtuberization of *Chlorophytum borivilianum*, a 15-min immersion every hour produced better results than did more or less frequent immersions (every 45 or 75 min) (Ashraf *et al.* 2013). For biomass accumulation in *Leucojum aestivum* shoots, the best conditions were a 15-min immersion every 8 h (Ivanov *et al.* 2011).

The shoots of *C. tenuiflora* grown under different immersion cycle treatments showed different rooting efficiencies. Shoots with the least frequent immersion (once every 24 h) showed the highest rooting efficiency (100%). There are other reported cases where roots developed from shoots during immersion culture. For instance, temporary immersion culture stimulated root development from somatic embryos of *Hevea brasiliensis* (Etienne *et al.* 1997) and from shoots of *A. comosus* (Ayenew *et al.* 2013). The conditions used here represent a promising alternative for bypassing the rooting phase, which lasts for 21 d in *C. tenuiflora* (Martínez-Bonfil *et al.* 2011). This enhanced rooting response may have occurred because plantlets growing in immersion culture assimilate more nutrients such as sugars, nitrate, and ammonium than do those growing in conventional conditions (semisolid culture) (Escalona *et al.* 2003). In addition, shoots growing in temporary immersion culture tend to accumulate higher levels of polyamines (Scherer *et al.* 2013) than those in permanent immersion culture. Polyamines have been reported to promote adventitious root formation in *Curcuma longa* (Viu *et al.* 2009) and *Vitis vinifera* (Neves *et al.* 2002) and to reverse hyperhydricity in *Thymus daenensis* (Hassannejad *et al.* 2012).

Among the six immersion cycles tested here, those with the longest total daily immersion times (240 and 60 min per d) resulted in shoots with hyperhydric symptoms. This phenomenon was characterized by an increase in shoot WC, a decreased tendency to form roots, abnormal stomata, and H_2O_2 accumulation. These results are consistent with those reported for *Dianthus caryophyllus*, in which hyperhydric shoots produced more H_2O_2 than did control shoots (Saher *et al.* 2004). Also, in *T. daenensis*, hyperhydricity was characterized by higher WC and lower rates of differentiation (Hassannejad *et al.* 2012). As well as showing morphological changes, hyperhydric *C. tenuiflora* shoots showed the lowest contents of phenolic compounds and flavonoids, and low free-radical scavenging and reducing activities. In *D. caryophyllus*, phenylalanine ammonia-lyase (PAL) activity was significantly lower in hyperhydric tissues than in control tissues (Saher *et al.* 2004). PAL is a key enzyme for the biosynthesis of phenolic compounds, so it is possible that lower PAL activity was responsible for the lower levels of phenolic compounds and flavonoids in hyperhydric *C. tenuiflora* tissues. Together, these results show that longer immersion times resulted in hyperhydricity and oxidative stress in *C. tenuiflora* shoots, leading to lower contents of secondary metabolites and lower survival rates.

Conclusions

The results reported here show that temporary immersion culture is a reliable and efficient methodology for *in vitro* micropropagation of *C. tenuiflora*. The plants produced using this method showed good survival rates.

Acknowledgments This work was supported by the Secretaría de Investigación y Posgrado del IPN-México (SIP–IPN Grants 20131786) and by the Fondo Mixto de Fomento a la Investigación Científica y Tecnológica CONACYT–Gobierno del Estado de Morelos (Grant MOR-2007-C01-79409). The funding sources were not involved in the preparation of this paper or in the decision to submit it for publication. RVT is indebted to the CONACYT and PIFI-IPN for the Master in Sciences fellowship awarded. GTT, JLTE, and ARLL are grateful to the Sistema de Becas por Exclusividad (IPN) and Programa de Estímulos al Desempeño de los Investigadores (IPN).

Conflict of interest The authors have no conflicts of interest to declare.

References

Albarran J, Bertrand B, Lartaud M, Etienne H (2005) Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (*Coffea arabica*) somatic embryos. Plant Cell Tissue Organ Cult 81:27–36

Alonso-Castro AJ, Villarreal ML, Salazar-Olivo LA, Gomez-Sanchez M, Dominguez F, Garcia-Carranca A (2011) Mexican medicinal plants used for cancer treatment: pharmacological, phytochemical and ethnobotanical studies. J Ethnopharmacol 133:945–972

Ashraf MF, Abd Aziz M, Stanslas J, Kadir MA (2013) Optimization of immersion frequency and medium substitution on microtuberization of *Chlorophytum borivilianum* in RITA system on production of saponins. Process Biochem 48:73–77

Ayenew B, Tadesse T, Gebremariam E, Mengesha A, Tefera W (2013) Efficient use of temporary immersion bioreactor (TIB) on pineapple (*Ananas comosus* L.) multiplication and rooting ability. J Microbiol Biotechnol Food Sci 2:2456–2465

Béjar E, Reyes-Chilpa R, Jiménez-Estrada M (2000) Bioactive compounds from selected plants used in the XVI century Mexican traditional medicine. In: Atta-ur-Rahman (ed) Studies in natural products chemistry, vol. 24. Elsevier, Amsterdam, pp 799–844

Biblioteca Digital de la Medicina Tradicional Mexicana (2011) UNAM, México City, México. http://www.medicinatradicionalmexicana.unam.mx/index.php. Cited 5 Feb 2014

Diouf PN, Stevanovic T, Cloutier A (2009) Antioxidant properties and polyphenol contents of trembling aspen bark extracts. Wood Sci Technol 43:457–470

Escalona M, Lorenzo JC, González B, Daquinta M, González JL, Desjardins Y, Borroto CG (1999) Pineapple (*Ananas comosus* L.

Merr) micropropagation in temporary immersion systems. Plant Cell Rep 18:743–748

Escalona M, Samson G, Borroto C, Desjardins Y (2003) Physiology of effects of temporary immersion bioreactors on micropropagated pineapple plantlets. In Vitro Cell Dev Biol Plant 39:651–656

Etienne H, Berthouly M (2002) Temporary immersion systems in plant micropropagation. Plant Cell Tissue Organ Cult 69:215–231

Etienne H, Lartaud M, Michaux-Ferriére N, Carron MP, Berthouly M, Teisson C (1997) Improvement of somatic embryogenesis in *Hevea brasiliensis* (Müll. Arg.) using the temporary immersion technique. In Vitro Cell Dev Biol Plant 33:81–87

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:150–158

Gómez-Aguirre YA, Zamilpa A, González M, Trejo-Tapia G (2012) Adventitious root cultures of *Castilleja tenuiflora* Benth. as a source of phenylethanoid glycosides. Ind Crop Prod 36:188–195

Hassannejad S, Bernard F, Mirzajani F, Gholami M (2012) SA improvement of hyperhydricity reversion in *Thymus daenensis* shoots culture may be associated with polyamines changes. Plant Physiol Biochem 51:40–46

Holmgren NH (1976) Four new species of Mexican *Castilleja* (subgenus *Castilleja*, Scrophulariaceae) and their relatives. Brittonia 28:195–208

Ivanov I, Georgiev V, Georgiev M, Ilieva M, Pavlov A (2011) Galanthamine and related alkaloids production by *Leucojum aestivum* L. shoot culture using a temporary immersion technology. Appl Biochem Biotechnol 163:268–277

López-Laredo A, Gómez-Aguirre Y, Medina-Pérez V, Salcedo-Morales G, Sepúlveda-Jiménez G, Trejo-Tapia G (2012) Variation in antioxidant properties and phenolics concentration in different organs of wild growing and greenhouse cultivated *Castilleja tenuiflora* Benth. Acta Physiol Plant 34:2435–2442

López-Laredo A, Ramírez-Flores F, Sepúlveda-Jiménez G, Trejo-Tapia G (2009) Comparison of metabolite levels in callus of *Tecoma stans* (L.) Juss. ex Kunth. cultured in photoperiod and darkness. In Vitro Cell Dev Biol Plant 45:550–558

Lorenzo J, González B, Escalona M, Teisson C, Borroto C (1998) Sugarcane shoot formation in an improved temporary immersion system. Plant Cell Tissue Organ Cult 54:197–200

Martínez-Bonfil B, Salcedo-Morales G, López-Laredo A, Ventura-Zapata E, Evangelista-Lozano S, Trejo-Tapia G (2011) Shoot regeneration and determination of iridoid levels in the medicinal plant *Castilleja tenuiflora* Benth. Plant Cell Tissue Organ Cult 107:195–203

Michoux F, Ahmad N, Hennig A, Nixon PJ, Warzecha H (2013) Production of leafy biomass using temporary immersion bioreactors: an alternative platform to express proteins in transplastomic plants with drastic phenotypes. Planta 237:903–908

Neves C, Santos H, Vilas-Boas L, Amâncio S (2002) Involvement of free and conjugated polyamines and free amino acids in the adventitious rooting of micropropagated cork oak and grapevine shoots. Plant Physiol Biochem 40:1071–1080

Prieto P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem 269:337–340

Saher S, Piqueras A, Hellin E, Olmos E (2004) Hyperhydricity in micropropagated carnation shoots: the role of oxidative stress. Physiol Plant 120:152–161

Salcedo-Morales G, Rosas-Romero G, Nabor-Correa N, Bermúdez-Torres K, López-Laredo AR, Trejo-Tapia G (2009) Propagation and conservation of *Castilleja tenuiflora* Benth. ("hierba del cáncer") through *in vitro* culture. Polibotánica 28:119–137

Scherer RF, Garcia AC, Fraga HPF, Vesco LLD, Steinmacher DA, Guerra MP (2013) Nodule cluster cultures and temporary immersion bioreactors as a high performance micropropagation strategy in pineapple (*Ananas comosus* var. *comosus*). Sci Hortic 151:38–45

Sreedhar RV, Venkatachalam L, Neelwarne B (2009) Hyperhydricity-related morphologic and biochemical changes in vanilla (*Vanilla planifolia*). J Plant Growth Regul 28:46–57

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. Plant J 11:1187–1194

Trejo-Tapia G, Rosas-Romero G, López-Laredo AR, Bermúdez-Torres K, Zamilpa A (2012) *In vitro* organ cultures of the cancer herb *Castilleja tenuiflora* Benth. as potential sources of iridoids and antioxidant compounds. In: Orhan I (ed) Biotechnological production of plant secondary metabolites. Bentham Science, Sharjah, pp 87–106

Viu AFM, Viu MAO, Tavares AR, Vianello F, Lima GPP (2009) Endogenous and exogenous polyamines in the organogenesis in *Curcuma longa* L. Sci Hortic 121:501–504

Zhao Y, Sun W, Wang Y, Saxena PK, Liu CZ (2012) Improved mass multiplication of *Rhodiola crenulata* shoots using temporary immersion bioreactor with forced ventilation. Appl Biochem Biotechnol 166:1480–1490