academicJournals

Vol. 14(7), pp. 569-581, 18 February, 2015
DOI: 10.5897/AJB2014.14313
Article Number: F582C1450501
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

*Full Length Research Paper*

# Polyamines and WOX genes in the recalcitrance to plant conversion of somatic embryos of Habanero pepper (*Capsicum chinense* Jacq.)

**Raúl Enrique Valle-Gough[1], Susana Alejandra Avilés-Viñas[1], Stephanie López-Erosa, Adriana Canto-Flick[1], Eunice Gómez-Uc[1], Luis Alfonso Sáenz-Carbonell[2], Neftalí Ochoa-Alejo[3] and Nancy Santana-Buzzy[1*]**

[1]Centro de Investigación Científica de Yucatán. Unidad de Bioquímica y Biología Molecular de Plantas. Calle 43 # 130, Chuburná de Hidalgo. 97200. Mérida, Yucatán, México.
[2]Centro de Investigación Científica de Yucatán. Unidad de Biotecnología. Calle 43 # 130, Chuburná de Hidalgo. 97200. Mérida, Yucatán, México.
[3]Departamento de Ingeniería Genética de Plantas. Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional. (CINVESTAV-Irapuato). Km 9.6 Libramiento Norte Carretera Irapuato-León, Gto, México.

Received 11 November, 2014; Accepted 9 February, 2015

**In order to determine the role of polyamines in the formation and development of the somatic embryos of *Capsicum chinense*, the effect of different concentrations (0, 0.01, 0.1, and 1.0 mM) of Putrescine, Spermidine and Spermine on the efficiency and morphology of the embryos was evaluated. The results show that none of the three polyamines evaluated had a significant effect on the number of embryos formed, except Spermidine (1 mM), which caused a significant reduction in their numbers, in comparison with the control treatment. However, the most noteworthy result was observed in the treatment containing 0.1 mM of Spermine. The embryos developed in this treatment showed harmonic apex-radicle development, pale-green coloration and the formation of two tiny cotyledonary leaves. Real-time PCR was used to analyze the differential expression of the *WUS*, *WOX1* and *WOX3* genes in somatic embryos treated with Spermine and untreated, including the zygotic embryo. The transcript levels of the genes analyzed were found to differ significantly between both types of embryos (somatic and zygotic), with the zygotic embryos presenting a higher level of transcripts; however, compared to the untreated somatic embryos, the somatic embryos treated with Spermine showed an increase in the transcript levels of the three genes analyzed (WUS, WOX1 and WOX3); the *WOX1* gene in particular presented an accumulation pattern similar to that of the zygotic embryo of the species.**

**Key words:** Somatic embryos, zygotic embryos, polyamines, transcript patterns and morphology.

## INTRODUCTION

Somatic embryogenesis (SE) is defined as a process in which a bipolar structure develops from a non-zygotic cell without vascular connection with the origin tissue (Merkle et al., 1995). As somatic embryos (SEs) are formed without any fertilization event they are genetically identical to the parent tissue and are therefore clones. Irrespective of the mode of production, the anatomical and physiological features of SEs are highly comparable

to zygotic embryos (ZEs) (Bandyopadhyay and Hamill, 2000; Deo et al., 2011; Karami et al., 2009; Karami and Saidi, 2010; Kumar-Sharma and Millam, 2004; Kumar-Sharma et al., 2008). The morphogenetic potential of *Capsicum* sp. has been reported earlier, but the process of regeneration is slow and generally produces low frequencies of shoot regeneration, thus it has been found to be highly recalcitrant (Heidmann et al., 2011; Kothari et al., 2010; Us-Camas et al., 2014). Development of a specific body plan during embryogenesis requires the coordination of cell fates according to their positions along the embryo axes (Breuninger et al., 2008). The shoot apical meristem (SAM) and cotyledons of higher plants are established during embryogenesis in the apex (Jurgens et al., 1995). The SAM is formed in the apex between cotyledons in dicotyledonous plants and generates stems, leaves, and floral organs in a set pattern (Angenent et al., 2005; Ueda and Laux, 2012). Thus, SAM formation during embryogenesis is a critical step to start subsequent vegetative and reproductive development (Aida et al., 1999; Mayer et al., 1998; Taiz and Zeiger, 2002; Takeda and Aida, 2010). However, fully functioning apical shoot meristems and dicotyledonous somatic embryos are rarely produced in the *Capsicum* sp. (Steinitz et al., 2003).

Defective somatic embryos have been reported in embryogenic cultures of different species (Halperin, 1966; Merkle et al., 1995; Schiavone and Cooke, 1987). Three categories of malformations have been described for somatic embryos of different species: fused embryos (Carraway and Merkle, 1997; Rodriguez and Wetzstein, 1994; Stipp et al., 2001); altered cotyledon morphology (Carraway and Merkle, 1997; Jayasankar et al., 2002); and lack of a shoot (abnormal apical meristem histodifferentiation) (Chengalrayan et al., 2001; Jayasankar et al., 2002; Stipp et al., 2001). High frequencies of these abnormalities and the most critical defect, the absence of a shoot in the regenerants have been reported in the *Capsicum* sp. (Lopez-Puc et al., 2006; Steinitz et al., 2003). The absence of a functional shoot and the embryo incapacity to convert into plants is how the recalcitrance in *Capsisum* sp. manifests (Aboshama, 2011; Heidmann et al., 2011; Kothari et al., 2010; Us-Camas et al., 2014).

Polyamines (PAs) are natural occurring compounds in eukaryotic cells and are essential for several processes such as growth and differentiation (Kevers et al., 2000; Kumar et al., 2007; Silveira et al., 2006; Steiner et al., 2007; Takeda et al., 2002; Yadav and Rajam, 1998). In plants, polyamines have been implicated in stress response response, fertilization, senescence, organogenesis and somatic embryogenesis (Kakkar et al., 2000; Slocum, 1991; Takahashi and Kakehi, 2010). The continual synthesis of PAs is an essential process during the SE for the differentiation and development of the embryos (Kakkar et al., 2000). Previous studies have shown that high concentrations of PAs are found in embryos after auxin removal, also the use of PAs biosynthesis inhibitors inhibits the SE process and the addition of PAs precursors increases the number of somatic embryos (Bais and Ravishankar, 2002). Other studies have stated that the differential distribution of PAs in cell lines are related to the embryogenic capacity, where lines with high levels of PAs showed an increase in the embryo formation (Kakkar et al., 2000; Takahashi and Kakehi, 2010; Yadav and Rajam, 1998). It has been suggested that the role of the PAs in SE is due to its polycationic nature, these compounds associates to molecules such as membranes and nucleic acids, exerting a protective role against free radicals and regulating the gene transcription (Ahmadi et al., 2014; Bais and Ravishankar, 2002; Kakkar et al., 2000; Karami and Saidi, 2010; Takahashi and Kakehi, 2010).

Extensive reprogramming of gene expression accompanies the transition from somatic cells into embryogenic competent cells in response to inductive signals. Extensive effort has been focused on the identification of “master” genes required for this, although it is now apparent that the induction of the embryogenic pathway is not governed by a single gene, but is under the control of an intrincated genetic network. Recent studies have revealed that the transcript accumulation of WUSCHEL-Homeobox (*WOX*) genes determines apical and basal dominions in different species (Gambino et al., 2011; Haecker et al., 2004; Lin et al., 2013; Palovaara et al., 2010 a,b; Vandenbussche et al., 2009; Van der Graaff et al., 2009; Zhang et al., 2011). One of the early expressed genes is WUSCHEL (WUS) which maintains a reservoir of undifferentiated cells in the SAM and its abnormal expression produces plants with discontinual growth and a compromised SAM identity (Gambino et al., 2011; Haecker et al., 2004; Ji et al., 2010; Stuurman et al., 2002; Su et al., 2009). Other genes of this family such as WOX1 and WOX3 are involved in the cotyledon determination; WOX1 is expressed in the central part of the cotyledon primordia meanwhile WOX3 is expressed earlier in the peripheral area of the cotyledon (Hacker et al., 2004; Vandenbussche et al., 2009). Mutants of WOX1 and WOX3 shows similar phenotypes like: dwarf

*Corresponding author. E-mail: buzzy@cicy.mx.



**Abbreviations: SE,** Somatic embryos; **ZEs,** zygotic embryos; **SAM,** shoot apical meristem; **WUS,** WUSCHEL; **WOX,** WUSCHEL-Homeobox; **PAs,** polyamines; **Put,** spermidine; **Spd,** spermdine; **Spm,** spermine; **MS,** Murashige and Skoog basal medium; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **$GA_3$**, gibberellic acid.

plants, reduced apical meristems and abnormal leaf morphology (Lin et al., 2013; Nakata et al., 2012; Vandenbussche et al., 2009; Zhang et al., 2011)

The aim of this study was to evaluate the effect of polyamines on the efficiency and morphology of the somatic embryogenesis in *Capsicum chinense*, and characterize the most frequent deformations in the somatic embryos of this species. The differential expression of *WUS*, *WOX1* and *WOX3* genes was analyzed through real-time PCR in zygotic embryos and in somatic embryos (with and without PAs) in the early and advanced stages of their development.

## MATERIALS AND METHODS

### Plant material

Orange Habanero pepper seeds (*C. chinense*) var. Mayan Ba´alche (2367-CHL-021-080110/C) were surface sterilized following the protocol reported by Santana-Buzzy et al. (2005). To induce seed germination, MS (Murashige and Skoog, 1962) medium supplemented with sucrose (3% w/v), Gelrite (0.22% w/v) and 1.14 µM $GA_3$ was used. The pH of the medium was adjusted to 5.8 prior to sterilization, which was performed in an autoclave at 121°C and 1.5 atm for 15 min. Once the seeds were placed on the germination medium, the plates were incubated in constant darkness for 14 days at 25 ± 2°C. The plantlets were then transferred to a growth chamber under a 16 h photoperiod with light intensity of 40 to 50 mol $m^{-2}$ $s^{-1}$ at 25 ± 2°C, for 7 days.

### Induction of somatic embryogenesis

The protocol reported by Avilés-Viñas et al. (2013) was used to induce and obtain somatic embryos. Hypocotyl segments (0.5 cm) measuring in length from aseptic plantlets were transferred onto MS medium containing 9.05 µM 2,4-D and then incubated at 25 ± 2°C, under constant light for 28 days. A total of 10 hypocotyls were transferred to flasks containing 50 ml MS medium supplemented with 4.5 µM 2,4-D and also various levels of putrescine (Put, 0, 0.01, 0.1 and 1 mM), spermidine (Spd, 0, 0.01, 0.1 and 1 mM) or spermine (Spm, 0, 0.01, 0.1 and 1 mM) for 15 days. The embryos formed were then subcultured in the same differentiation medium, without PAs, where they remained for 30 days, until completing their development. All the liquid cultures were kept in constant agitation (100 rpm), under continuous light at 25 ± 2°C. Only the treatments that showed SEs with a morphological improvement or increased efficiency were used for further analysis. Three flasks were used for each treatment and the efficiency was evaluated by taking three samples of 10 ml from each flask containing the embryo culture. The embryos were counted with the aid of a stereoscopic microscope (Nikon SMZ800). The data were submitted to variance analysis (ANOVA) and for the comparison of means; the Tukey test ($P \leq 0.05$) was used with the SPSS 16.0.0 software.

### Histological analysis

Samples of embryos were fixed in FAA (37% formaldehyde: acetic acid: 96% ethanol, v/v/v) taken to a final volume of 100 ml with distilled water, from 24 to 48 h at 10°C. The procedures of dehydration, embedment and section were performed according to the method of Berlin and Miksche (1976). The sections were transferred on glass slides, deparaffinated with Ultraclear (Baker) and stained with tholuidine blue (0.05%) for 5 min.

### Scanning electron microscopy

For the preparation of samples, somatic embryos in cotyledonary stages and mature zygotic embryos were selected. Samples of both types were submerged in a solution of glutaraldehyde (25%) and a buffer of cacodylate (3%, 0.1 M, pH = 7.2), followed by three rinses with same buffer, for 30 min each one. Dehydration was achieved by gradual exposure of the samples to different solutions of ethanol (50, 70 and 90% v/v), 1 h for each solution. The dehydrated explants were then submerged in absolute alcohol for 1 h and dried in a Sandri-795 Tousimis drier, substituting the ethanol with liquid $CO_2$ and finally with gaseous $CO_2$. Finally, the samples were metalized with a gold layer 21 nm thick using a Denton Vacuum Desk II metalizer. The metalized samples were observed using a JSM-6360LV JEOL scanning electron microscope.

### RNA extraction and cDNA synthesis

Samples (1 g) were taken from: immature zygotic embryos (IZE), mature zygotic embryos (MZE), induced hypocotyls (IH), somatic embryos in early stages of development: globular-heart stages (GH), and somatic embryos in advanced stages: torpedo-cotyledonary stages (TC), in both cases, treated and untreated with 1 mM Spm. Extraction of total RNA was performed following the protocol reported by Chomczinsky and Sacchi (1987). The samples were treated with DNAse (Ambion) and the concentration was quantified in Nanodrop (Thermo Scientific). The cDNA synthesis was carried out using the Oligo-dT primer (Invitrogen) with the SuperScriptIII enzyme (Invitrogen), following the manufacturer's instructions. RNA integrity was observed in agarose gel (1.5%) stained with ethidium bromide.

### Primer design and PCR conditions

WUS (NM_001247086.2), WOX1 (XM_0042235751.1) and WOX3 (XM_004251238.1) primers were designed from homolog sequences of *Solanum lycopersicum*, available at the NCBI. The sequences were aligned using the MEGA v 6.0. program and the primers were designed with the Primer3plus program (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The constitutive genes previously reported in *C. annuum* (Wan et al., 2011): Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ubiquitin 3 (UBI-3) and beta-Tubulin (β-Tub) were considered to be used as normalization genes after the analysis of gene stability in the geNorm (http://medgen.ugent.be/;jvdesomp/genorm/) software. Relative expression was processed with the method of Ct ($2^{-\Delta\Delta Ct}$) (Livak and Schmitgen, 2001), in a StepOne thermal cycler (Applied Biosystems). The real-time PCR reaction mixture comprised the following components at a final volume of 20 µl containing: 10 µl of SYBR-GREEN Master Mix (Applied Biosystems), 0.12 µM of each primer and 4 µl of cDNA. Reaction conditions were 94°C (3 min); 40 cycles of 94°C (15 s), 55 to 65°C (30 s) varying the alignment temperature for each primer (Table 1). The PCR was performed in six replicates per sample using three biological samples. The results were submitted to ANOVA and the means were compared with the Tukey test ($P \leq 0.05$) using the SPSS 16.0.0 software.

## RESULTS AND DISCUSSION

### Effect of polyamines on the efficiency and developmental morphology of embryogeny

The addition of Put and Spm to the culture media had no significative effect on the number of embryos formed in

**Table 1.** List of primers used for real time PCR.

| Gene | Primer sequence | Tm | Fragment |
|---|---|---|---|
| *WUS* | F:5´-AGGTCTCCAACTGCTGAACA- 3´<br>R:5´-CACGAGCTTTATGGTTCTGAAA- 3´ | 60°C | 110 bp |
| *WOX1* | F:5´-TGCAACAAAGAAGGAGCCTACTAG- 3´<br>R:5´-TTCCATAGTTCTTGGTGTTGATGGAC- 3´ | 65°C | 195 bp |
| *WOX3* | F:5´-AACAAGGTGGAGTCCAACCC-3´<br>R:5´- CCCTCAATTTTCCCCCTATCC-3´ | 60°C | 192 bp |
| *GAPDH* | F: 5´- ATGATGATGTGAAAGCAGCG- 3´<br>R: 5´-TTTCAACTGGTGGCTGCTAC-3´ | 55°C | 276 bp |
| *UBI-3* | F: 5´- GAGGGTGAGTGAGCAGTTC- 3´<br>R: 5´-CTTCATCGTCATCTGCTGTC- 3´ | 55°C | 167 bp |
| *β-TUB* | F: 5´-TGTCCATCTGCTCTCTGTTG-3´<br>R: 5´-CACCCCAAGCACAATAAGAC-3´ | 55°C | 204 bp |

the treatments evaluated (Figure 1A to C), while the addition of Spd causes a marked decrease in the efficiency (somatic embryos per litre) along with the increase of the Spd concentration, with significant differences when compared to the control treatment (Figure 1B). However, the addition of 0.1 mM Spm improved the embryo morphology. In the Figure 2A we observe germinated somatic embryos treated with 0.1 mM Spm; different stages of development were observed before germination (Figure 2B and C). Under these culture conditions (0.1 mM Spm) it was observed, for the first time, a harmonic apical-radical development and the emission of two tiny pale green cotyledonary leaves (Figure 2C and D), although these embryos did not subsequently evolve into plants due to a deformed shoot apex. It is likely that this change in coloration of the embryo could be attributed to the fact that PAs have been detected in vacuoles, mitochondria and chloroplasts (Slocum, 1991). Borrell et al. (1995) also detected these compounds in spinach thylakoid membranes, associated with light-reception complexes of the photosystem II. They concluded that the PAs maintain photosynthetic activity, thus preventing senescence due to osmotic stress.

Independently of the applied treatment, the embryos always emerged through the epidermis of the explant (Figure 3A, B and C) originating in the perivascular zone of the hypocotyl. The first embryos (globular-stage) were visible approximately 12-15 days after induction culture (Figure 3C and D), followed by heart, torpedo and cotyledonary embryo stages (Figure 3E, F and G) through an elongated shape and showing both poles clearly: shoot and root, with no apparent morphological differences observed among the treatments during the first stages of development (Figure 3D and E). Shoot meristem differentiation usually begins at the late globular stage and structurally organized meristems are visible at the heart or cotyledonary stage of somatic embryo development (Taiz and Zeiger, 2002). However, the evolution from torpedo stage to cotyledonary stage failed in the majority of the embryos developed (Figure 3F and G), indicating a deformed shoot apex, incapacitated for subsequent germination.

The most frequent deformations in the shoot meristem were manifested either by the absence of cotyledons (Figure 4A and B) and deformed cotyledons (Figure 4D and 4E). Embryos with exposed shoot apical area were also observed (Figure 4C and F). All of these abnormalities incapacitated the somatic embryos to germinate and subsequently convert into plants. Our results corroborate those reported by Steinitz et al. (2003), who attributed this incapacity of *C. annum* embryos for plant conversion due to the absence of a shoot, resulting in failure for the establishment of a normal functioning shoot meristem. The radical apex of the SEs always formed normally (Figure 4A, 4B, 4D and 4E) and radicle emission occurred spontaneously in most of the embryos formed. In contrast with the SEs, it was possible to observe the formation of well-defined cotyledons in the ZEs (Figure 5A and 5D), showing both meristems, apical and radical (Figure 5B, C and E) and the formation of provascular bundles along the apical-basal axis (Figure 5B).

The addition of PAs favored embryogenic responses in different species (Ahmadi et al., 2014; Kevers et al., 2000; Paul et al., 2009; Steiner et al., 2007; Yadav and Rajam, 1998). In *Hevea brasiliensis,* the addition of PAs increased the amount of embryogenic callus and the number of embryos per gram of callus using 50 µM Spd (El-Hadrami et al., 1989a, b) and also in *Brassica napus* the addition of 2.5 µM Put for 48 h increased three-fold the embryo formation and 5.56 µM Put increased four-fold the embryo conversion (Ahmadi et al., 2014). PAs have also been found to reduce the responses of embryogenic cultures. Silveira et al. (2006) observed a reduction in the growth of *Araucaria angustifolia* suspension cultures when Spd and Spm (1.0 mM) were added to the culture media. Working on *Coffea canephora* and

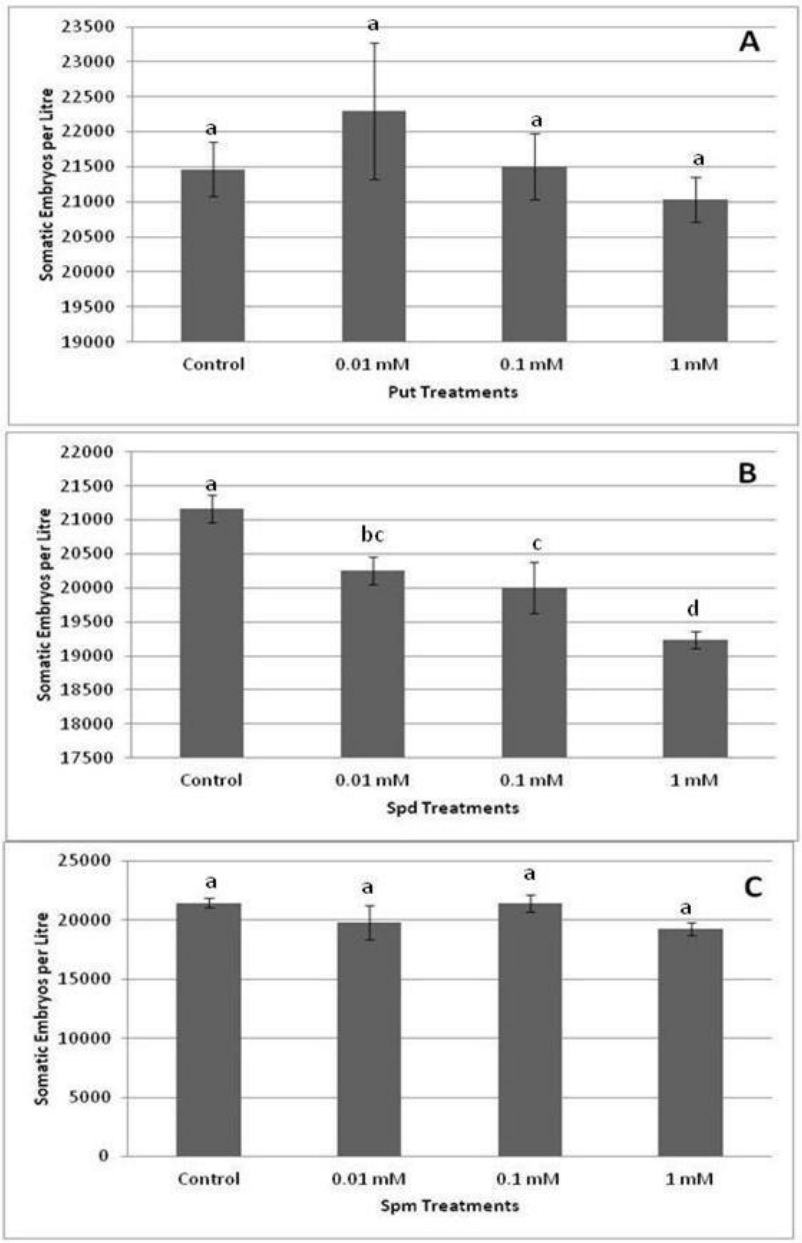


**Figure 1.** Effect of polyamines on somatic embryogenesis efficiency of *Capsicum chinense*. **A)** Effect of Put. **B)** Effect of Spd. **C)** Effect of Spm. Control: Liquid MS + 4.5 µM 2,4-D. Samples taken after 30 days of culture differentiation. Mean ± E.S. (n = 3, $p < 0.05$). Different letters reveals mean differences.

*Coffea arabica*, Calheiros et al. (1994) noted that among exogenously applied PAs, Spd significantly reduced the number of SEs in both species.

Similar results were also reported in *Solanum melongena* where concentrations of 0.5 mM Spd and Spm caused a decrease in the number of embryos formed (Yadav and Rajam, 1998). In *B. napus* microspores increased concentrations up to 23 to 56 µM; Put reduced the embryo formation and increased the callus formation (Ahmadi et al., 2014). Spm is capable of capturing free radicals and interacting with DNA to protect it from the action of these compounds. It has also been reported that, in *Arabidopsis thaliana*, high concentrations of Spd and Spm are able to regulate the S-adenosylmethyonine decarboxylase (SAMDC) (Kakkar et al., 2000; Takashi and Kakehi, 2010).

In *Picea glauca*, increased levels of PAs during the initial developmental stages of somatic embryogenesis resulted in an increase in SAM synthetase, coinciding with high levels of stress related proteins (Lippert et al.,

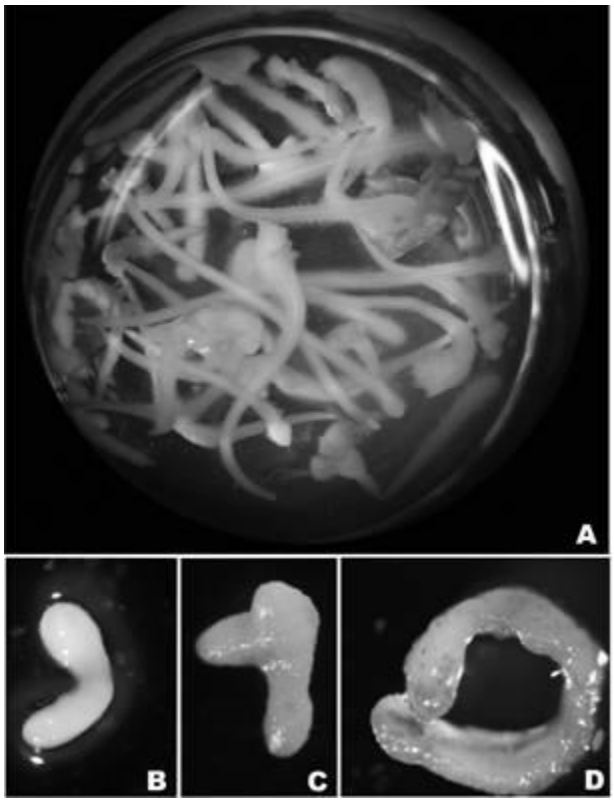


**Figure 2.** Effect of Spm on the morphology of *Capsicum chinense* somatic embryos. **A)** Somatic embryos with 0.1 mM Spm. **B)** Somatic embryo at torpedo stage. **C)** Somatic embryo at cotyledonary stage. **D)** Germinated somatic embryo.

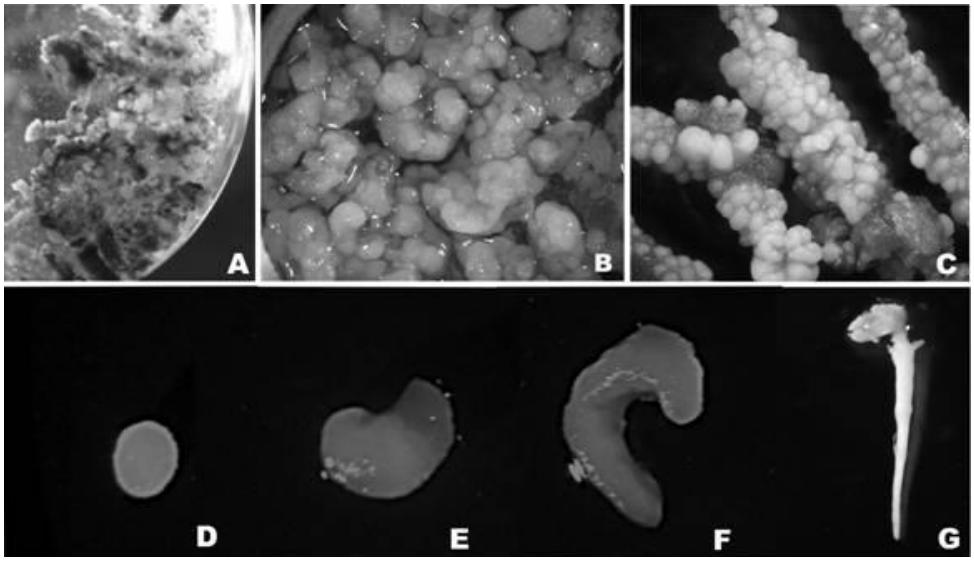


**Figure 3.** Microphotography of somatic embryos in *Capsicum chinense*. **A)** Hypocotyls with somatic embryos in liquid medium after 14 days of culture (MS + 4.5 µM 2,4-D). **B)** Somatic embryos clusters formed in liquid medium after 28 days of culture (MS + 4.5 M 2,4-D), **C)** Hypocotyls after with 14 days of culture initiation (MS + 9.0 µM 2,4-D). **D)** Globular somatic embryos. **E)** Heart-shaped somatic embryo. **F)** Torpedo somatic embryos and G) Cotyledonary somatic embryos.

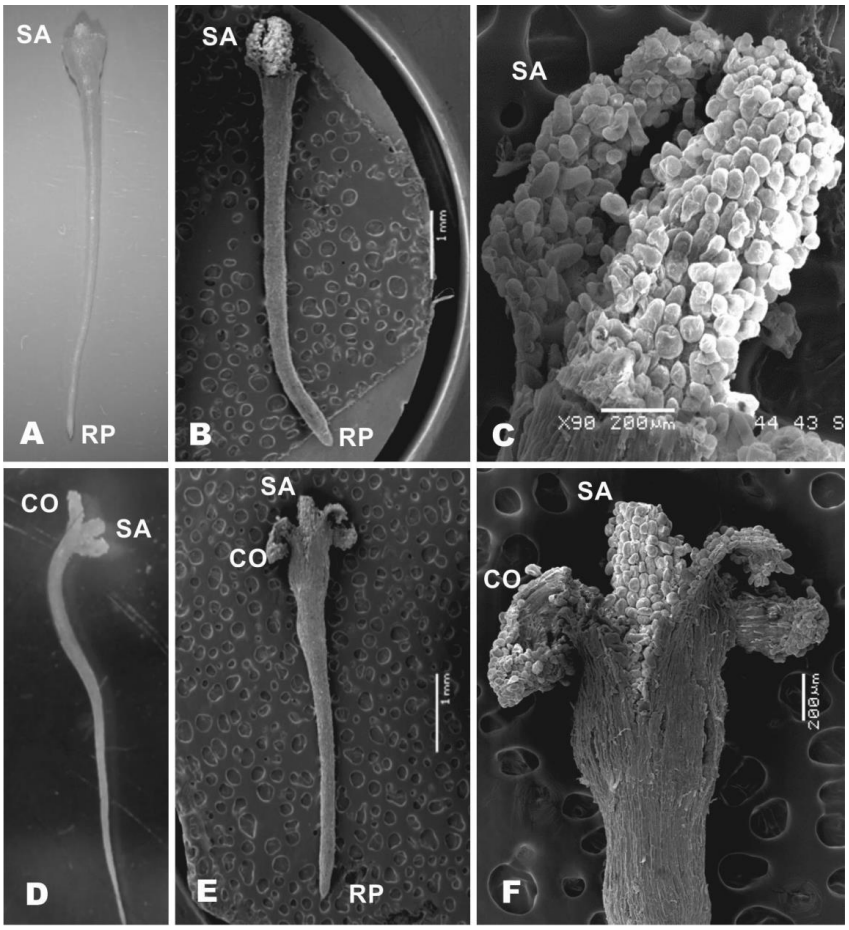


**Figure 4.** Characterization of deformed somatic embryos in *Capsicum chinense*. Microphotography (A, D). Scanning electron micrographs (B, C, E, F). A) Somatic embryo with absence of cotyledons. B) Somatic embryo without cotyledons. C) Close-up of the apical shoot from embryos without cotyledons with an exposed shoot area. D) Somatic embryos with deformed cotyledons. E) Somatic embryo with deformed cotyledons. F) Close-up to the deformed cotyledons and exposed shoot apex. SA, Shoot Apex; RP, Root Pole; CO, cotyledons.

2005). This allow us to infer that these compounds play a protective role during the embryogenesis due to the stress produced during this process (Karami and Saidi, 2010; Takahashi and Kakehi, 2010). This concurs with observations made by Regla-Márques (2011; Unpublished data), who detected significantly higher contents of PAs in SEs, in comparison with ZEs of *C. chinense*.

## WUS transcript levels in somatic and zygotic embryos of *C. chinense*

WUS transcripts were detected in the IH, increasing significantly in the GH and diminishing drastically (up to seven-fold) in the TC (Figure 6A). A similar pattern was observed in the ZEs, showing a higher level of WUS transcripts in the IZE which also diminished significantly in the MZE; however, this decrease was considerably lower than that observed in embryos at the TC. When SEs were treated with 0.1 mM Spm, the WUS transcript levels in GH+Spm were significantly lower to those observed in TC+Spm (Figure 6B). This behavior differed when compared to ZEs and untreated SEs, where the WUS transcript levels were significantly lower in GH+Spm compared to GH with a significant increase in TC+Spm when compared to TC. The presence of WUS has been detected in the early stages of embryogenesis, and is seen to diminish as the embryo develops (Gambino et al., 2011; Su et al., 2009), this behavior was observed in the ZEs and SEs of *C. chinense*. When the SEs were treated with Spm the WUS transcript levels showed an opposite behavior to previous reports. Alterations in the genes responsible for maintaining the shoot meristem have been observed in Arabidopsis mutants with defective SAM, deformed cotyledons and

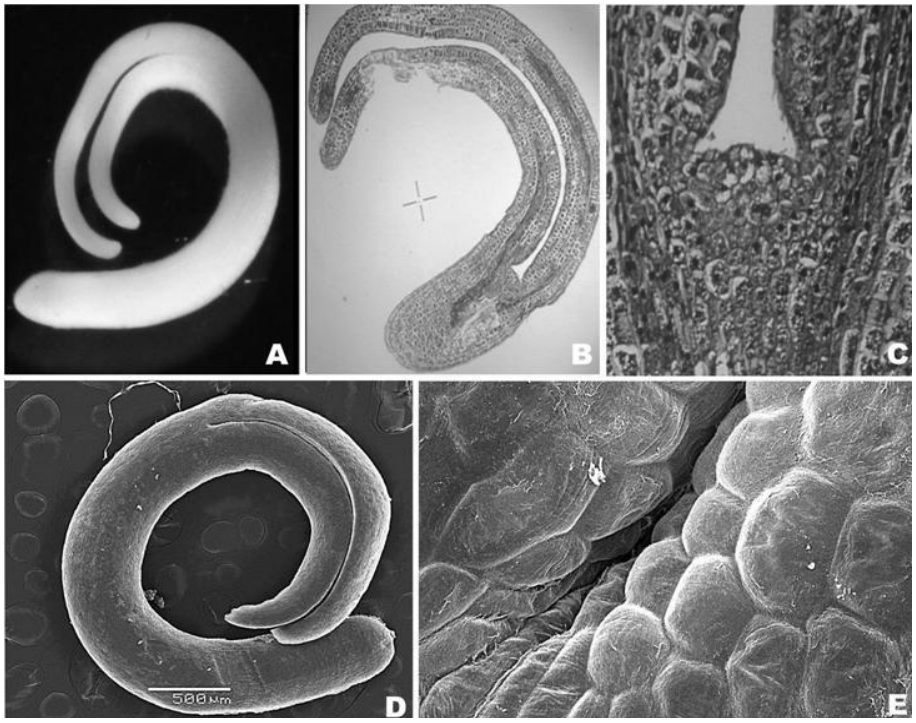


**Figure 5.** Ultrastructural and histological analysis of zygotic embryos in *Capsicum chinense.* **A)** Microphotography of a mature zygotic embryo. **B)** Histology of a complete mature zygotic embryo. **C)** Histology of a shoot apical meristem from a mature zygotic embryo. Scanning electron microscopy in zygotic embryos. **D)** Mature zygotic embryo. **E)** Close-up of the shoot apex in mature zygotic embryo.

lack of shoot development (Budziszewski et al., 2001; Mayer et al., 1998). Other authors have reported *wu*s mutants in which interrupted growth and lack of apical dominance have been observed (Kieffer et al., 2006; Laux et al., 1996; Stuurmann et al., 2002). In *ham* (Hairy Apical Meristem) mutants of *Petunia hybrid,* a similar behavior in the expression patterns of WUS was observed in early developmental stages when compared to wild-type. However, when the plant development advances, the WUS expression reduced sharply and a disorganized shoot meristem became more evident (Stuurmann et al., 2002). This behavior of WUS was also observed in our study in which the absence of the shoot meristem or the presence of a deformed shoot meristem is also observed. A number of reports have associated these deformations in the shoot meristem with alterations in auxin transport during embryonic development (Palovaara et al., 2010a; 2010b; Vanneste and Friml, 2009), WUS can interact with auxin responsive genes (Busch et al., 2010; Kieffer et al., 2006) and is found to be involved in the establishment in the auxin transport by its association with PIN1 (Su et al., 2009). The morphological improvement with 0.1 mM Spm in the SEs could be associated with a four-fold increase in the WUS transcript levels in TC+Spm, maybe due to an improved auxin transport in these stages of development.

**WOX1 transcript accumulation in somatic and zygotic embryos of *C. chínense***

During somatic embryogenesis, WOX1 transcript levels were detected in every stage of the embryos formed, including the IH phase, with a significant increase in GH decreasing significantly in TC (Figure 7A). The WOX1 transcript levels in ZEs were significantly higher in MZE when compared to IZE (Figure 7A); the behavior of the SEs was different to those observed in ZEs showing an opposite behavior between these embryos. When the SEs were treated with 0.1 mM Spm, mean differences were detected when compared with the untreated SEs, where a significant decrease was observed in GH+Spm when compared to GH (Figure 7B). The TC+Spm showed a significant increase in the WOX1 transcripts when compared to TC (Figure 7B). In general, the Spm treated SEs showed a different behavior from the SEs without Spm, in this aspect the 0.1 mM Spm treated SEs behaved similar to the ZEs, where the WOX1 transcript levels IZE were significantly lower when compared to MZE (Figure 7A); this same pattern were observed in Spm treated embryos were the WOX1 transcript levels were lower in GH+Spm when compared to TC+Spm (Figure 7B).

According to reports on other species, WOX1 transcript

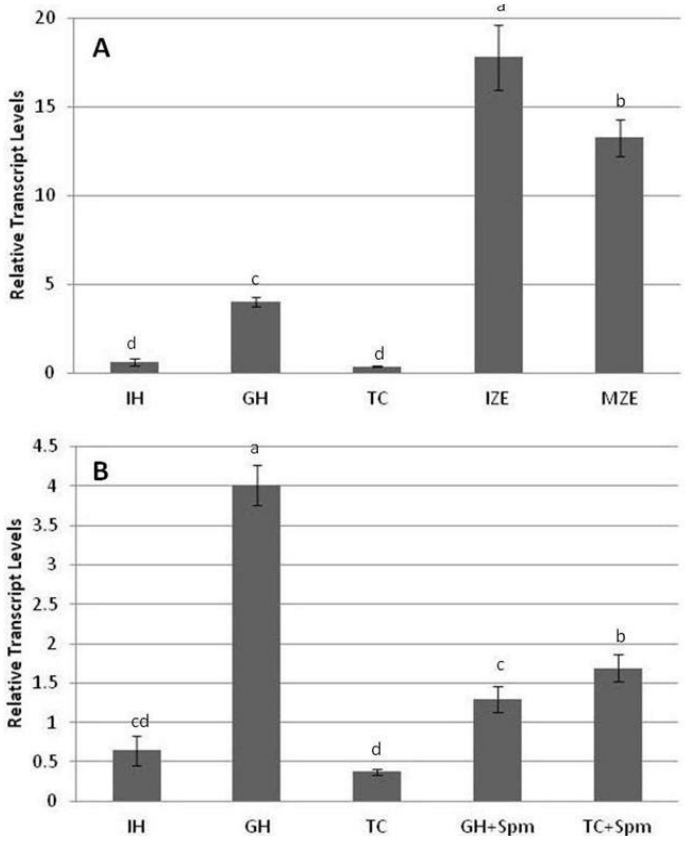


**Figure 6.** Quantification of WUS transcripts levels in *Capsicum chinense*. A) WUS transcript levels in zygotic and somatic embryos. B) Effect of Spm on WUS transcripts levels in somatic embryos of *C. chinense*. The PCR signals were normalized to β-TUB. Values are mean ± S.E. (n =6, $p < 0.05$). Different letters indicate significant statistical differences. IH, hypocotyls in induction; GH, SEs at globular and heart stages; TC, SEs at torpedo and cotyledonary stages; IZE, immature zygotic embryos; MZE, mature zygotic embryos GH+Spm, SEs at globular and heart stages treated with Spermine and TC+Spm, SEs at torpedo and cotyledonary stages treated with Spermine.

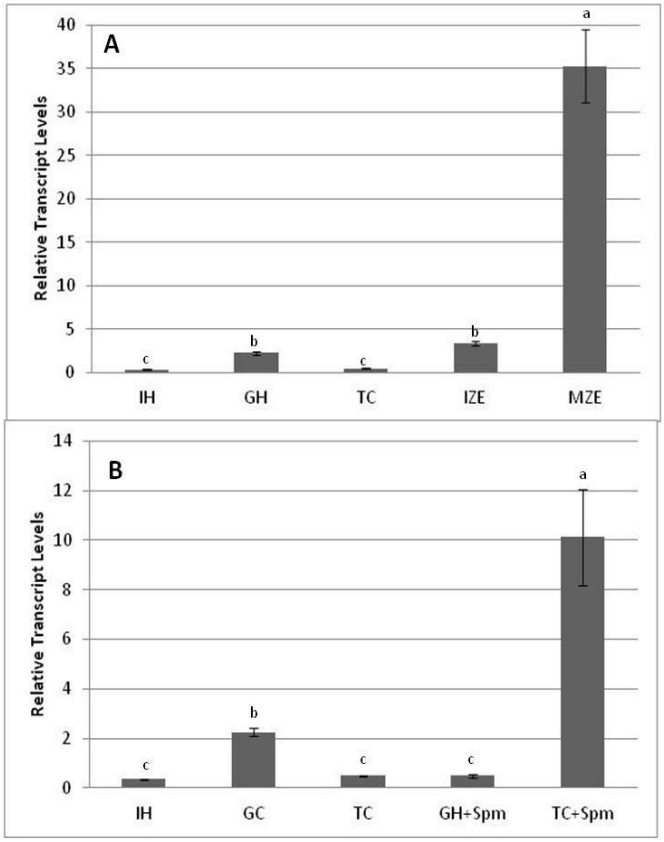


**Figure 7.** Quantification of WOX1 transcripts levels in *C. chinense*. A) WOX1 transcripts levels in zygotic and somatic embryos. B) Effect of Spm on WOX1 transcripts levels in somatic embryos of *C. chinense*. The PCR signals were normalized to β-TUB transcripts. Mean ± S.E. (n =6, $p < 0.05$). Different letters indicate significant statistical differences. IH, hypocotyls in induction; GH, SEs at globular and heart stages; TC, SEs at torpedo and cotyledonary stages; IZE, immature zygotic embryos; MZE, mature zygotic embryos GH+Spm, SEs at globular and heart stages treated with Spermine and TC+Spm, SEs at torpedo and cotyledonary stages treated with Spermine.

patterns show greater presence of transcripts in the more advanced stages of embryonic development (Gambino et al., 2011; Haecker et al., 2004; Lin et al., 2013). Previous reports have demonstrated that over-expression and under-expression of WOX1 can cause alterations in plant phenotype due to the fact that this gene is associated with the organization of cellular division in leaves, organs and carpels (Lin et al., 2013; Vandenbussche et al., 2009; Zhang et al., 2011). In *Nicotiana tabacum wox1,* a significant reduction in the WOX1 transcripts levels was observed, fully complemented plants showed a major increase in WOX1 transcripts similar to the wild-type (Lin et al., 2013). Moreover, the addition of PAs, particularly Spm, is capable of reverting the mutant phenotype, most likely because WOX1 is associated with the modulation of SAMDC1, a key enzyme in the biosynthesis of PAs (Ge et al., 2006; Zhang et al., 2011). The addition of Spm increased the WOX1 transcript levels in TC+Spm in *C. chinense*; this suggests that the morphological improvement in the SEs of this species could be attributed to the role of WOX1 in the PAs biosynthetic pathway and its possible interactions with WUS and other member of the WOX family like WOX3.

### Accumulation of WOX3 transcripts in somatic and zygotic embryos of *C. chinense*

The quantification of WOX3 transcripts showed no significant differences in the different developmental stages of the SEs (Figure 8A), whereas, with the ZEs, transcript levels of this gene (WOX3) was significantly higher in the MZE (Figure 8A). With the addition of 0.1 mM Spm, the quantity of transcripts increased significantly,

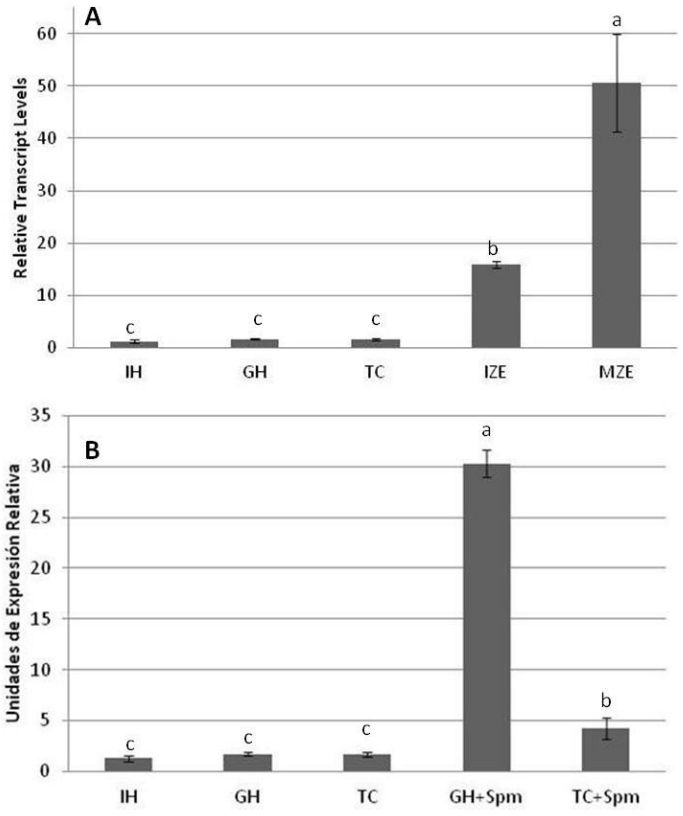


**Figure 8.** Quantification of WOX3 transcripts levels in *C. chinense*. A) WOX3 transcripts levels in zygotic and somatic embryos. B) Effect of Spm on WOX3 transcripts levels in somatic embryos of *C. chinense*. The PCR signals were normalized to β-TUB transcripts. Mean ± S.E. (n =6, $p < 0.05$). Different letters indicate significant statistical differences. IH, hypocotyls in induction; GH, SEs at globular and heart stages; TC, SEs at torpedo and cotyledonary stages; IZE, immature zygotic embryos; MZE, mature zygotic embryos GH+Spm, SEs at globular and heart stages treated with Spermine and TC+Spm, SEs at torpedo and cotyledonary stages treated with Spermine.

in comparison with the untreated SEs (Figure 8B), but with a different accumulation pattern to those observed in the ZEs where the GH+Spm showed greater transcript levels than the TC+Spm (Figure 8B).

Our results differ from reports on the behavior of WOX3 transcript accumulation for other species, in which the transcripts have been observed to accumulate in advanced stages of embryonic development and to diminish significantly in the phases prior to germination (Haecker et al., 2004; Gambino et al., 2011). There is evidence to support the argument that WOX3 malfunction can be related to malformations of the aerial part, as this gene is associated with early leaf formation (Nakata et al., 2012; Lin et al., 2013; Shimizu et al., 2009; Vandenbussche et al., 2009). It is likely that, with the addition of Spm, WOX3 could be acting as a marker prior to organ formation. The significant increase in WOX3 transcripts in the GH+Spm could be attributed to the high similarity existing between the proteins, WOX1 and WOX3, and thus WOX3 could be substituted by WOX1 under these culture conditions. A number of reports to this effect indicate that, due to the fact that the mutant phenotypes *wox1* and *wox3* are similar, these genes can act redundantly in embryonic development (Lin et al., 2013; Vandenbussche et al., 2009).

Shoot architecture in higher plants is highly dependent on the activity of the SAM, which maintains pluripotent stem cells at its tip (Steeves and Sussex, 1989). During the development of postembryonic shoots, the SAMs continuously produce organs in a regular pattern, for example, stems, leaves and flowers. Therefore, the subtle variations in primordia initiated by the SAM account for all of the remarkable differences and diversity in shoot architecture that we observe in nature. Recent analyses of *A. thaliana* mutants have identified several key genes that play essential roles in the specific function of SAMs (Geier et al., 2008; Kinoshita et al., 2010; Laux et al., 1996; Lohmann et al., 2001; Lenhard et al., 2002; Lenhard and Laux, 2003).

Studies on the biological function of plant Homeodomain proteins, designated WOX proteins, have revealed that they are transcription factors and are involved in the regulation of various developmental processes (Haecker et al., 2004; Lohmann et al., 2001; Qu and Zhu, 2006; Van der Graaff et al., 2009; Zhang et al., 2011). The WOX genes were all involved in maintaining a balance between cell division and differentiation (Gallois et al., 2004). WUS is an early gene expressed in the apical embryo region, while WOX1 is expressed in the central zone of the cotyledon and WOX3 in the peripheral zone (Haecker et al., 2004).

It has been observed that altered patterns of these genes have shown deformations in the apical region and can affect the function of other genes (Laux et al., 1996; Lin et al., 2013; Shimizu et al., 2009; Stuurman et al., 2002; Su et al., 2009; Zhang et al., 2011). This could suggest that the deformations observed could be the result of an anomalous accumulation of WUS, WOX1 and WOX3 transcripts during somatic embryogenesis.

In our study, the alterations detected in the levels of WUS, WOX1 and WOX3 transcripts in SEs of *C. chinense*, which are affected by the apical meristem syndrome (Steinitz et al., 2003), suggests that their incapacity to germinate and convert to plants is of a genetic nature. However, the addition of 0.1 mM Spm to the culture medium improved the embryo morphology of these deformed embryos, facilitating embryos with an adequately harmonious development of both radical and apical zones, a change in coloration from creamy white to pale green and the emission of two tiny, albeit very rudimentary, cotyledonary leaves. From the analysis of transcript levels in these embryos after Spm treatments, it was possible to observe a significant increase in WOX1 in TC+Spm (Figure 7B). The effect of polyamines has been documented previously (Galston and Shawney, 1990;

Ge et al., 2006; El-Hadrani et al., 1989a 1989b; Kevers et al., 2000; Slocum, 1991; Walden et al., 1997; Yadav and Rajam, 1998) and in the case of Spm, it has been found to be capable of modulating the activity of SAMDC1 (Zhang et al., 2011) and transcription through its association with nucleic acids (Galston and Shawney, 1990; Lippert et al., 1995; Walden et al., 1997), which would be reflected in a higher level of WUS, WOX1 and WOX3 transcripts. Even though the phenotype of the somatic embryos was improved with the addition of Spm, their inability to convert to plants persisted. SAMDC is involved in the final steps of the plant polyamine biosynthetic pathway and is essential for plant shoot architecture (Ge et al., 2006; Friedman et al., 1982; 1985; Smith et al., 1985; Rastogi and Sawhney, 1990; Thu-Hang et al., 2002).

Our results coincide with those proposed by Zhang et al. (2011) who have suggested that WOX1 plays an important role in meristem development, possibly via modulation of SAMDC1 activity and polyamine homeostasis. However, the inability of *Capsicum* embryos to convert to plants is clear evidence of the complexity of this phenomenon in which numerous genes and/or other factors do not allow the germination to follow a normal pattern during its development, perhaps because the shoot meristem is not functionally capacitated for this physiological process.

## Conflict of interests

The authors did not declare any conflict of interest.

## ACKNOWLEDGEMENT

We wish to thank the CONACYT for providing the financial support for this project.

### REFERENCES

Aboshama HMS (2011). Direct Somatic Embryogenesis of Pepper (*Capsicum annuum* L.). World J. Agric. Sci. 7(6):755-762.

Ahmadi B, Shariatpanahi ME, Ojaghkandi MA, Heydari AA (2014) Improved microspore embryogenesis induction and plantlet regeneration using putrescine, cefotaxime and vancomycin in Brassica napus L. Plant Cell Tissue Organ Cult. 118:497-505

Aida M, Ishida T, Tasaka T (1999). Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interaction among the cup-shaped cotyledon and shoot meristemless genes. Development 126:1563-1570.

Angenent GC, Stuurman J, Snowden KC, Koes R (2005). Use of Petunia to unravel plant meristem functioning. Trends Plant Sci. 10(5):243-250.

Avilés-Viñas SA, Lecona-Guzmán CA, Canto-Flick A, López-Erosa S, Santana-Buzzy N (2013). Morpho-histological and ultrastructural study on direct somatic embryogenesis of *Capsicum chinense* Jacq. in liquid medium. Plant Biotechnol. Rep. 7:277-286.

Bais HP, Ravishankar GA (2002). Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue Organ Cult. 69:1-34.

Bandyopadhyay S, Hamill JD (2000). Ultrastructural studies of somatic embryos of *Eucalyptus nitens* and comparisons with zygotic embryos found in mature seeds. Ann. Bot. 86:237-244.

Berlin GP, Mischke JP (1976). Botanical microtechnique and cytochemistry. 3rd edn. Iowa State University Press, Ames.

Borrell A, Culianez-Macia FA, Altabella T, Besford RT, Flores D, Tiburcio AF (1995). Arginine decarboxylase is localized in chloroplasts. Plant Physiol. 98:105-110.

Breuninger H, Rikirsch E, Hermann M, Ueda M, Laux T (2008). Differential Expression of WOX Genes Mediates Apical-Basal Axis Formation in the Arabidopsis Embryo. Dev. Cell. 14:867-876.

Budziszewski GJ, Lewis SP, Glover LW, Reineke J, Jones G, Ziemnik LS, Lonowski J, Nyfeler B, Aux G, Zhou Q, McElver J, Patton DA, Martiennensen R, Grossniklaus, Ma H, Law M, Levin JZ (2001). Arabidopsis Genes Essential for Seedling Viability: Isolation of Insertional Mutants and Molecular Cloning. Genetics 159(4):1765-1778.

Busch W, Miotk A, Ariel FD, Zhao Z, Forner J, Daum G, Suzaki T, Schuster C, Schultheiss SJ, Leibfried A, Haubeiss S, Ha N, Chan RL (2010). Transcriptional Control of a Plant Stem Cell Niche. Dev. Cell. 18:841-853.

Calheiros MBP, Vieira LGE, Fuentes SRL (1994). Effects of exogenous polyamines on direct somatic embryogenesis in cofee. Rev. Bras. Fisiol. Veg. 6:109-114.

Carraway DT, Merkle SA (1997). Plantlet regeneration from somatic embryos of American chestnut. Can. J. For. Res. 27(11):1805-1812.

Chengalrayan K, Hazra S, Gallo-Meagher M (2001). Histological analysis of somatic embryogenesis from mature zygotic embryo-derived leaflets of peanut (*Arachis hypogaea* L.). Plant Sci. 161(3):415-421.

Chomczinsky P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thyocianate-phenol-chloroform extraction. Ann. Biochem. 162(1):156-159.

Deo PC, Tyagi PT, Taylor M, Harding R, Becker D (2010). Factors affecting somatic embryogenesis and transformation in modern plant breeding. South Pac. J. Nat. Appl. Sci. 28:27-40.

El-Hadrami I, Carron MP, D'Azauc J (1989a). Clonal variability of embryogenic potential in *Hevea brasiliensis*: relations with polyamines and peroxidases in calli. Compt. Ren. Acad. Sci. 308:299-305.

El-Hadrami I, Michaux-Ferriere N, Carron MP, D'Azauc J (1989b). Polyamines, a possible limiting factor in somatic embryogenesis of *Hevea brasiliensis*. Compt. Ren. Acad. Sci. 308:205-211.

Friedman R, Altman A, Bachrach U (1982). Polyamines and root formation in mung bean hypocotyl cuttings: I. effects of exogenous compounds and changes in endogenous polyamine content. Plant Physiol. 70:844-848.

Friedman R, Altman A, Bachrach U (1985). Polyamines and root formation in mung bean bypocotyl cuttings: II. incorporation of precursors into polyamines. Plant Physiol. 79:80–83.

Gallois JL, Nora FR, Mizukami Y, Sablowski R (2004). WUSCHEL induces shoot stem cell activity and developmental plasticity in the root meristem. Genes Dev. 18:375-380.

Galston AW, Shawmey RK (1990). Polyamines in Plant Physiology. Plant Physiol. 94:406-410.

Gambino G, Minuto M, Bocacci P, Perrone I, Vallania R, Gribaudo I (2011). Characterization of expression dynamics of WOX homeodomain transcription factors during somatic embryogenesis in Vitis vinifera. J. Expt. Bot. 62(3):1089-1101.

Ge C, Cui X, Wang Y, Hu Y, Fu Z, Zhang D, Cheng Z, Li J (2006). BUD2, encoding a S-adenosylmethionine decarboxylase, is required for Arabidopsis growth and development. Cell Res. 16:446-456.

Geier F, Lohmann JU, Gerstung M, Maier AT, Timmer J, Fleck C (2008). A quantitative and dynamic model for plant stem cell regulation. PLoS One 3(10):e3553.

Haecker A, Gross-Hardt R, Geiges B, Sarkar A, Breuninger H, Hermann M, Laux T (2004). Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in Arabidopsis thaliana. Development 131(3):657-668.

Halperin W (1966). Alternative Morphogenetic Events in Cell Suspensions. Am. J. Bot. 55(5):443-453.

Heidmann I, de Lange B, Lambalk J, Angenent GC, Boutilier K (2011).

Efficient sweet pepper transformation mediated by the Baby Boom transcription factor. Plant Cell Rep. 30(6):1107-1115.
Jayasankar S, Bondada B, Gray LD (2002). A unique morphotype of grapevine somatic embryos exhibits accelerated germination and early plant development. Plant Cell Rep. 20(10):907-911.
Jurgens G, Mayer U, Busch M, Laux T (1995). Pattern Formation in the Arabidopsis Embryo: A Genetic Perspective. Phil. Trans. R. Soc. Lond. B. 350(1331):19-25.
Kakkar RK, Nagar PK, Ahuja PS, Rai VK (2000). Polyamines and Plant morphogenesis. Biol. Plant. 43(1):1-11.
Karami O, Aghavaisi B, Pour AM (2009). Molecular aspects of somatic-to-embryogenic transition. J. Chem. Biol. 2:177-190.
Karami O, Saidi A (2010). The molecular basis for stress-induced acquisition of somatic embryogenesis. Mol. Biol. Rep. 37:2493-2507.
Kevers C, Le Gal N, Monteiro M, Dommes J, Gaspar T (2000). Somatic embryogenesis in *Panax ginseng* in liquid cultures: a role for polyamine and their metabolic pathways. Plant Growth Regul. 31: 209-214.
Kieffer M, Stern Y, Cook H, Cleric E, Maulbetsch C, Laux T, Davies B (2006). Analysis of the Transcription Factor WUSCHEL and Its Functional Homologue in Antirrhinum Reveals a Potential Mechanism for Their Roles in Meristem Maintenance. Plant Cell 18:560-573.
Kinoshita A, Betsuyaku S, Osakabe Y, Mizuno S, Nagawa S, Stahl Y, Simon R, Yamaguchi-Shinozaki K, Fukuda H, Sawa S **(**2010). RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in Arabidopsis. Development 137:3911-3920.
Kothari SL, Joshi A, Kachhwaha S, Ochoa-Alejo N (2010). Chili peppers- A review on tissue culture and transgenesis. Biotechnol. Adv. 28(1):35-48.
Kumar V, Sharma A, Narasimha-Prasad BC, Gururaj HB, Giridhar P, Ravishankar G (2007). Direct shoot bud induction and plant regeneration in *Capsicum frutescens* Mill.: influence of polyamines and polarity. Acta Physiol. Plant. 29:11-18.
Kumar-Sharma S, Millam S (2004). Somatic embryogenesis in *Solanum tuberosum* L.: a histological examination of key developmental stages. Plant Cell Rep. 23:115-119.
Kumar-Sharma S, Millam S, Hedley PE, McNicol J, Bryan GJ (2008). Molecular regulation of somatic embryogenesis in potato: an auxin led perspective. Plant Mol. Biol. 68(1-2):185-201.
Laux T, Mayer KFX, Berger J, Jurgens G (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development 122:87-96.
Lenhard M, Jurgens G, Laux T (2002). The wuschel and shoot meristem less genes fulfill complementary roles in Arabidopsis shoot meristem regulation. Development 129:3195-3206.
Lenhard M, Laux T (2003). Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. Development 130:3163-3173.
Lin H, Niu L, McHale NA, Ohme-Takagi M, Mysore KS and Tadege M (2013). Evolutionary conserved repressive activity of WOX proteins mediates leaf blade outhgrowth and floral organ development in plants. PNAS 110(1):366- 371.
Lippert D, Zhuang J, Ralph S, Ellis DE, Gilbert M, Olafson R, Ritland K, Ellis B, Douglas CJ, Bohlmann J (2005). Proteome analysis of early somatic embryogenesis in *Picea glauca*. Proteomics 5:461-473.
Livak K, Schmittgen T (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-[delta]-[delta] Ct mehod. Methods 25(4):402-408.
Lohmann JU, Hong RL, Hobe M, Busch MA, Parcy F, Simon R, Weigel D (2001). A molecular link between stem cell regulation and floral patterning in Arabidopsis. Cell 105:793-803.
Lopez-Puc G, Canto-Flick A, Barredo-Pool F, Zapata-Castillo P, Montalvo-Peniche MC., Barahona-Pérez F, Santana-Buzzy N (2006). Direct somatic embryogenesis: a highly efficient protocol for in vitro regeneration of habanero pepper (*Capsicum* chinense Jacq.). HortScience 41:1645-1650.
Mayer KFX, Schoof H, Haecker A, Lenhard M, Jurgens G, Laux T (1998). Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell 95:805-815.
Merkle SA, Parrott WA, Flinn BS (1995). Morphogenetic aspects of somatic embryogenesis. *In vitro* Embryogenesis Plants 20:155-203.
Murashige T, Skoog L (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.
Nakata M, Matsumoto N, Tsugeki R, Rikirsch E, Laux T, Okada K (2012). Roles of the middle domain-specific WUSCHEL-REALTED HOMEOBOX genes in early development of leaves in Arabidopsis. Plant Cell 24(2):519-535.
Palovaara J, Halberg H, Stasolla C, Luit B, Hakman I (2010b). Expression of a gymnosperm PIN homologous gene correlates with auxin immunolocalization pattern at cotyledon formation and in demarcation of the procambium during *Picea abies* somatic embryo development and in seedling tissues. Tree Physiol. 30:479-489.
Palovaara J, Hallberg H, Stasolla C, Hakman I (2010a). Comparative expression patterns analysis of WUSCHEL-related homeobox 2 (WOX2) and WOX8/9 in developing seeds and somatic embryos of the gymnosperm *Picea abies*. New Phytol. 188:122-135.
Paul A, Mitter K, Sen-Raychaudhuri S (2009). Effect of polyamines on in vitro somatic embryogenesis in *Momordica charantia* L. Plant Cell Tissue Organ Cult. 97:303-311.
Qu LJ, Zhu YX (2006). Transcription factor families in Arabidopsis: Major progress and outstanding issues for future research. Curr. Opin. Plant Biol. 9:544-549.
Rastogi R, Sawhney VK (1990). Polyamines and flower development in the male sterile stamenless-2 mutant of tomato (*Lycopersicon esculentum* Mill.): II. effects of polyamines and their biosynthetic inhibitors on the development of normal and mutant floral buds cultured in vitro. Plant Physiol. 93:446-452.
Rodriguez APM, Wetzstein HY (1994). The effect of auxin type and concentration on pecan (*Carya illinoinensis*) somatic embryo morphology and subsequent coversion into plants. Plant Cell Rep. 13(11):607-611.
Santana-Buzzy N, Canto-Flick A, Barahona-Pérez F, Montalvo-Peniche MC, Zapata-Castillo P, Solís-Ruíz A, Zaldívar-Colli A, Gutiérrez-Alonso O and Miranda-Ham ML (2005). Regeneration of habanero pepper (*Capsicum chinense* Jacq.) via organogenesis. HortScience 40:1-3.
Schiavone FM, Cooke TJ (1987). Unusual patterns of somatic embryogenesis in the domesticated carrot: developmental effects of exogenous auxins and auxin transport inhibitors. Cell Differ. 21(1):53-62.
Shimizu R, Ji J, Kelsey E, Ohtsu K, Schnable PS, Scanlon MJ (2009). Tissue Specific and Evolution of Meristematic WOX3 Function. Plant Physiol. 149:841-850.
Silveira V, Santa-Catarina C, Tun NN, Scherer GFE, Handro W, Guerra MP, Floh EIS (2006). Polyamine effects on the endogenous polyamines contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O Ktze. Plant Sci. 171:91-98.
Slocum RD (1991) Tissue and subcellular localization of polyamines and enzymes of polyamines metabolism. In: R.D. Slocum, H.D. Flores (eds). Biochemistry and Physiology of Poliamines in Plants. CRC Press, Boca Ratón R.L. pp. 93- 104.
Smith MA, Davies PJ, Reid JB (1985). Role of polyamines in gibberellin-induced internode growth in peas. Plant Physiol. 78:92-99.
Steeves TA, Sussex IM (1989). Patterns in plant development. 2nd Ed. Cambridge University Press. p. 388.
Steiner N, Santa-Catarina C, Silveira V, Floh EIS, Guerra MP (2007). Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. Plant Cell Tissue Organ Cult. 89: 55-62.
Steinitz B, Kusek M, Tabib Y, Paran I, Zelcer A (2003). Pepper (*Capsicum annuum* L.) regenerants obtained by direct somatic embryogenesis fail to develop a shoot. *In Vitro* Cell. Dev. Biol. 39: 296- 303.
Stipp LCL, Januzzi- Mendes BM, Stefano-Piedade SMD, Martinelli-Rodriguez AP (2001). In vitro morphogenesis of *Cucumis melo* var. Indorus. Plant Cell Tissue Organ Cult. 65(1):81-89.
Stuurmann J, Jäggi F, Kuhlemeier C (2002). Shoot meristem maintenance is controlled by a GRAS-gene mediated signal from differentiating cells. Genes Dev. 16:2213-2218.
Su YH, Zhao XY, Liu YB, Zhang CL, O´Neill SD, Zhang XS (2009). Auxin-induced WUS expression is essential for embryogenic stem cell renewal during somatic embryogenesis in Arabidopsis. Plant J. 59:448-460.

Taiz L, Zeiger E (2002). Plant Physiology. 3rd Ed. Sinauer Associates.
Takahashi T, Kakehi J (2010). Polyamines: ubiquitous polycations with unique roles in growth and stress responses. Ann. Bot. 105:1-6.
Takeda S, Aida M (2010). Establishment of the embryonic shoot apical meristem in Arabidopsis thaliana. J. Plant. Res. 124:211-219.
Takeda T, Hayawaka F, Oe K, Matsuoka H (2002). Effects of exogenous polyamines on embryogenic carrot cells. Biochem. Eng. J. 12:21-28.
Thu-Hang P, Bassie L, Safwat G, Trung-Nghia P, Christou P, Capell T (2002). Expression of a heterologous S-adenosylmethionine decarboxylase cDNA in plants demonstrates that changes in S-adenosyl-L-methionine decarboxylase activity determine levels of the higher polyamines spermidine and spermine. Plant Physiol. 129:1744-1754.
Ueda M, Laux T (2012). The origin of the plant body axis. Curr. Opin. Plant Biol. 15:578-584.
Us-Camas R, Rivera-Solís G, Duarte-Aké F, De-la-Peña C (2014). In vitro culture: an epigenetic challenge for plants. Plant Cell Tissue Organ Cult. 18(2):187-201.
Van der Graaff E, Laux T, Rensing S (2009). The WUS homeobox containing (WOX) protein family. Genome Biol. 10(12):248.
Vandenbussche M, Horstmann A, Zethof J, Koes R, Rijpkerna AS, Gerats T (2009). Differential Recruitment of WOX Transcription Factors for Lateral Development and Organ Fusion in Petunia and Arabidopsis. Plant Cell 21:2269-2283.
Vanneste S, Friml J (2009). Auxin: A Trigger for Change in Plant Development. Cell 136:1005- 1016.
Walden R, Cordeiro A, Tiburcio AF (1997). Polyamines: Small Molecules Triggering Pathways in Plant Growth and Development. Plant Physiol. 113:1009-1013.
Wan H, Yuan W, Ruan M, Ye Q, Wang R, Li Z, Zhou G, Yao Z, Zhao J, Liu S, Yang Y (2011). Identification of reference genes for reverse transcription quantitative real-time PCR normalization in pepper (*Capsicum* annuum L.). Biochem. Biophys. Res. Commun. 416:24-30.
Yadav JS, Rajam MV (1998). Temporal regulation of somatic embryogenesis by adjusting cellular polyamine content in eggplant. Plant Physiol. 116: 617-625.
Zhang Y, Wu R, Qin G, Chen Z, Gu H, Qu LJ (2011). Over-expression of WOX1 Leads to Defects in Meristem Development and Polyamine Homeostasis in Arabidopsis. J. Int. Plant Biol. 53(6):493-506.