Physiol Mol Biol Plants (April–June 2013) 19(2):283–288
DOI 10.1007/s12298-012-0160-4

SHORT COMMUNICATION

TDZ pulsing evaluation on the in vitro morphogenesis of peach palm

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Published online: 29 January 2013

Abstract Peach palm (*Bactris gasipaes* Kunth.) cropping is an excellent alternative to native species exploitation; nevertheless, the problems with seed germination and conventional propagation justify the use of in vitro culturing. Aiming to asses TDZ pulsing effect on *B. gasipaes* morphogenesis, explants obtained from unarmed microplants were maintained in two treatments, half of them in MS free medium (without growth regulator) and the other half in MS with TDZ (0.36 μM). Both groups were transferred to growth regulator-free MS medium following 14 days of culture. After 84 days of culture, TDZ pulsing increased the growth and development of the shoots, restricted the growth and development of the roots, with no influence on adventitious bud induction or somatic embryogenesis. Furthermore, development of prickles, thickening of roots and chlorotic leaves were noted under TDZ pulsing. Leaf sheath histological analysis showed an epidermal origin and no vascularization of these prickles.

Keywords *Bactris gasipaes* · Micropropagation · Thidiazuron · Organogenesis · Prickles

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Introduction

Peach palm (*Bactris gasipaes* Kunth.) cropping for heart-of-palm and fruit production has relevance in Tropical America, for both subsistence and commercial purposes (Clement and Manshardt 2000). Moreover, it is an alternative to the exploitation of native species.

Although palms are considered recalcitrant (Sarasan et al. 2002; Steinmacher et al. 2007), *B. gasipaes* in vitro regeneration through organogenesis and/or direct somatic embryogenesis has provided satisfactory results (Almeida and Kerbauy 1996; Almeida and Almeida 2006; Almeida et al. 2012). However, further research is needed to optimize the propagation techniques.

Thidiazuron (TDZ) shows similar activity to cytokinins (Mok et al. 1982; Mok and Mok 2001) and also induces auxin-like responses (Murthy et al. 1995; Jones et al. 2007). It enhances in vitro vegetative propagation in several species, both by the development of adventitious buds (Wilhelm 1999;

Almeida et al. 2012) and by the induction of somatic embryos (Hutchinson et al. 1996; Zhang et al. 2001; Almeida et al. 2012). Nevertheless, in vitro usage of this cytokinin is unfavorable to shoot elongation (Gill and Ozias-Atkins 1999; Ahmad et al. 2006), normal development of leaves (Nieuwkerk and Zimmerman 1986) and rooting (Hutchinson et al. 1996; Jaiswal and Sahwney 2006).

The unfavorable action of TDZ on shoot and root elongation is caused by shifting the metabolism of endogenous growth regulators (Hutchinson et al. 1996), particularly natural endogenous cytokinins, by inhibiting their degradation (Hare and Van Staden 1994) and by promoting their expression (Mok et al. 1987). This highlights the need to transfer explants cultured in the presence of TDZ to growth regulator-free medium to promote shoot elongation and root induction (Ahmad et al. 2006).

Pulsing techniques involve reducing the explants' exposure time to growth regulators (Andrade et al. 2006; Aasim 2010), minimizing the required time to produce micropropagated plants (Pullman et al. 2003; Andrade et al. 2006). This study aimed to evaluate the effect of TDZ pulsing on the in vitro peach palm morphogenesis.

Material and methods

Plant material and culture conditions

Peach palm (*Bactris gasipaes* Kunth-Araceae) plants, obtained of seeds from the Yurimáguas provenance, were used as explants. Twenty seedlings were selected and cultivated in basal medium (MS – Murashige and Skoog 1962, modified by Almeida and Almeida 2006), followed by direct organogenesis induction on basal medium supplemented with α-naphthaleneacetic acid (NAA, 12.9 μM) and 6-benzylaminopurine (BAP, 3.55 μM). Subcultures were done twice, every 90 days, alternating with basal medium (Graner 2009). Seedling selection was based on the absence of prickles (unarmed), uniform development, 8.0±0.1 cm of shoot length, two pinnate leaves, a developed root system and no noticeable propagules. Aerial portions and roots were removed, preserving only the shoot apical meristems and keeping the explant size between 1.0 and 2.5 cm. These explants were grown in 2.5×15 cm test tubes containing 10 ml of liquid basal medium (control) or liquid basal medium supplemented with TDZ (0.36 μM) for 14 days of pulsing, followed by subculture in liquid basal medium. All medium was renewed every 28 days. The culture medium pH was adjusted to 5.8 and autoclaved at 121 °C for 20 min (≈1 kgfcm^{-2}). All plant material was kept in a growth chamber at 25±2 °C under a 16 h photoperiod at 42 μmol$m^{-2}s^{-1}$ light irradiance.

Data collection

Data were collected at 84 days of culture. The root number (RN), root length (RL), rooting (RT), adventitious bud number (ABN), somatic embryo number (SEN), shoot length (SL) and leaf number (LN) were measured for each explant. SL measurement was performed from the two largest expanded leaves to the proximal region of the stem base.

Histological analyses

Leaf sheath samples taken from microplants developed under TDZ pulsing were analyzed following the procedures detailed in Almeida et al. (2012).

Experimental design and data analysis

The experiment was carried out under a completely randomized design with two treatments, with ten replicates. The collected data were analyzed by Wilcoxon-Mann–Whitney test and exact logistic regression test for RT.

Results and discussion

The TDZ pulsing promoted RT in only 10 % of the explants (P=0.0198), a very low number compared to the 70 % rooting observed in the control treatment. For both RN and RL, pulsing was also inferior (P=0.0126 and 0.0198, respectively). Additionally, the formation of thick and unbranched roots was noted. In the control treatment, fine and branched roots were developed (Figs. 1 and 2a–c), which, according to Sommer and Caldas (1981) and Mohammed et al. (1992), promotes survival during the acclimatization process. This inhibitory effect on rooting was previously observed in *B. gasipaes* with the continued presence of TDZ in the culture medium for 140 days (Graner 2009).

Because TDZ is a highly stable compound and is resistant to oxidases (Mok et al. 1987; Murthy et al. 1995), it most likely caused residual effects on the tissues of the proximal region of the explants, inhibiting rooting when transferred to growth regulator-free medium (Figs. 1 and 2b). Therefore, it is possible that *B. gasipaes* requires a shorter exposure to the TDZ to promote root development.

Although TDZ application inhibits root elongation (Murthy et al. 1995), we observed the thickening of roots during in vitro culture (Fig. 2b–d), a phenomenon not reported to date and related to a low survival of adventitious roots during acclimatization (Smith and McClelland 1991; Barry-Etienne et al. 2002).

Both shoot length (SL) (P<0.001) and leaf number (LN) (P<0.001) were higher in explants grown under exposure to

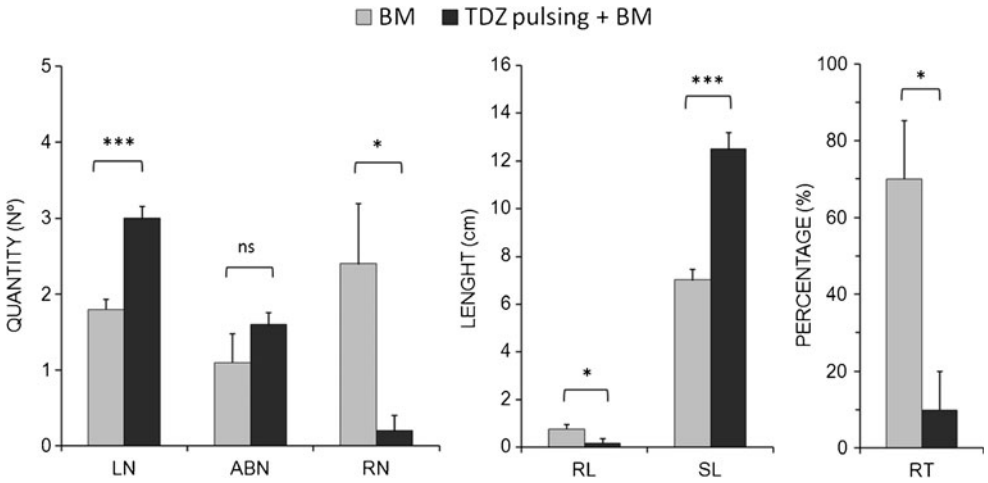

Fig. 1 Mean values and standard error of the mean for leaf number (*LN*), adventitious bud number (*ABN*), root number (*RN*), root length (*RL*) and shoot length (*SL*) in centimeters (cm) and rooting (*RT*) as a percentage (%). ns, not significant, * $P \leq 0.05$, ** $P \leq 0.01$ and ***, $P \leq 0.001$ by the Wilcoxon-Mann–Whitney or exact logistic regression test. BM = basal medium, TDZ pulsing = 14-day TDZ pulsing (0.36 mM)

TDZ compared to those grown in basal medium (Fig. 1). Furthermore, the results were nearly double those obtained by Graner (2009) for these parameters, under continued presence of TDZ.

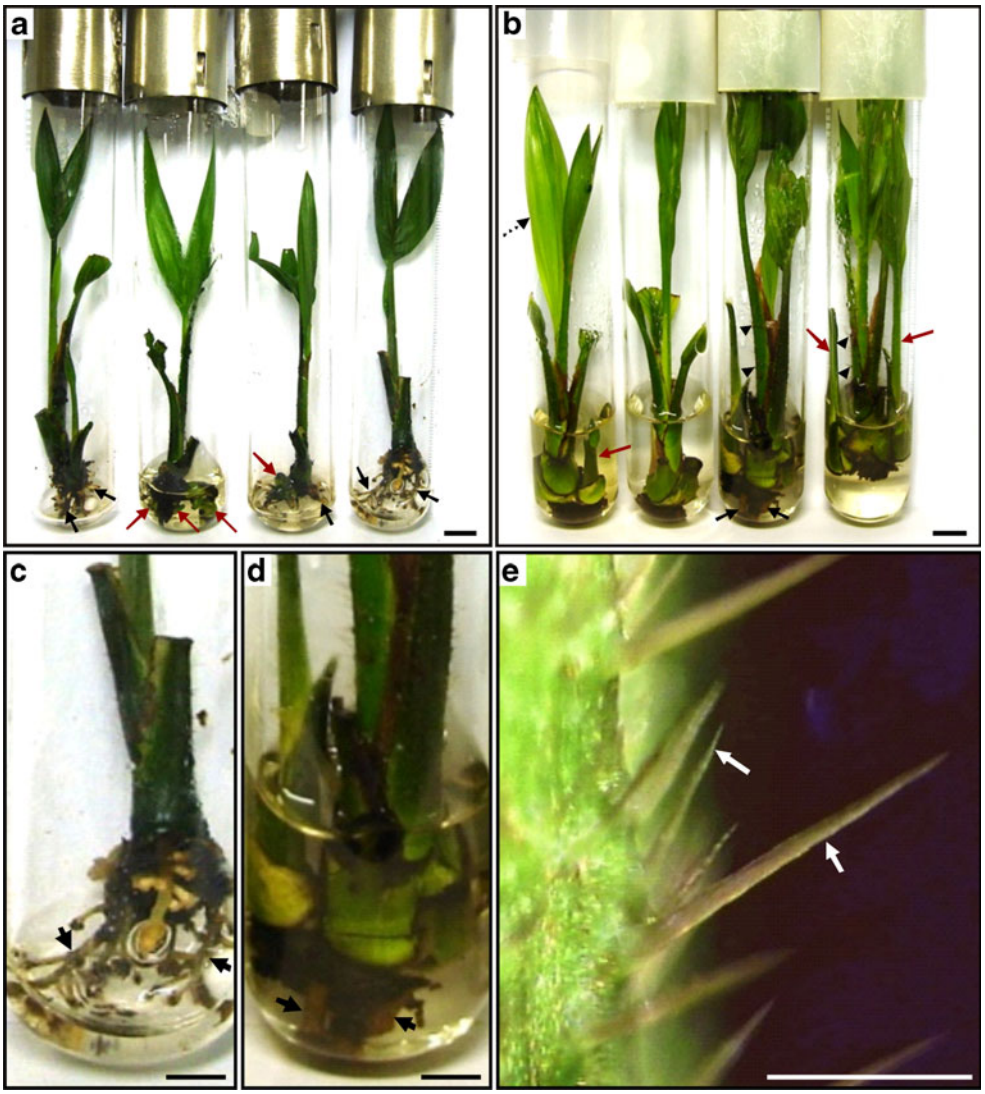

Fig. 2 *B. gasipaes* explants at the 84th day of in vitro cultivation. **a** In TDZ-free medium, showing reduced quantity of leaves without morphological abnormalities, small bud induction (*red arrows*) and elongated, thin, branched adventitious roots (*black arrows*).**b** TDZ-pulsed explants showing a large number of leaves with large dimensions and chlorosis (*dashed black arrow*), elongated buds (*red arrows*) and poorly developed root systems. Thick, unbranched roots (*black arrows*) and prickles on the outer sheath of the petiole (*arrow heads*). **c** Root system (*black arrows*) developed in TDZ-free medium. **d** Root system (*black arrows*) developed under TDZ pulsing. **e** Detail of prickles (*white arrows*). *Bars*: **a** and **b**=1.0 cm; **c** and **d**=0.5 cm; **e**=0.1 cm

Adventitious bud induction by TDZ has been reported in others species (Wilhelm 1999; Nitnaware et al. 2011); however, in this experiment, significant differences for this characteristic were not found between treatments (P= 0.1606) (Fig. 1), showing that the induction time was not enough to stimulate development. Nonetheless, the TDZ pulsing did not promote the development of short shoots, minimizing the negative effects caused on ABN by continuous exposure to TDZ (Graner 2009) (Fig. 2b).

Even though the use of TDZ in culture medium for somatic embryo induction via direct morphogenesis has been described for several species, (Hutchinson et al. 1996; Zhang et al. 2001) in our study, and as previously reported by Graner (2009), no somatic embryos were observed in either culture condition.

Several in vitro morphological changes in the leaves caused by TDZ addition to culture medium have been reported. In apple (*Malus domestica* Borkh), high concentrations promote tissue necrosis, vitrification and abnormal growth of leaves (Nieuwkerk and Zimmerman 1986). In peach palm under prolonged TDZ exposure, Graner (2009) noted the presence of large chlorotic or dark-green leaves with deformation of veins. Under our TDZ pulsing treatment, large and sometimes chlorotic leaves were noticed (Fig. 2b), the latter probably related to nutritional deficiencies (Deenik et al. 2000) and possibly promoted by the use of the cytokinin, as observed by Murthy et al. (1995).

All external sheaths and buds of the explants developed prickles when grown under TDZ exposure (Fig. 2b–d), while control treatment remains unarmed (Fig. 2a). Prickle development in *B. gasipaes* progenies of unarmed origins in in vivo conditions was reported by Chavez Flores et al. (1990), who suggested that endogenous factors affect prickle expression during plant development.

Even though the term "spines" is usually used in the literature, the leaf sheath histological analysis showed an epidermal origin and no vascularization (Fig. 3a–b), as previously reported by Tomlinson (1962), justifying its replacement by the term "prickle". Histological analysis in TDZ-pulsed explants showed that these structures are multicellular, unbranched (Fig. 3a) and originate from the meristematic activity of leaf sheath epidermal dedifferentiation by anticlinal cell divisions (Fig. 3b). While in TDZ-free medium no meristematic activity was observed (Fig. 3c).

Histone modification and DNA methylation are epigenetic mechanisms that have a key role in the regulation of gene activation and silencing (Jenuwein and Allis 2001; Gan et al. 2007). These events are often induced by stress (Boyko and Kovalchuk 2011), an effect that has been ascribed to TDZ in vitro (von Aderkas and Bonga 2000; Mamaghani et al. 2009). Therefore, we can infer that TDZ activated a silenced gene (or a group of genes according to Clement and Manshardt 2000) responsible for prickle development in unarmed peach palm microplants, featuring an epigenetic event.

The results of our study show that the exposure of peach palm explants to TDZ pulsing for 14 days promoted the growth and development of shoots, greatly restricted the growth and development of roots but had no influence on adventitious bud or somatic embryo induction. Moreover, prickle development, root thickening and leaf chlorosis under TDZ exposure were observed.

Keeping in mind that poor root development and prickle development should be avoided in TDZ application, the testing of shorter pulsing times could help to solve these problems while keeping its potential to promote shoot growth and development.

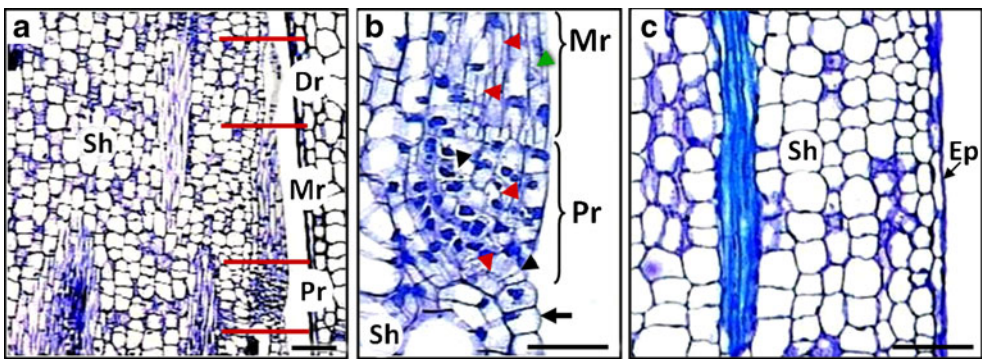

Fig. 3 Leaf sheath longitudinal histological sections of *B. gasipaes* explants at the 84th day of in vitro cultivation. **a** In TDZ pulsing treatment showing an unbranched multicellular prickle with the proximal multiseriate, middle multiseriate and distal uniseriate region denoted by red bars, **b** A magnified view of the proximal and middle region displaying no vascularization, the epidermal origin of the prickle (*black arrow*), anticlinal divisions (*black arrowheads*), periclinal divisions (*red arrowheads*) and oblique divisions (*green arrowhead*). **c** In TDZ-free medium with no meristematic activity in epidermal cells. Sheath (*Sh*), distal region (*Dr*), middle region (*Mr*), proximal region (*Pr*), Epidermis (*Ep*). *Bars*: **a**=100 μm; **b** and **c**= 50 μm

Acknowledgements The authors thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the Escola Superior de Agricultura"Luiz de Queiroz" - University of São Paulo (ESALQ/USP), FINEP (Financiadora de Estudos e Projetos), Inaceres Agrícola and *InVitro Palm* (Consulting, Study and Biological Development Ltda) for support.

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