

A protocol for rapid in vitro propagation of genetically diverse pitaya

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Abstract An efficient propagation system of pitaya with diverse genetic background was established by using tender stem as explants. Various types of plant growth regulators (PGRs) were used to determine the most effective hormone combination for shoot proliferation. Zeatin (ZT), thidiazuron (TDZ), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 6-benzylaminopurine (6-BA) alone could induce multiple shoots. In that case, the most shoots per explant i.e. 4.6 (13.68 μM ZT), 4.8 (0.11 μM TDZ), 4.3 (17.76 μM 6-BA) and 3.2 (0.23 μM 2, 4-D) were obtained. The best PGRs combination was MS medium with 3.0 μM ZT and 0.5 μM IBA producing the most shoots per explant and the most vigorous shoots. Of the two varieties and six superior selections cultured on the best PGRs combination, more than 6.0 shoots per explant were obtained. No polymorphism was detected among in vitro-derived plantlets selected at random after 11 sub-cultures. Those results showed that MS media with 13.68 μM ZT and 2.46 μM IBA was suitable for shoot propagation of diverse pitaya varieties and selections. The protocol can be used for large-

scale propagation of pitaya to meet the demand of increasing commercial cultivation.

Keywords *Hylocereus* · Genetic diversity · Propagation · Genetic fidelity · SCoT

Pitaya (dragon fruit) is the fruit produced by the plant with the same name belonging to the genus *Hylocereus* of the Cactaceae. It contains glucose, betalains, vitamins, organic acids, soluble dietary fiber, phyto albumins and minerals (Stintzing and Carle 2007; Nurliyana et al. 2010; Wichienchot et al. 2010; Liaotrakoon, 2013a). Pitaya is not only utilized as fruit, but also can be taken as flower, vegetable, health products and medicine. The fruit is delicious and can be processed into juice, jam, ice cream, pastries, vinegar and wine. Dragon fruit seed oils are a good source of essential fatty acids and tocopherols, with a high oxidative stability (Liaotrakoon et al. 2013b). Pitaya flowers and vines can be used as vegetables and herbs while peels are used to extract color ingredient for food and cosmetic industries (Esquivel et al. 2007; Harivandaran et al. 2008). Pitaya is also considered a potential economic crop for harsh conditions such as drought, heat, and poor soil (Mizrahi and Nerd 1999). It has drawn much attention and growing areas of pitaya are increasing rapidly in many countries due to its economic potential and nutritional benefits (Le Bellec et al. 2006; Esquivel et al. 2007; Ortiz-Hernández and Carrillo-Salazar 2012).

Pitaya is mainly propagated by cuttings (Elobeidy 2006; Cavalcante and Martins 2008). However, the method is inefficient, time-consuming and susceptible to damping-off. Tissue culture is an efficient method for rapid propagation of plants, and to obtain healthy and pathogen-free plants in a relatively short time and minimal space using

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very few starting materials. Currently, several researchers have established protocols for propagation of pitaya (Infante 1992; Drew and Azimi 2002; Mohamed-Yasseen 2002; Pelah et al. 2002; Viñas et al. 2012; Fan et al. 2013). However, pitaya with diverse genetic backgrounds respond differently to the same culture medium, probably due to genetic conditioning (Drew and Azimi 2002; Mohamed-Yasseen 2002; Fan et al. 2013). Improved protocols are needed to better suit the genetic diversity of this crop. Therefore, it is essential to develop an efficient propagation procedure for pitaya with genetic diversity. In this study, an efficient protocol for in vitro propagation and acclimatization was developed for eight genetically diverse pitaya. Genetic fidelity of in vitro-derived plantlets was assessed by Start Codon Targeted (SCoT) marker.

Two varieties and six superior selections with four flesh colors and excellent quality were used as materials (Supplementary Fig. 1). Young vines, without removing thorns, were washed in running tap water for 30 min and cut into segments (4.0–5.0 cm). Segments were surface disinfected in 75 % (v/v) ethanol for 1 min, then 0.1 % (w/v) HgCl₂ for 10 min followed by rinsing five times with sterile water. After discarding both ends, segments were vertically inserted in MS medium (Murashige and Skoog 1962) without plant growth regulators (PGRs). New shoots were subcultured on the same medium through a few cycles to produce materials for experiments. Explants (0.5 cm) were placed vertically on MS media containing 3 % sucrose, 0.8 % agar, and 0–31.93 μM ZT (Genview, USA), 0–13.62 μM TDZ (Sigma, USA); 0–13.57 μM 2, 4-D (Beijing Dingguo Changsheng Biotechnology Co. Ltd, China); 0–31.08 μM 6-BA (Beijing Dingguo Changsheng Biotechnology Co. Ltd, China). Unless otherwise specified, superior selection ‘10–2’ was used as a model system to screen for the optimum PGRs combination. Responses of two varieties and six superior pitaya selections were subsequently characterized on the optimum PGRs combination. All cultures were maintained at 25 ± 2 °C under a light–dark cycle of 16:8 with a light intensity of 50 μE m⁻² s⁻¹. The elongated shoots (approximately 2.5 cm) were rooted in MS media having 1.23–2.46 μM IBA (Sigma, USA) or 0.54–1.34 μM NAA (Beijing Dingguo Changsheng Biotechnology Co. Ltd, China) (data not published yet). Rooted plantlets were removed after 30 days and kept in large Petri dishes with 10 ml for 7–10 days. Thereafter they were transferred to peat moss and kept in room temperature for 90 days and finally transferred to soil for acclimatization.

Thirty randomly-selected plantlets from 11 sub-cultures cycles, as well as the parental plants (control), were used to assess their genetic fidelity by SCoT marker. The PCR parameters were performed according to the procedure of Xia et al. (2014). PCR products were examined by ethidium bromide staining 2.0 % agarose gels.

All experiments were carried out in a completely randomized design thrice. Data analyses were performed using DPS 7.05 software. Differences of compared sets were considered significant using LSD test at $P \leq 0.05$.

TDZ, ZT, 6-BA and 2, 4-D are commonly used for shoot regeneration in many plant species (Ellis et al. 1991; Huettelman and Preece 1993). Micropropagation of pitaya has been achieved using TDZ or 6-BA alone (Mohamed-Yasseen 2002; Viñas et al. 2012; Fan et al. 2013), however, the role of ZT and 2, 4-D in pitaya propagation is not reported yet. In this study, we found that pitaya explants were more sensitive to TDZ than ZT, 6-BA and 2, 4-D (Supplementary Tables 1–4 and Supplementary Fig. 2). TDZ at as low as 0.11 μM resulted in formation of green compact callus at the base of the proximal part of explants which significantly inhibited shoot growth already formed (Supplementary Table 2 and Supplementary Fig. 2b). By increasing 6-BA, TDZ or ZT concentrations, shoot number per explant increased significantly while shoot heights were reduced significantly. Dual effects of 2, 4-D on pitaya propagation were detected. Lower concentrations of 2, 4-D (<0.45 μM) is beneficial to shoot propagation while higher concentration (≥ 0.45 μM) induced friable callus and hairy roots (Supplementary Table 4 and Supplementary Fig. 2d). The most shoot number per explant i.e. 4.6 (13.68 μM ZT), 4.8 (0.11 μM TDZ), 4.3 (17.76 μM 6-BA) and 3.2 (0.23 μM 2, 4-D) were obtained. However, the highest shoot production is not always the best and might lead to somaclonal variation. Higher concentrations of 6-BA, TDZ, ZT and 2, 4-D had negative effects on pitaya shoot propagation (Supplementary Tables 1–4 and Supplementary Fig. 2). ZT had a greater effect than that TDZ, 6-BA and 2, 4-D for the activation of axillary buds. Explants cultured on MS medium containing ZT gave better shoot production and elongation without callus induction, reducing the time and labor involved.

Table 1 Effects of plant growth regulators on shoot propagation of superior selection ‘10–2’

Plant growth regulators (μM)			Shoot height (cm)	Shoot number per explant	Callus induction (%)
ZT	NAA	IBA			
0.00	0.00	0.00	2.2 a	1.3 d	0.0 b
13.68	0.00	0.00	1.1 c	4.7 b	0.0 b
13.68	0.54	0.00	1.3 c	3.3 c	14.3 a
13.68	1.34	0.00	1.2 c	3.6 bc	0.0 b
13.68	0.00	1.23	2.3 a	6.4 a	0.0 b
13.68	0.00	2.46	2.0 b	7.1 a	0.0 b

Different letters within the same column indicate significant differences (LSD test; $P < 0.05$)

Table 2 Responses of eight genetically diverse pitaya to 13.68 μM ZT and 2.46 μM IBA

Varieties/ superior lines*	Species	Pulp color	Average shoot number per explant	Average shoot height (cm)
Guangming no. 2	<i>H. undatus</i>	Red	6.3	2.7
81-1	<i>H. polyrhizus</i> ×		6.2	2.6
10-2	<i>H. undatus</i>		7.6	2.6
Hongbaoshi	<i>H. polyrhizus</i>	White	6.8	3.3
49-2	<i>H. polyrhizus</i> ×		6.0	2.7
93-2	<i>H. undatus</i>		7.1	2.6
97-3		Red without and white within	7.4	2.4
69-1		Pink	7.0	2.4

* 49-2, 93-2, 81-1, 10-2, 97-3 and 69-1 are superior selections from seedlings of Guangming no. 2 × Hongbaoshi

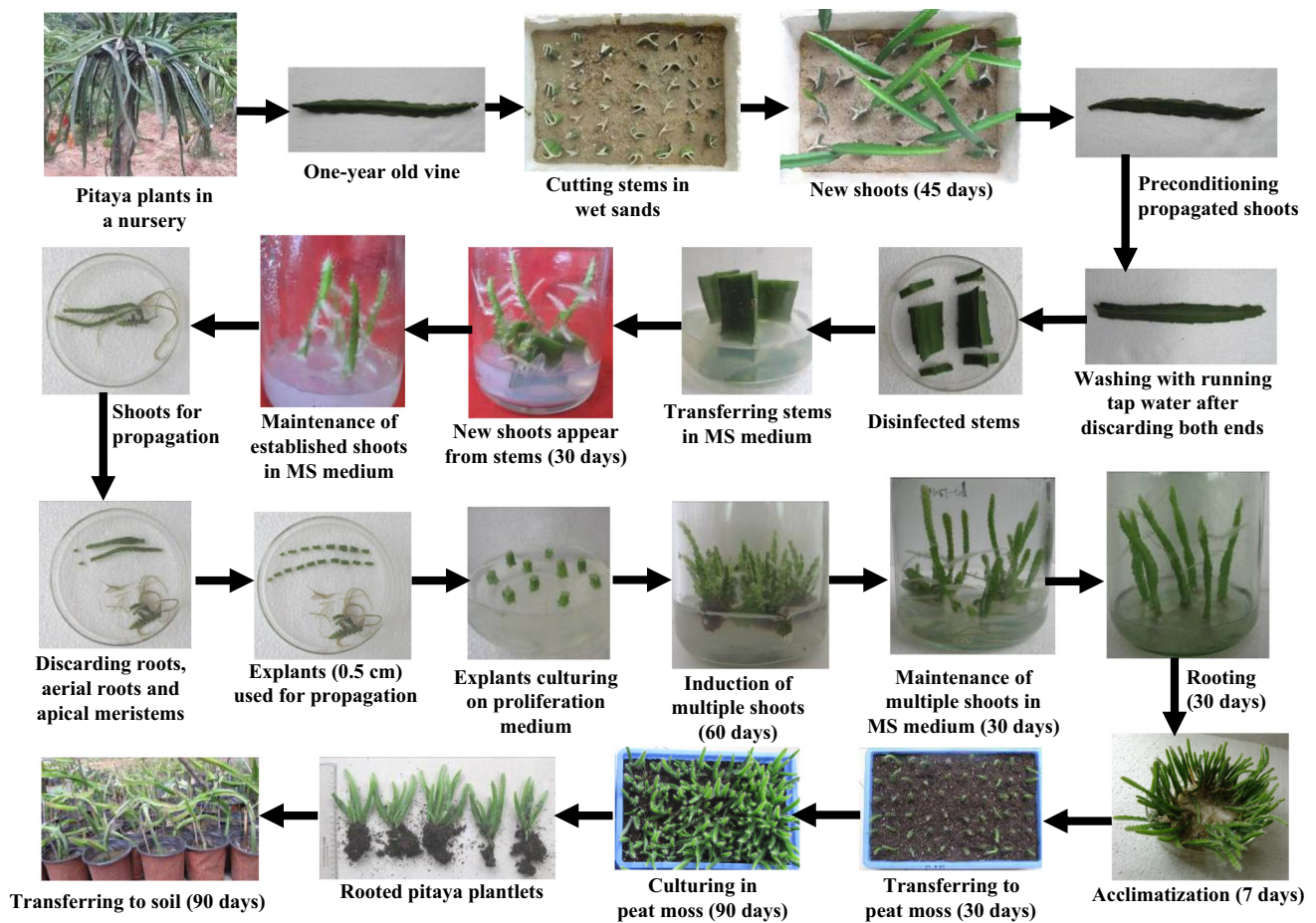


Fig. 1 A schematic representation of the pitaya in vitro propagation procedure

The formation of adventitious shoots is regulated by interactions between cytokinins and auxins. Mohamed-Yasseen (2002) reported that the best shoot proliferation of *H. undatus* was 0.5 mM TDZ and 0.5 mM NAA. However, Fan et al. found that the best medium for micropropagation of *H. undatus* was MS with 2.0 mM BA and 0.5 mM NAA. In this study, the highest shoot number per explant was obtained with 13.68 μM ZT and 2.46 μM IBA (Table 1

and Supplementary Fig. 3). Areoles began to swell after 5 days of culture (Supplementary Fig. 4b) and multiple buds emerged directly from areoles after 10 days of culture on the best PGRs combination, without an intermediate callus phase (Supplementary Fig. 4 c). Average shoots elongated to 2.0 cm and shoot number per explant was 7.1 after 60 days of culture (Supplementary Fig. 4 d–f). Of the two varieties and six superior pitaya selections tested, more

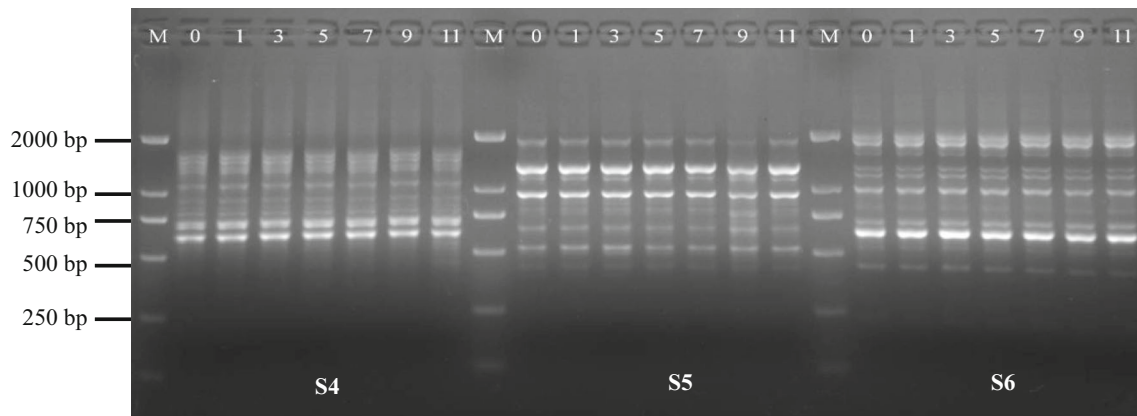


Fig. 2 SCoT analysis of the parental plant (superior selection ‘10–2’) and its in vitro-propagated plants after 11 subculture cycles using primer S4, S5 and S6. *MDL* 2000 DNA marker; *0* the parental plants;

1, 3, 5, 7, 9, 11 in vitro-propagated plants from subculture for *1, 3, 5, 7, 9* and *11* cycles

than 6.0 vigorous shoots per explant were obtained in 60 days on the best PGRs combination (Table 2 and Supplementary Fig. 5). Rooted plantlets were initially maintained in normal conditions then transferred to the field with 100 % survival rate. Figure 1 is a schematic representation of the pitaya in vitro propagation procedure, including the approximate time required for each step.

SCoT, a simple and novel DNA marker, has been used for genetic diversity analysis of micropropagated plants due to its high reproducibility and sensitivity (Agarwal et al. 2014; Rathore et al. 2014). Results from SCoT analyses showed that there was no polymorphic bands among propagated plants and parental plants (Fig. 2 and Supplementary Table 5), implying that high genetic fidelity can be guaranteed using the in vitro protocol.

This is the first report on the effects of ZT and 2, 4-D in pitaya propagation. The protocol of pitaya propagation in our experiments is to avoid shoot production from callus, and production of multiple shoots directly from areoles. The protocol has been applied in large-scale propagation of pitaya (Supplementary Fig. 6).

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