

# TDZ-induced Callus and Adventitious Shoot Formation from Leaf, Petiole and Calyx Explants of *Tigridiopalma magnifica* (Chen)

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## ABSTRACT

*Tigridiopalma magnifica* Chen is an endemic, critically endangered species found only in Guangdong Province, China. Unfortunately, economic exploitation has impacted severely on its natural environments in mountain areas in recent years, disrupting fragile plant communities and sharply decreasing distribution of the species. To better protect but also allow sustainable utilization of this critically endangered ornamental plant, we report on an efficient *in vitro* mass propagation and plant regeneration protocol from original *in vivo* explants. Callus and adventitious shoot formation was induced on calyx explants incubated on induction media (Murashige and Skoog) containing thidiazuron (TDZ) alone or in combination with 6-benzyladenine (BAP). Leaf and petiole explants from *in vitro* grown plants also induced callus and adventitious shoot formation on induction media containing 4.4-22.0  $\mu\text{M}$  TDZ alone or in combination with 2.25  $\mu\text{M}$  BAP or 5.37  $\mu\text{M}$   $\alpha$ -naphthaleneacetic acid (NAA), respectively. Our study shows that TDZ plays a crucial role in the induction of adventitious shoots from calyx, leaf and petiole explants. Once adventitious shoots were induced, shoots were able to be propagated and subcultured for mass proliferation on MS media containing 1.07  $\mu\text{M}$  NAA and 4.45  $\mu\text{M}$  BAP or 1.07  $\mu\text{M}$  NAA and 4.6  $\mu\text{M}$  cytokinin, respectively. Root formation was achieved on  $\frac{1}{2}$ MS medium containing 0.49-4.92  $\mu\text{M}$  3-indolebutyric acid or 0.54-5.37  $\mu\text{M}$  NAA; 99% of rooted plants survived establishment in soil.

**Keywords:** *Tigridiopalma magnifica* (Chen), thidiazuron, plant regeneration

**Abbreviations:** BAP, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; NAA,  $\alpha$ -naphthaleneacetic acid; KT, cytokinin; TDZ, thidiazuron

## INTRODUCTION

*Tigridiopalma magnifica* Chen is also called 'bear's paw' or 'large lotus pod' and was first found in the 1970's in Guangdong Province, China (Chen 1978). *Tigridiopalma* (Melastomaceae) is a monospecific genus and *T. magnifica* is the only known example of this Chinese endemic species (Xu 1998). It is found only in the Big Fog Mountain/Ehuangzhang Natural Protection Zones between Yangchun County and Xinyi County in Guangdong Province (Chen 1978; Wang *et al.* 2005). *T. magnifica* is currently listed as a critically endangered species (Chen 1984; Xing 2005), partly because its natural distribution area is very narrow (it is only found in an area of  $< 1.0 \text{ km}^2$  in the core zone of Ehuangzhang) but also due to increasing exploitation of adjacent original forest in recent years for cultivation of *Citrus* (orange) and fast-growing eucalypts. Cultivation of crops has resulted in a sharp decrease in the extremely fragile natural environment of *T. magnifica*; consequently, its continued existence in the wild can no longer be guaranteed. This unusual plant is highly adapted to its natural environment; it only grows on rocks in moist shadowy closed forested valleys where other understory plant species can seldom grow. Leaves of *T. magnifica* are large (up to 100 cm in diameter) when fully grown; and its flowers are small but brightly mauve coloured. *T. magnifica* has a very high ornamental and landscape value and can be used as an indoor ornamental plant (Zeng 2005; Zhao 2006). Propagation and regeneration of *T. magnifica* via seed sowing and immature leaf *in vivo* has been reported (Li *et al.* 2006; Zeng *et al.* 2008). However, adventitious shoot formation induced from

leaf, petiole or floral explants of *T. magnifica* has never been reported before. In this report we present details of an efficient shoot mass propagation and plant regeneration system in *T. magnifica* developed from calyx, leaf and petiole explants respectively via callus induction and adventitious shoot formation.

## MATERIALS AND METHODS

### Establishment of an adventitious shoot propagation system from floral explants

Plants of *T. magnifica* were grown in pots at the Introduction and Domestication Nursery of South China Botanical Garden, Guangzhou. Immature flowers (3-4 mm long) were collected in September (2006) and sterilized in 70% (v/v) alcohol for 10 s and 0.1% (w/v) mercuric chloride for 9 min then rinsed in sterilized water 3 times. Flowers were taken off the perianth and petal and inoculated on MS (Murashige and Skoog 1962) basal medium containing different plant growth regulators (PGRs) (Table 1). Each treatment consisted of 5 calyx explants/container (total of 30 explants/treatment) and the experiment was repeated within a week. Data from both experiments were pooled and statistically analyzed by one-way ANOVA with a *post-hoc* test (PLSD,  $P \leq 0.05$ ) to separate treatment means. Culture jars were placed in a culture chamber at  $25 \pm 2^\circ\text{C}$  with low lighting (PPFD =  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 14-h photoperiod). All media contained 30  $\text{g l}^{-1}$  sucrose with pH adjusted to 5.6 and solidified with 0.6% (w/v) agar (Guangdong Huankai Microbiological Scientific Company, Guangzhou city, China), then autoclaved at  $121^\circ\text{C}$  for 15 min.

**Table 1** Effects of PGR combination in induction media on callus and adventitious shoot formation from calyx explant of *T. magnifica*.

PGR combination in the induction media ( $\mu\text{M}$ )	Observed result	Number of shoots per explant
0	No response	0 a*
BAP 4.45	No callus, no shoot formation	0 a
TDZ 4.40	Few callus, shoot formation	11.8 c
TDZ 0.88 + BAP 4.45	Few callus, shoot formation	7.4 b
NAA 1.07+ BAP 4.45	Few callus, no shoot formation	0 a
2, 4-D 4.5+ BAP 4.45	Few callus, no shoot formation	0 a
NAA 5.37	Few callus, no shoot formation	0 a
2, 4-D 4.5	Few callus, no shoot formation	0 a

\* Means followed by the same letter in a column are not significantly different by the LSD test ( $P \leq 0.05$ ).

**Table 2** Adventitious shoot propagation of *T. magnifica* in different propagation media after culturing for 25 days and 45 days, respectively.

PGR combination in propagation media ( $\mu\text{M}$ )	Propagation index in different culture period (days)	
	25	45
BAP 4.45	2.6 b	6.0 b
BAP 4.45 + NAA 1.07	3.6 a	10.9 a
KT 4.6 + NAA 1.07	3.4 a	10.5 a
TDZ 0.88 + BAP 4.45	2.4 b	4.6 b
TDZ 0.88 + KT 4.6	2.5 b	5.1 b

Means followed by the same letter in a column are not significantly different by the LSD test ( $P \leq 0.05$ ).

### Propagation of multiple shoots

Adventitious shoots were transferred to the shoot propagation MS medium containing 1.07  $\mu\text{M}$   $\alpha$ -naphthaleneacetic acid (NAA) and 4.45  $\mu\text{M}$  6-benzyladenine (BAP) maintained in low light (as above) and subcultured every 45 days. Approximately 3 months later (2 subculture periods) shoot clumps were divided into smaller pieces and inoculated to different propagation media and subcultured for 45 days. The propagation indices of different culture media (Table 2) were investigated and calculated after a further 25 and 45 days, respectively. This procedure was repeated three times in total with treatment data pooled and analyzed statistically as before with mean propagation indices calculated.

### Induction of adventitious shoot formation from leaves and leafstalks *in vitro*

Immature leaves and petioles *in vitro* were cut into approx. 0.5  $\text{cm}^2$  in size (0.6-0.7 cm long) and inoculated on various media (Table 3). There were 5 culture jars/treatment, each containing 10 explants. Jars were incubated in the dark for 30 days and then transferred to light culture (as above). After a total of 50 days callus induction or adventitious shoot formation was investigated. The experiments were repeated twice and mean number of adventitious shoot was recorded and statistically analyzed as previously stated.

### Root formation and plantlet establishment in potting mixture

Adventitious shoots (2-3 cm high) regenerated on MS medium containing 1.07  $\mu\text{M}$  3-indolebutyric acid (NAA) and 4.45  $\mu\text{M}$  BAP were divided into a single shoots and placed in various rooting media containing  $\frac{1}{2}$  (half micro- and macronutrients) MS basal medium with zero PGR (control treatment), zero PGR + 0.2% (w/v) activated charcoal (AC), 1.07 or 5.37  $\mu\text{M}$  NAA, 0.98 or 4.92  $\mu\text{M}$  3-indolebutyric acid (IBA), or a combination of 1.07  $\mu\text{M}$  NAA + 2.46  $\mu\text{M}$  IBA (Table 3). Root formation was investigated after 2 months of culture. Plantlets were removed from jars and agar was rinsed off with tap water, then plantlets were transplanted into plastic pots containing humus soil and vermiculite mixture (1: 1). Potted plantlets were grown under greenhouse conditions (23-33°C) at the South China Botanical Garden without air condition and moistened with water every day. Plant survival rate was recorded after 40 days culture.

## RESULTS

### Induction of adventitious shoot formation from flower explants

Medium containing 4.4  $\mu\text{M}$  thidiazuron (TDZ) induced more compact white callus from calyx bases after 32-38 days while less of the same type of callus induction was observed on media containing 0.88  $\mu\text{M}$  TDZ and 4.4  $\mu\text{M}$  BAP during the same period. Over the next 1-2 weeks adventitious shoots were produced from this basal callus (Fig. 1A). Medium containing 4.4  $\mu\text{M}$  TDZ induced more adventitious shoots than medium containing 0.88  $\mu\text{M}$  TDZ and 4.45  $\mu\text{M}$  BAP (Table 1). Medium containing 1.07  $\mu\text{M}$  NAA and 4.45  $\mu\text{M}$  BAP induced some callus on explants; however no adventitious shoots were observed. On basal media containing 4.45  $\mu\text{M}$  BAP, 4.5  $\mu\text{M}$  2,4-D, 5.37  $\mu\text{M}$  NAA or 4.45  $\mu\text{M}$  BAP and 4.5  $\mu\text{M}$  2,4-D, respectively, neither callus nor adventitious shoots were induced and explants became brown and died gradually after 30 days in culture.

### Propagation of multiple shoots

Callus with adventitious shoots was transferred to medium containing 1.07  $\mu\text{M}$  NAA and 4.45  $\mu\text{M}$  BAP for further culture where more adventitious shoot formation occurred. Three months later enough multiple shoots were induced to be cut into small pieces and then inoculated on various propagation media. Media containing 1.07  $\mu\text{M}$  NAA and 4.45  $\mu\text{M}$  BAP or 1.07  $\mu\text{M}$  NAA and 4.6  $\mu\text{M}$  cytokinin (KT) induced more multiple shoots on media containing 4.45  $\mu\text{M}$  BAP, 0.88  $\mu\text{M}$  TDZ and 4.45  $\mu\text{M}$  BAP or 0.88  $\mu\text{M}$  TDZ and 4.6  $\mu\text{M}$  KT, respectively (Table 2). It was evident that calli were induced more frequently on medium containing 0.88  $\mu\text{M}$  TDZ and 4.45  $\mu\text{M}$  BAP and while some adventitious shoots gradually grew from callus tissue, somatic embryos were not observed.

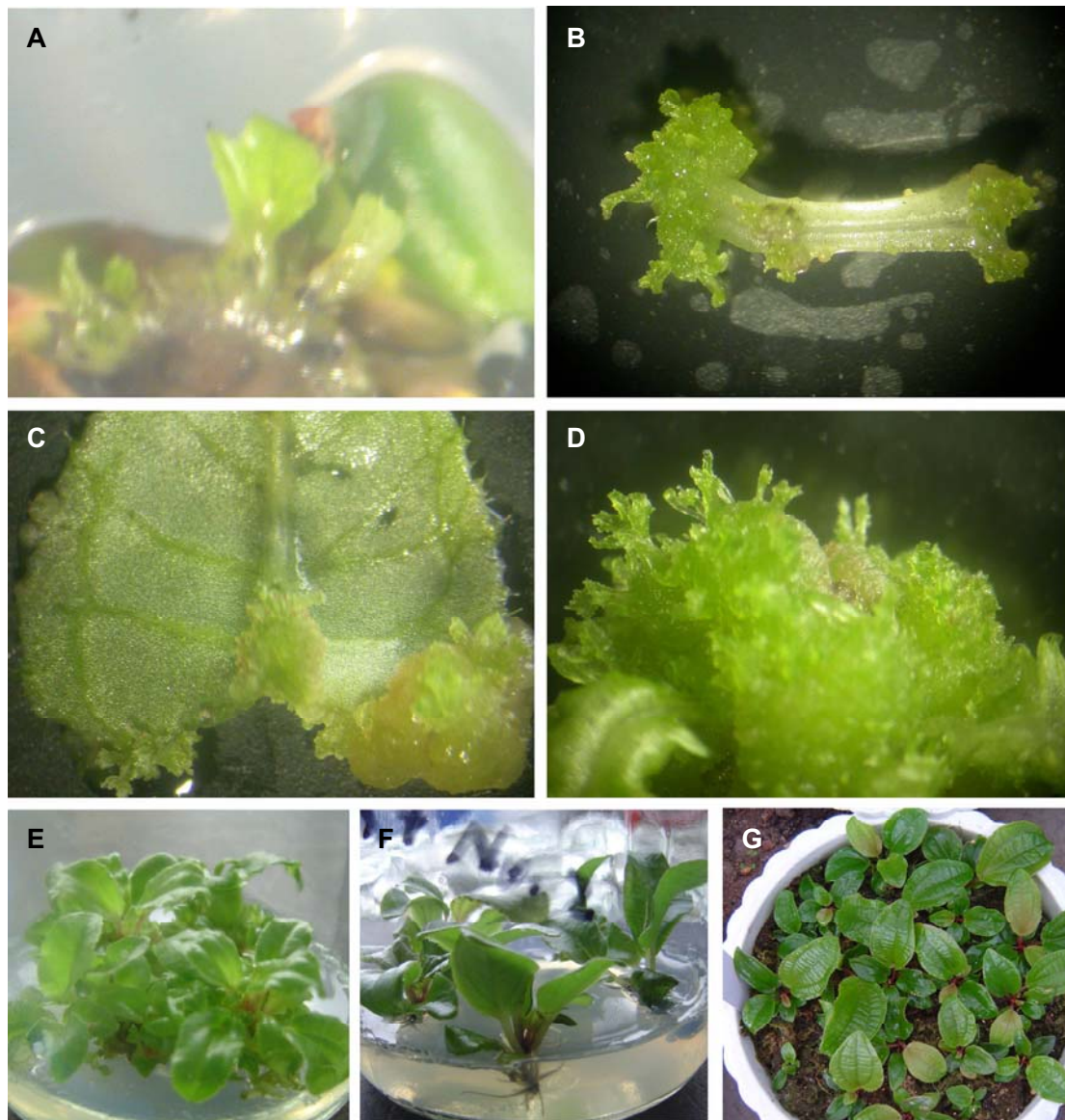
### Propagation of multiple shoots

Callus cultures with adventitious shoots were grown on

**Table 3** Effect of PGR combination in induction media on callus and adventitious shoot formation from immature leaf and petiole explants of *T. magnifica*.

PGR combination in the induction media ( $\mu\text{M}$ )	Leaf explants		Petiole explants	
	Observation result	Number of shoots/explant	Observation result	Number of shoots/explant
TDZ 4.4	Callus and adventitious shoots	10.9 b	Callus and adventitious shoots	19.6 b
TDZ 4.4 + NAA 5.37	Callus and adventitious shoots	15.2 b	Callus and adventitious shoots	16.2 b
TDZ 4.4 + BA 2.22	Callus and adventitious shoots	12.5 b	Callus and adventitious shoots	16.8 b
TDZ 22.0	Callus and adventitious shoots	28.5 c	Callus and adventitious shoots	18.6 b
NAA 5.37 + BA 8.90	Leaf expansion	0 a	Leaf stalk expansion	0 a
NAA 16.11	Root formation	0 a	Leaf stalk expansion and root formation	0 a
2,4-D 18.0	Color turned to be yellow	0 a	Leaf stalk expansion	0.1 a

\* Means followed by the same letter in a column are not significantly different by the LSD test ( $P \leq 0.05$ ).



**Fig. 1** Adventitious shoot formation induced from different explants of *T. magnifica*. (Bars = 0.2 mm). (A) Adventitious shoot formation induced from a calyx explant on MS + 0.88  $\mu\text{M}$  TDZ + 4.4  $\mu\text{M}$  BAP; (B) Callus and adventitious shoot formation on a petiole explant on MS + TDZ 4.4+NAA 5.37; (C, D) Callus and adventitious shoots induction on a leaf explant on MS + TDZ 4.4+BA 2.22; (E) Multiple shoot proliferation on MS + 1.07  $\mu\text{M}$  NAA +4.45  $\mu\text{M}$  BAP; (F) Root formation after culturing for two months on  $\frac{1}{2}$ MS medium containing 1.07  $\mu\text{M}$  NAA; (G) Rooted plantlets established in potting mixture (humus soil + vermiculite, 1: 1).

**Table 4** Root formation of *T. magnifica* on the different rooting media.

PGR combination in the rooting media ( $\mu\text{M}$ )	Rooting time (weeks)	Root growth
No PGRs	-*	No roots
No PGRs + 0.2% w/v activated charcoal	-	No roots
NAA 1.07	6	Few roots
IBA 0.98	6	Few roots
NAA 5.37	5	More roots
IBA 4.92	5	More roots
NAA 1.07 + IBA 2.46	5	More roots

\* no root formation within 2 months.

medium containing 1.07  $\mu\text{M}$  NAA and 4.45  $\mu\text{M}$  BAP to stimulate shoot proliferation. After 4 weeks 2-3 cm high shoot clumps (Fig. 1E) were divided into single shoots and placed in various rooting media. All media containing 0.54-5.37  $\mu\text{M}$  NAA or 0.49-4.92  $\mu\text{M}$  IBA induced roots in 5-6 weeks (Fig. 1F). Among the rooting media, those containing high concentrations of NAA (5.37  $\mu\text{M}$ ) or IBA (4.92  $\mu\text{M}$ ) induced earlier rooting with longer roots compared to media containing lower concentrations of NAA (0.54  $\mu\text{M}$ ) or IBA (0.49  $\mu\text{M}$ ) (Table 4). Media containing only 0.2% active carbon or no PGRs did not induce any root formation (up to 2 months after shoots were placed in these media).

Rooted plantlets were transferred into plastic pots containing humus soil and vermiculite (1: 1) with 99% of 300 plantlets surviving after 40 days in the greenhouse. All plantlets grew normally and no obvious aberrations were observed (Fig. 1G).

## DISCUSSION

Our results revealed that only TDZ could induce adventitious shoots of *T. magnifica* from different explants including calyx explants from plants grown *in vivo* or leaves and petioles from *in vitro* grown shoots. However other cytokinins

(BAP, KT) or auxins (2,4-D, NAA) seemed not to play any observable role in enhancing induction of adventitious shoot formation from the explants. This indicated that TDZ plays a crucial role in the induction process leading to adventitious shoot formation in *T. magnifica*, similar to observations reported for other plant species where TDZ has been used to induce shoot organogenesis from leaf explants (Mohamed *et al.* 1991; Dai and Zhang 2007; Nhut *et al.* 2007; Xu *et al.* 2007; He *et al.* 2009). Once adventitious shoots of *T. magnifica* were induced, these shoots could in turn be propagated by multiple shoot proliferation, rooted and established as whole plantlets with high success. This protocol will be highly valuable for *ex situ* conservation, mass propagation, cultivation and for possible reintroduction of this critically endangered endemic Chinese plant.

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