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TDZ-Induced *in Vitro* Shoot Regeneration of *Aerides maculosum* Lindl. from Shoot Tip Thin Cell Layers

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ABSTRACT

Efficient shoot regeneration of *Aerides maculosum* Lindl. was achieved using transverse thin cell layers (tTCLs) of shoot tips in the presence of thidiazuron (TDZ). Protocorm-like bodies (PLBs) or proliferating shoot buds were observed when tTCLs were cultured on Mitra *et al.* (1976) basal medium supplemented with 13.62 µM TDZ. A high percentage (81%) of PLBs survived and ultimately produced healthy shoots with 2-3 leaves. Shoots rooted when cultured on the same basal medium supplemented with 12.25 µM indole-3-butyric acid. The regenerated plantlets grew normally with a 90% survival rate. This is the first ever report of *in vitro* propagation of this orchid species.

Keywords: conservation, endangered orchids, *in vitro* regeneration, Karnataka state, Western Ghat Forests
Abbreviations: IAA, indole-3-acetic acid; PLB's-protocorm-like bodies; **IBA**, indole-3-butyric acid; **NAA**, α-naphthaleneacetic acid; **PGR**, plant growth regulator; **TDZ**, thidiazuron (N-phenyl-N-1,2,3-thidiazuron-5'-ylurea)

INTRODUCTION

Aerides maculosum Lindl. (Orchidaceae) is one of the important epiphytic native orchid species of Western Ghat forests of Karnataka). Multiplication of this species in nature is through seeds and only 0.3% of seeds germinated in the presence of suitable mycorrhiza (Rao 1998). Over-exploitation and slow growth rate of plants has reduced the number of native orchids in these forests. Furthermore, there are no reports of *in vitro* propagation of this orchid species in the literature. So it is essential to take measures such as plant tissue culture for propagating this native orchid species since these methods have also played an important role in the micropropagation of several commercially important orchids to meet the demands of the growing market throughout the world (Wimber 1963; Morel 1964; Rao 1977; Wang 1988; Sharma and Tandon 1990; Sharma *et al*. 1991; Lakshmanan *et al*. 1995; Ichihashi 1997; Nayak *et al*. 1997; Ichihashi 1998; Chang and Chang 1998; Kanjilal *et al*. 1999; Nayak *et al*. 2002; Malabadi *et al*. 2004, 2005; Malabadi and Nataraja 2007a, 2007b; Huan *et al*. 2004; Teixeira da Silva *et al*. 2006; Malabadi *et al.* 2008a, 2008b, 2008c; Malabadi *et al*. 2009). In orchids, plantlets are usually regenerated through protocorms following seed germination (Kauth *et al*. 2008), occasionally from protocorm-like bodies (PLBs) that arise spontaneously *in vitro* from plant parts such as the shoot base, or direct shoot organogenesis. Transverse thin-cell layers (tTCLs) of plant tissues such as apical meristems, stem nodes, and PLBs have been successfully used as explants for plant regeneration in a few orchids as well as other plant species (e.g., Begum *et al.* 1994; Nayak *et al*. 2002; Malabadi *et al.* 2004, 2005; Teixeira da Silva *et al*. 2006; Malabadi and Nataraja 2007a, 2007b; Zhao *et al.* 2007; Malabadi *et al.* 2008a). This culture system was first developed by Tran Thanh Van for programming different patterns of morphogenesis in *Nicotiana tobacum* (Tran Thanh Van 1973a, 1973b, 1980). Therefore, in order to save time, and plants in nature, simple, but efficient improved method such as tTCLs could be used as an efficient propagation tool for the fast multiplication of *A. maculosum*. The application of tTCL technology is very efficient in terms of using a small number of plants as an explant source but resulting in a high production of plantlets.

Due to the success of this system for the *in vitro* regeneration of several other orchids, the objective of this study was to develop an *in vitro* propagation method using tTCL technology in *A. maculosum*. Murthy and Pyati (2001) developed a micropropagation system using young leaf segments of *in vitro* and *ex vitro* plants in basal MS medium with coconut endosperm and N^6-benzyladenine (BA). This could help in the conservation of this native orchid. Moreover, this *in vitro* multiplication method can be used for large-scale production to meet the growing demands of native Indian orchids.

MATERIALS AND METHODS

Ten *Aerides maculosum* Lindl. plants, collected from the Western Ghat Forests of Karnataka state, India were established in pots (potting mixture of charcoal chips, coconut husks and broken tiles (2: 2: 1), and grown under greenhouse conditions at the Department of Botany, Karnatak University, Dharwad, India. The *in vitro* regeneration protocol used in this paper was adopted from our previous work on *Eria dalzelli* (Malabadi *et al.* 2008a), also an indigenous Indian epiphytic orchid. Shoot tips of *A. maculosum* (0.5-0.8 cm) harvested from mother plants were carefully washed in double distilled water (DDW). They were surface decontaminated sequentially with 0.1% streptomycin (1 min), 70% (v/v) ethanol (5 min) and 0.1% (w/v) $HgCl_2$ (2 min) (Sigma-Aldrich, St. Louis, USA), and thoroughly rinsed with sterilized DDW. tTCLs 1-5 mm thick were cut from shoot tips (approx. 0.5 cm in length) and these sections were cultured on Mitra *et al.* (1976) basal medium with 3.0% sucrose, 0.7% agar, 0.5 gl^{-1} *myo*-inositol, 1.0 gl^{-1} casein

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hydrolysate, 0.5 gl^{-1} L-glutamine, 250 mgl^{-1} peptone, 0.2 gl^{-1} *p*-aminobenzoic acid, and 0.1 gl^{-1} biotin, all purchased from Sigma. The medium was supplemented with a range of thidiazuron (TDZ) concentrations (0. 04, 0.22, 0.45, 2.27, 4.54, 9.08, 11.35, 13.62, 18.16, 22.71, 27.24, 31.78, 36.32, 40.86 and 45.41 µM) without any other plant growth regulators (PGRs) in 25 mm × 145 mm glass culture tubes (Borosil, Mumbai, India) containing 15 ml of the above basal medium under cool white fluorescent light (Mysore lamps, India) at 100 µmol m^{-2} s^{-1} and 25 ± 3°C with a relative humidity of 55-60%. The pH of the media was adjusted to 5.8 with 1 N NaOH or HCl before agar was added. Media without TDZ served as the control. The media were then sterilized by autoclaving at 121°C at 1.04 Kg cm^{-2} for 15 min. L-glutamine, biotin, *p*-aminobenzoic acid and TDZ were filter sterilized (Whatman filter paper, pore size = 0.45 µm; diameter of paper = 25 mm) and added to the media after autoclaving when the medium had cooled to below 50°C.

The cultures were maintained for 8-10 weeks to initiate PLBs or proliferating shoot buds. The freshly initiated individual PLBs were transferred to the above basal medium containing 13.62 µM TDZ. Healthy shoots with 2-3 leaves developed within 10-12 weeks. They were subcultured on the same medium for another 2 weeks for further shoot development. All experiments contained 30 cultures per replicate, with four replicates (120 cultures) per experimental treatment, and each treatment was repeated three times (120 × 3 = 360). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA and the differences detected using Duncan's multiple range test. All statistical analyses were performed at the 5% level using the SPSS (Microsoft Windows ver. 13.0.1.1) statistical software package.

Well-developed shoots were further transferred to fresh basal medium supplemented with various concentrations of auxins (indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA)) to test rooting capacity. All shoot buds were cultured on basal medium supplemented with IAA (0.57, 8.56, 11.42, 14.27, 17.13, 19.98, 22.84 µM), IBA (0.49, 2.45, 4.9, 7.35, 9.8, 12.25, 14.7, 19.6 µM) and NAA (0.53, 2.68, 5.37, 8.05, 10.74, 13.42, 16.11 µM) for assessing rooting efficiency (**Table 2**). Shoots with well developed roots on 12.25 µM IBA-supplemented basal medium were washed thoroughly under running tap water and transplanted into 15 cm diameter plastic pots containing a potting mixture of charcoal chips, coconut husks and broken tiles (2: 2: 1). Three to four plants were planted in each pot and the plants were watered daily and fertilized at weekly intervals with a foliar spray of a mixture of commercial DAP (di ammonium phosphate) and NPK (Mangalore Fertilizers Ltd., India) (nitrogen 20: phosphorous 10: potassium 10).

RESULTS

In the present study, the highest number of responsive explants (81.0 ± 3.9) showing the maximum number of PLBs or proliferating shoot buds was observed at 13.62 µM of TDZ (**Table 1; Fig. 1**). Therefore, 13.62 µM of TDZ is the optimum concentration for inducing PLBs or proliferating shoot buds in *A. maculosum* (**Table 1**). The shoot tip tTCLs remained green and developed small bud-like structures when cultured on 13.62 µM of TDZ supplemented basal medium after two weeks (**Fig. 1A**). These structures were maintained for 4 weeks and then further subcultured on the same fresh medium for another 4 weeks. After 4-8 weeks, these structures formed healthy shoots with 2-3 leaves. Lower concentrations (0.04, 0.22, 0.45, 2.27, 4.54 µM) of TDZ could not effectively initiate PLBs or proliferating shoot buds (**Table 1**). All the explants (shoot tip tTCLs) remained green for 5 weeks, eventually turned brown, and finally died. Incorporation of higher concentrations (22.71, 27.24, 31.78, 36.32, 40.86, 45.41 µM) of TDZ lead to the browning of explants, and ultimately resulted in their death (**Table 1**). Few explants remained green for 2-3 weeks and failed to induce any organogenesis. All the explants cultured on basal medium without TDZ (control) failed to induce PLBs. On the other hand low initiation of PLBs or proliferating shoot buds was noticed when TDZ at 9.08, 11.35, 13.62 and 18.16 µM was incorporated into basal medium. The shoots regenerated on 13.62 µM of TDZ-supplemented basal medium were tested for rooting efficiency with different concentrations of auxins such as IAA, IBA and NAA (**Table 2**). The shoots failed to produce roots with lower concentrations of IBA (from 0.24 to 7.35 µM). Rooting efficiency was satisfactory by increasing the con-

Table 1 Effect of different concentrations of TDZ on the initiation of protocorm-like bodies (PLBs) or proliferating shoot buds in *A. maculosum* Lindl.

TDZ (µM)	Responsive explants (%)	№ of PLBs or shoot buds per explant	№ of shoot s produced per explant
Control*	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
0.04	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
0.22	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
0.45	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
2.27	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
4.54	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
9.08	6.0 ± 0.2b	2.0 ± 0.1b	1.0 ± 0.1b
11.35	10.0 ± 2.5b	4.0 ± 0.1b	2.0 ± 0.1b
13.62	**81.0 ± 3.9a**	**54.0 ± 2.0a**	**36.0 ± 5.0a**
18.16	3.0 ± 0.1b	2.0 ± 0.1b	1.0 ± 0.1b
22.71	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
27.24	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
31.78	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
36.32	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
40.86	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
45.41	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c

*Control=Basal medium without TDZ

Data scored after 14 weeks and represent the mean ± SE of at least three different experiments. In each column, the values with different letters are significantly different (P<0.05)

Table 2 Effect of different concentrations of auxin (IAA, IBA, NAA) on rooting of shoots regenerated with 13.62 µM TDZ treatment.

IAA (µM)	Rooting (%)	IBA (µM)	Rooting (%)	NAA (µM)	Rooting (%)
Control*	0.0 ± 0.0c	control	0.0 ± 0.0c	control	0.0 ± 0.0c
0.28	0.0 ± 0.0c	0.24	0.0 ± 0.0c	0.26	0.0 ± 0.0c
0.57	0.0 ± 0.0c	0.49	0.0 ± 0.0c	0.53	0.0 ± 0.0c
2.85	0.0 ± 0.0c	2.45	0.0 ± 0.0c	2.68	0.0 ± 0.0c
5.71	0.0 ± 0.0c	4.90	0.0 ± 0.0c	5.37	0.0 ± 0.0c
8.56	0.0 ± 0.0c	7.35	0.0 ± 0.0c	8.05	0.0 ± 0.0c
11.42	0.0 ± 0.0c	**9.8**	16.0 ± 2.1b	10.74	0.0 ± 0.0c
14.27	0.0 ± 0.0c	**12.25**	**72.0 ± 3.1a**	13.42	0.0 ± 0.0c
17.13	0.0 ± 0.0c	14.7	0.0 ± 0.0c	16.11	0.0 ± 0.0c
19.98	0.0 ± 0.0c	17.15	0.0 ± 0.0c	18.79	0.0 ± 0.0c
22.84	0.0 ± 0.0c	19.6	0.0 ± 0.0c	21.48	0.0 ± 0.0c
25.69	0.0 ± 0.0c	22.05	0.0 ± 0.0c	24.16	0.0 ± 0.0c
28.55	0.0 ± 0.0c	24.5	0.0 ± 0.0c	26.85	0.0 ± 0.0c

*Control= Basal medium without IAA, IBA and NAA

Data scored after 4 weeks and represent the mean ± SE of at least three different experiments. In each column, the values with different letters are significantly different (P<0.05) according to DMRT (Duncan's multiple range test).

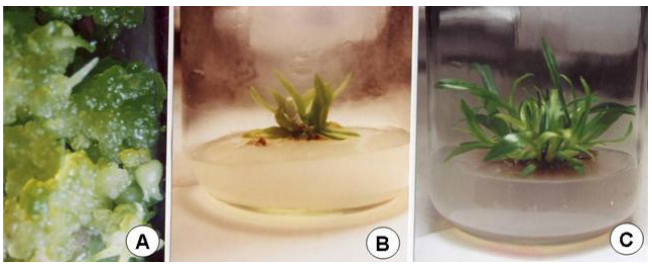

Fig. 1 ***In vitro*** **multiplication of** ***A. maculosum*** **Lindl.** (**A**) Initiation of protocorm-like bodies or proliferating shoot buds in tTCL of shoot tip after 3-4 weeks of culture on Mitra *et al*. (1976) basal medium supplemented with 13.62 µM TDZ. (**B**) Healthy shoots formed rooting on Mitra *et al*. (1976) basal medium supplemented with 13.62 µM TDZ. (**C**) Well developed hardened plants ready for transfer to field conditions.

centration of IBA from 9.08 to 12.25 µM (**Table 2**). Highest percentage of rooting (72%) was with 12.25 µM IBA-supplemented basal medium (**Table 2; Fig. 1B, 1C**). The shoots cultured on basal medium supplemented with various concentrations of IAA (0.28-28.55 µM) and NAA (0.26-26.85 µM) failed to produce roots (**Table 2**). The lowest percentage of rooting (2.0-6.0%) was observed with 9.8 µM IBA.

DISCUSSION

In orchids, an efficient shoot regeneration of *Vanda coerulea* was achieved using TCLs and TDZ (Malabadi *et al.* 2004a). PLBs or proliferating shoot buds was observed when thin shoot tip sections were cultured on Vacin and Went (1949, VW) basal medium supplemented with 11.35 µM TDZ. The highest percentage of PLBs (95%) survived and ultimately produced healthy shoots with 2-3 leaves when subjected to a 4-week TDZ treatment. A culture period longer than 8 weeks with TDZ resulted in the formation of fascinated or distorted shoots, a feature often associated with the culture of hybrid *Cymbidium* (Teixeira da Silva, unpublished observations). Shoots produced roots when cultured on half-strength VW medium supplemented with 11.42 µM IAA in *V. coerulea* (Malabadi *et al.* 2004a). A protocol for induction of direct somatic embryogenesis, secondary embryogenesis and plant regeneration of *Dendrobium* cv. 'Chiengmai Pink' was developed using TDZ (Chung *et al.* 2007). 5-25% of leaf tip segments of *in vitro*-grown plants directly formed somatic embryos on half-strength MS medium supplemented with 0.3, 1 and 3.0 mg/l TDZ (Chung *et al.* 2007). TDZ was effective in the induction of *in vitro* morphogenesis, shoot regeneration and multiplication (Ernst 1994; Chen and Piluek 1995; Nayak *et al.* 1997; Chen and Chang 2000) and direct somatic embryogenesis (Chen *et al*. 1999; Chen and Chang 2000) of several orchids (*Phalaenopsis*, *Doritaenopsis*, *Cymbidium aloifolium*, *Dendrobium aphyllum*, *Dendrobium moschatum*, *Oncidium*). Moreover, TDZ combined with 2,4-D are required for callus induction in *Cymbidium ensifolium* var. *misericors* (Chang and Chang 1998), *Oncidium* (Chen and Chang 2000a, 2000b), *Phalaenopsis* (Chen *et al.* 2000) and *Paphiopedilum* (Lin *et al*. 2000). However, Huang *et al*. (2001) reported that TDZ inhibits shoot proliferation and rooting in *Paphiopedilum*. In addition, Chen *et al*. (2002) reported that the best treatment of 4.54 µM TDZ induced only 20% of leaf explants to form shoots in *Paphiopedilum*. Very recently Hong and coworkers (2008) reported induction of totipotent callus on half strength MS medium supplemented with 22.60 µM 2,4-D and 4.54 µM TDZ in darkness in a *maudiae*-type slipper orchid, *Paphiopedilum* 'Alma Gavaert' (Hong *et al*. 2008). The callus was proliferated more and maintained without any morphogenesis on the same medium over a 2-month interval. When callus was transferred to half-strength MS medium supplemented with 26.85 µM NAA, an average of 4.7 PLBs/shoot bud formed from each explant after 120 days of culture. Nayak *et al.* (1997) induced high frequency shoot proliferation in *Dendrobium moschatum*, *Cymbidium aloifolium* and *Dendrobium apyllum* using 4.0 µM TDZ. Stem nodal explants of *Paphiopedilum philippinense* hybrids (hybrid PH59 and PH60) directly formed shoots when cultured on modified half-strength MS basal medium supplemented with a combination of 4.52 µM 2,4-D and 0.45 µM TDZ (Chen *et al*. 2002). On PGR-free basal medium, the percentage of explants with shoots was 33.3 and 0% and the 1 and 0 shoots/explant in hybrid PH59 and hybrid PH60, respectively. In hybrid PH59, 4.52 µM 2,4-D + 0.45 µM TDZ induced a higher percentage of explants with shoots and shoot number/explant than did the PGR-free treatment. In hybrid PH60, although 4.52 µM 2, 4-D and 0.45 µM TDZ promoted shoot formation, the highest shoot number was found with 4.52 µM 2, 4-D alone (Chen *et al*. 2002). An efficient shoot regeneration of *Eria dalzelli* (Dalz.) Lindl. for the first time was achieved using shoot tip tTCLs and TDZ (Malabadi *et al*. 2008a). As in this study, PLBs or proliferating shoot buds were observed when shoot tip tTCLs were cultured on the same basal medium supplemented with 9.08 µM TDZ. The highest percentage (96%) of PLBs survived and ultimately produced healthy shoots with 2-3 leaves. Shoots rooted when cultured on this basal medium supplemented with 11.42 µM IAA. The regenerated plantlets grew normally and a 90% survival rate was achieved. This simple protocol will be useful for the large-scale propagation of *E. dalzelli* (Malabadi *et al*. 2008a).

TDZ is a substituted phenyl urea with cytokinin-like activity (Mok *et al.* 1982) and therefore, stimulates rapid shoot differentiation. TDZ aids in rapid plant regeneration of a number of plant species through organogenesis (Malik and Saxena 1992). The potential of TDZ to stimulate shoot formation is very common in dicotyledonous plants such as *Pisum sativum* (Massimo *et al.* 1996), *Cajanus cajan* (Eapen *et al.* 1998), *Arachis hypogaea* (Kanyand *et al.* 1994) as well as in monocotylednous plants such as *Dendrocalamus strictus* Nees (Singh *et al.* 2001). In *Costus speciosus*, rhizome thin sections cultured on B_5 basal medium without TDZ (control) or with low concentrations 0.45 and 4.54 µM TDZ completely failed to produce shoot buds. Higher concentrations of TDZ, particularly 36.32, 40.86 and 45.41 µM, resulted in the browning of explants which finally necrosed (Malabadi *et al.* 2004b). On the other hand, initiation of shoot buds was observed in the range 11.35-27.34 µM TDZ with highest percentage of rhizome thin sections (92%) producing shoot buds (12 ± 2.01) at 18.16 µM TDZ (Malabadi *et al.* 2004b). 87% of these shoot buds elongated and gave rise to shoots. On this medium the explants remained green for 3 weeks and developed small bud-like structures from the central as well as peripheral regions during this period. After 2 weeks, the shoot buds showed further elongation and formed 2 to 3 leaves. A sharp decrease in the number of shoot bud formation was also noticed when the concentration of TDZ was increased from 18.16 to 31.78 µM (Malabadi *et al*. 2004b). Least and poor growth of shoot buds (1.8 ± 0.02) was noticed at 31.78 µM TDZ in *Costus speciosus* (Malabadi *et al.* 2004b). Wilhelm (1999) reported successful micropropagation of juvenile Sycamore maple (*Acer pseudoplatanus*) *via* adventitious shoot formation by the use of 0.04 µM TDZ. In the case of pea cvs. 'Sugar Ann' and 'Patriot', an average of 20 shoots formed on MS basal medium supplemented with 0.5 or 1.0 µM TDZ (Massimo *et al.* 1996). TDZ either alone (4.54 or 9.08 µM) or in combination with IAA (5.71 µM) on MS-supplemented medium induced a high frequency of shoot regeneration from primary leaf segments of three pigeonpea (*Cajanus cajan* L.) cultivars (Eapen *et al.* 1998). Singh *et al.* (2001) also reported TDZ-induced shoot multiplication in bamboo (*Dendrocalamus strictus*) and maximum number of shoots (14.8 ± 1.0) were obtained from shoot explants cultured in 2.27 µM TDZ supplemented half strength MS basal medium. Treatment of soybean callus with TDZ stimulated cytokinin accumulation (Thomas and Katterman 1986). TDZ was also effective in shoot formation from leaf explants or stem tTCLs of a number of chrysanthemum cultivars, although the level of fasciation and abnormal shoot formation was high (Teixeira da Silva and Fukai 2003).

Only one other study on the micropropagation of *Aerides maculosum* exists (Murthy and Pyati 2001) and in that study BA was used to induce PLBs from leaf explants. The acclimatization rate was a little lower than that of this study, i.e. 84%. Murthy (2005) did make a brief report on the induction of PLBs from protocorm and leaf segments of *Aerides crispum*, but none of the claims were quantified.

These studies all agree with the present findings, i.e., that TDZ is a potent inducer of organogenesis, in this case, PLBs and shoots.

PERSPECTIVES

As the flowers of *A. maculosum* are used in various festivals of the region, this orchid thus forms an integral part

of tradition and culture. Indiscriminate collection of this orchid from the wild, therefore, poses a threat to its survival. Therefore, for conservation as well as for commercial purposes, production of plants using tissue culture techniques is important, but only holds value if a large number of plantlets can be produced within a short time frame and with minimum input expenses. The added benefit of this protocol is that it can be transferred to a rural community surrounded by rich orchid diversity and with less technical know-how where small-scale tissue culture units can be set up to generate employment for rural people as well as for conservation of biodiversity. Orchid biotechnology is relevant to the needs of orchid growers in any country and the supply of uniform clonal planting material comes to market mainly from *in vitro culture*. The demand for micropropagated orchids also explains the recent rapid increase in the number of commercial orchid laboratories and private companies operating in South India, particularly in Bangalore, Karnataka state.

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