Combinatorial Effects of Thidiazuron and Gibberellic Acid on iv vitro Propagation of an Endangered Tree: Cane Palm (Dypsis lutescens)

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Abstract. Dypsis lutescens (H. Wendl.) Beentje and Dransf., commonly known as areca palm, golden yellow palm, or cane palm belong to the Arecaceae family and is an evergreen endangered ornamental palm tree. An effective protocol for in vitro conservation of D. Lutescens under various regimes of thidiazuron and gibberellic acid is standardized in the present study. The combined effect of thidiazuron and gibberellic acid at an appropriate level (0.5 µM) in MS culture media improved the morphogenic growth responses from apical meristem with the highest number of shoots per culture (4.52) with an average shot length of 6.6 cm in 96% cultures after 8 weeks inoculation of explants. Thereafter, well proliferated pretreated micro shoots with a high dose (100 µM) of indole-3-butyric acid (IBA) weresub-cultured on ½ MS media supplemented with various combinations of IBA and gibberellic acid. Of these, a combination of 0.5 µM of both IBA and GA_3 was found to be the most effective for in vitro root formation, where 4.5 roots with an average root length of 3.8 cm per micro shoot in 88% cultures were recorded after 4 weeks. During acclimatization, photosynthetic traits and their attributes such as chlorophyll contents, stomatal conductance, intercellular CO_2 concentration, and net photosynthetic rate were evaluated. These physiological attributes were decreased initially during 14 days, thereafter, were steadily increased up to 90 days of acclimatization. Successfully acclimatized plantlets were shifted to fields where they were normally grown without any morphogenic changes with 96.7% of survival. This is the first study regarding high throughput regeneration of D. lutescensfrom apical meristem which could be a useful alternative approach for mass propagation and conservation of woody ornamental trees under appropriate regimes of plant growth regulators.

Keywords: Cane Palm; Dypsis Lutescens, In-Vitro Conservation; Micropropagation, PGRs.

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Highlights

- Dypsislutescensis an endangered ornamental palm tree,
- Combine application of thidiazuron and gibberellic acid was most effective to improve in vitro propagation of D. lutescens,
- Pre-treatment of micro shoots with a high dose of IBA stimulated the several root initials at the basal end of micro shoots of D. lutescens,
- This is the first study regarding high throughput regeneration of D. lutescensfrom apical meristem.

1. Introduction

Floriculture with its four major components (cut flowers, cut foliage, potted green plants, and bedding plants) is the most flourishing industry and ranks among the top four profitable exports worldwide (Sharnouby et al., 2021). It generates aesthetic beauty and food for soul peace and higher profitability for producers compared to fruit and vegetable farming. Hence, more than 120 countries are actively involved in the floriculture trade and Japan is leading with an annual worth of $3.7 billion (Ahmad et al., 2020). Cut flowers are the leading product as they share more than 50% of the total world floriculture trade value (Darras, 2020).

Potted green plants such as ornamental shrubs and trees are 2nd to cut flowers with a 43% share in the worldwide floriculture trade (Burger et al., 2020). Perennial palms of the Arecaceae tribe are ornamental plants and a most economic source of various bioactive compounds, rattan, thatch, lumber, tannins, wine, beverages, fiber, oil, and food as well (Bisht, 2020). This diverse and complex palms tribe is comprised of more than 20 genera and 4000 species, naturally distributed in tropical and sub-tropical regions of the world (Koppetsch et al., 2020). Nowadays, palms are considered a symbol of tourism and are an important part of evergreen vegetation, interiorscapes, landscapes, national gardens, and monuments in every region of the world such as The Palm House at the Royal Botanical Gardens, and Kew Gardens in England. Fifteen palm species including Dypsis lutescens are most commonly found across the world including in Pakistan (Newman and Driver, 2020).

Dypsis lutescens (Wendl.) Beentje and Dransf., commonly known as areca palm, golden cane palm, butterfly palm, the yellow palm is the most economic ornamental plant (Ahmad, Koppetsch et al., 2020). Species derived its name from the Latin word "lutescens" meaning yellow growing refers to its yellowish stem. It is native to moist areas of Madagascar and the Philippines (Goodman and Jungers, 2021) but it has been adapted to grow in subtropical and tropical areas of South India, Reunion, southern Florida, Canary Islands, El-Salvador, Cuba, Puerto Rico, Leeward Islands, Jamaica, the Dominican Republic, Andaman Islands, Haiti, and the Venezuelan Antilles. This palm tree has the potential to grow up to 12m high with several shoots and 2 to 3m long curved pinnate leaves having 40 to 60 leaflet pairs and yellow flowers in panicles. It provides aesthetic beauty and gives a graceful look to indoor as well as outdoor locations in homes, offices and shopping centers. Hence, the palm tree was awarded the Garden Merit of the Royal Horticultural Society (Ahmad et al., 2020).

Cane palm has large-scale economic importance due to an important specimen of landscaping, as well as its medicinal, antiseptic, and antioxidant uses. The decoction of roots of D. lutescens promotes urine flow and is also used to cure the diseases of the liver, bronchitis, dysentery, and sore throat. The salt obtained from the pith of the trunk of D. lutescens is also used for the treatment of intestinal worms and the treatment pancreatic disorders caused by malaria. The ash of bark is antiseptic and used for scabies and toothache and ripened fruits are eaten by several birds. Moreover, D. lutescens efficiently cleans the air by filtering various pollutants such as benzene, formaldehyde, toluene, etc. (Ahmad et al., 2020). Cane palm can grow in diverse climatic ranges but best thrives in clay soil with no overwatering and relatively low-temperature ranges from 4.4 to 16°C. It is sensitive to humidity, overwatering, elevated air temperature, and fungal diseases, therefore, it is ranked as the most endangered ornamental plant even in its native environments.

Cane palm is propagated by vegetative as well as sexual means of reproduction i.e., shoot offsets and seeds respectively (Khan et al., 2020). Propagation through vegetative means is limiting, time-consuming, and laborious while the seed germination rate is very poor through sexual reproduction. Environmental factors like fluoride toxicity (which causes foliar necrosis), and deficiency of iron, manganese, and zinc also affect the growth of cane palm but fungal diseases are a major threat to its propagation (Haq et al., 2020). Leaf spot, leaf blight, Ganoderma butt rot, bud, rot, and welfare are common fungal diseases of cane palm caused by Annellophora, Exserohilum, Phaeotrichoconis, Stigmina, Bipolaris, Cercospora, Gliocladium, Pestalotiopsis, Pestalotia, Phyllachora, Colletotrichum, Pseudocercospora, Calonectria, Ganoderma, Phytophthora, and Fusarium (Elliott and Uchida, 2021). Although several control measures and conservation techniques are used by growers to enhance the growth rate and control the diseases but results are not satisfactory. Hence, there is a dire need to develop novel and alternative means of propagation to conserve the most endangered palm tree.

Ex-situ conservation techniques are considered the most appropriate for the protection of plants' diversity beyond their natural habitat rather than in-situ strategies (Ramon et al., 2020). Conservation of woody plant species has been done in seed banks by storing seeds at low-temperature regimes over long periods (Silva et al., 2021) but this approach is not appropriate for the conservation of cane palm due to the poor viability of seeds and successful propagation through seeds is not possible. Therefore, propagation through in vitro techniques is an efficient and innovative approach to protecting and conserving plant biodiversity. Efficient and Standard protocols for in vitroseed germination, micropropagation, callogenesis, and somatic and zygotic embryo formation have been reported for the conservation of many woody trees (Gaidamashvili and Benelli, 2021). Among in vitro techniques, a micropropagation is a potent tool and an effective practice for rapid propagation, true-to-type, disease-free, and premium quality planting material of threatened and endangered plant species (Prasad et al., 2021). However, no study has yet been reported for in vitro propagation and conservation of cane palm. Therefore, there is a dire need to standardize the specific in vitro conditions (media and explant type, plant growth regulators, and concentration) for the growth and propagation of cane palm.

Plant growth regulators generally enhance the growth rate but their prolonged use is linked with several morphological and physiological deformations. A synthetic phenylurea-derived *PGR N*-phenyl *N'*-1,2,3-thiazol-5-yl urea (thidiazuron: *TZD*) suppresses the morphological growth and physiological processes but has widely been used in combination with gibberellic acid (GA_3) in the micropropagation of many woody trees (Javed et al., 2019; Ahmad et al., 2020). Previous studies suggested that GA_3 works antagonistically with cytokinins (thidiazuron) at a lower dose and promotes in vitro shoot and root formation, elongation and multiplication by regulating the biosynthesis of signaling messengers and signal transduction. These investigations reported the normal and disease-free production of true-to-type plantlets.

Hence, the present study determines an efficient protocol for micropropagation and histogenesis through apical meristem of *D.* lutescens under a different regime of GA_3 and TDZ. Changes in physiological traits like Chlorophyll (*Chla/b*) contents, stomatal conductance (*gs*), intercellular CO_2 concentration (*Ci*), and photosynthetic rate (*PN*) were also evaluated during the acclimatization of in vitro generated plantlets. Up to now, this is the first study of in vitro conservation of *D.* lutescens through micropropagation using GA_3 and *TDZ.* This study would also be useful to propagate other recalcitrant woody trees.

2. Materials and Methods

2.1. Explant Establishment and Culture Conditions

One-year-old plants of *D. lutescens* were collected from the Plant Nursery, University Road Sargodha (22° 23′ 25″ N, 84° 21′ 44″ E). Naturally protected and wrapped apical meristems were excised from the mother plant with a sterile stripper. Surface sterilization of explants was carried out under disinfected and sterile conditions. Apical meristem was initially treated with a drop of surfactant (Tween-20) for five minutes and was washed with double-distilled water (ddH_2O) for ten minutes followed by treating with 0.1% $HgCl_2$ for 5 minutes and repeatedly washed with ddH_2O to remove the traces. Finally, apical meristems were inoculated in autoclaved agar-solidified MS nutrient media supplemented with various doses of GA_3 and TDZ with a pH of 5.8 in aseptic culture vials. All the cultures were maintained at 24 ± 2°C under a 16/8day-night cycle with a photosynthetic photon flux density (PPFD) of 50–100 μmol $m^{-2}s^{-1}$. Sub-culturing was accomplished onto the fresh MS media after every 2 weeks and data was recorded on the percentage of culture response, shoot numbers, and shoot and root length per culture after 4 and 8 weeks.

2.2. Shoot multiplication of in vitro plantlets

Disinfected apical meristems were inoculated on a sterilized MS medium supplemented with various doses of GA_3 and TDZ (0.10, 0.25, 0.50, 0.75, and 1.0 μM) to evaluate the single and combined effect of both PGRs (Table 1 and 2).

After every two weeks, plantlets were sub-cultured on the same media. Data on the percentage of culture response shoots number, shoot length and root length per culture were recorded after 4 and 8 weeks.

2.3. Rhizogenesis

The 4 cm long micro shoots with 2 or 3 leaves from 8 weeks old culture were cultured in liquid MS media supplemented with indole butyric acid (IBA) and GA_3 (0.1, 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5) on filter paper bridge. Data regarding percent rooting, root number, and root length per micro shoot were recorded after 4 weeks of sub-culture.

2.4. Acclimatization and hardening

Healthy rooted micro shoots were removed from culture vials and were gently washed with running tap water and transplanted in a mixture of sand, peat, and soil (0.5: 1: 1) in thermo-porecups covered with transparent polybags. The transplanted plantlets were watered with ¼ MS liquid solution (without sucrose and organic salts) for 4 weeks and thereafter with tap water. Polythene bags were gradually removed to harden the plantlets and transferred to pots after 60days. Thereafter, plantlets were successfully shifted to the open field after 90 days.

2.5. Estimation of physio-biochemical traits

During the acclimatization period, various important physio-biochemical traits such as the photosynthetic traits and their attributes such as chlorophyll (*Chl*a/b) contents, stomatal conductance (*g*s), intercellular CO_2 concentration (*C*i), and photosynthetic rate (*P*N) were of *in vitro* generated plantlets were measured during acclimatization period on different days (14, 28, 42, 56, 70 and 90) after the transfer (*DAT*). The leaf gas exchange parameters were measured from fully expanded intact leaves using a portable Infra-Red Gas Analyzer (IRGA, LI-COR 6400, Lincoln, NE, USA) while chlorophyll contents were measured using UV-spectrophotometer following the protocol described by Mackinney (1941).

2.6. Statistical Analysis

Data recorded in the present study from different treatments were subjected to statistical analysis using RStudio 3.4.4. ANOVA was performed to evaluate the influence of treatments and error during the study using a fundamental R function "l.mr". The significant disparity among various treatments was analyzed to compare treatments by applying LSD at the confidence interval of ($P \leq 0.05$) using R Package "Agricolae". Bar plots were drawn for physio-biochemical traits using R Package "ggplot-2".

3. Results

3.1. Single effect of TDZ and GA_3 on in vitro shoot formation

Surface sterilized apical meristems of *D. lutescens* were inoculated on MS media supplemented with various doses of TDZ and GA_3 to evaluate the shoot initiation and multiplication response of explants (Fig. 1). No shoot was formed when apical meristem was cultured on PGR-free MS basal media while a remarkable differential response was observed when apical meristem was cultured on MS media supplemented with various doses (0.1, 0.25, 0.50, 0.75, and 1.0 µM) of TDZ and GA_3 (Table 1A). Of these concentrations, 0.50 µM of GA_3 induced a maximum number of shoots (1.6 shoots) with an average shot length of 3.0 cm and 3.8 leaves per culture vail after 4 weeks of inoculation.

Whereas, 1.62 shoots per culture with an average shot length of 3.4 cm and 3.7 leaves per culture were recorded in 68% of cultures after 8 weeks of inoculation on the same dose (Table 1). On the other hand, the highest number of shoots (1.67) with an average shot length of 2.81 cm and 2.82 leaves per culture were recorded after 4 weeks of inoculation (Figure 1B), whereas 1.91 shoots with an average shot length of 4.6 and 3.2 leaves per culture in 92% cultures were recorded after 8 weeks of inoculation (Figure 1C) on MS media supplemented with equimolar concentration of TDZ (Table 1). Overall, the shoot response was relatively higher in TDZ-raised cultures as compared to GA_3-raised cultures.

Table 1. Effect of GA_3 and TDZ on shoot formation of *D. lutescens* from the apical meristem

PGR		Culture Response (%)	4 weeks			8 weeks		
GA_3	TDZ		No. of Shots Per Culture Vail	Shoot Length (cm)	Number of Leaves per Culture Vail	No. of Shots Per Culture Vail	Shoot Length (cm)	Number of Leaves per Culture Vail
0.0	**0.0**	0	0.0±0.00d	0.0±0.00e	0.0±0.00f	0.0±0.00e	0.0±0.00f	0.0±0.00e
0.1		16	0.15±0.02cd	0.8±0.33d	0.8±0.17e	0.15±0.02d	0.8±0.21e	0.9±0.11d
0.25		28	1.31±0.04ab	1.4±0.66cd	2.0±0.13c	1.30±0.11c	2.0±0.63d	2.2±0.17b
0.5		68	1.60±0.14a	3.0±0.45a	3.8±0.40a	2.52±0.16a	3.87±0.84b	3.7±0.10a
0.75		52	1.07±0.05b	2.6±0.72b	2.0±0.28c	1.62±0.31bc	3.40±0.56bc	1.6±0.25c
1.0		36	0.80±0.03bc	1.8±0.52c	1.4±0.21d	0.82±0.01d	2.2±0.76d	1.2±0.11c
	0.1	48	0.46±0.01c	1.02±0.36d	1.2±0.17d	0.46±0.01de	2.6±0.36d	1.8±0.17c
	0.25	64	1.29±0.09ab	1.71±0.33c	2.13±0.21bc	1.40±0.09c	3.2±0.33c	2.6±0.21b
	0.5	92	1.67±0.08a	2.81±0.22ab	2.82±0.33b	1.91±0.08b	4.6±0.22a	3.6±0.33a
	0.75	76	0.42±0.07c	2.05±0.18bc	1.31±0.21d	0.87±0.07d	3.8±0.18b	1.6±0.21c
	1.0	52	0.10±0.04cd	0.93±0.48d	0.65±0.09e	0.40±0.04de	2.1±0.48d	1.1±0.09d

Each value represents the mean of ten replicates for every treatment along with standard deviation. Means within a column followed by the dissimilar letter are significantly different at $P < 0.05$.

3.2. The combined effect of TDZ with GA_3 on Shoot proliferation

Shoot proliferation response of in vitro generated culture was improved by supplementation of MS media with various combinations of both PGRs i.e., GA_3 and TDZ. The maximum number of shoots (4.52) with the highest shoot length (6.6) and several leaves (4.2) was recorded in 96% cultures after 8 weeks of inoculation in MS media supplemented with a combination of optimum concentrations (0.50 μM) of GA_3 and TDZ (Table 2, Figure 1 D-F). Shoot multiplication response on in vitro raised cultures was significantly reduced at higher doses of TDZ and also exhibited abnormal growth like callus induction, stunted growth, and shoot tip necrosis within 8 weeks of sub-culturing (Table 2, Figure 1 G).

Table 2. Effect combination of GA_3 and TDZ on shoot multiplication of *D. lutescens*

PGR		Culture Response (%)	8 weeks		
GA_3	TDZ		No. of Shots Per Culture Vail	Shoot Length (cm)	Number of Leaves per Culture Vail
0.0	**0.0**	0	0.0±0.00e	0.0±0.00d	0.0±0.00f
0.1	**0.5**	70	1.11±0.02d	1.8±0.21e	0.9±0.11e
0.25	**0.5**	85	2.30±0.11b	3.4±0.63c	3.2±0.17b
0.5	**0.5**	96	4.52±0.16a	6.6±0.22a	4.2±0.33a
0.75	**0.5**	80	2.62±0.31b	4.87±0.56b	2.6±0.25c
1.0	**0.5**	75	1.82±0.01c	3.2±0.76c	2.21±0.11d
0.5	**0.1**	75	1.46±0.01	3.6±0.36c	2.8±0.17c
0.5	**0.25**	70	2.40±0.09b	4.2±0.33b	3.6±0.21b
0.5	**0.5**	96	4.52±0.16a	6.6±0.22a	4.2±0.33a
0.5	**0.75**	76	1.87±0.07c	4.8±0.18b	2.6±0.21c
0.5	**1.0**	84	1.40±0.04cd	2.1±0.48d	1.1±0.09e

Each value represents the mean of ten replicates for every treatment along with standard deviation. Means within a column followed by the dissimilar letter are significantly different at $P < 0.05$.

3.3. Root Induction of in Vitro Generated Micro Shoots

The in vitro generated micro shoots were taken out from 8 weeks old cultures and isolated from plantlets and pretreated with a 100 µM solution of IBA for 24 hours to induce the root initiation. After 24 hours, IBA treated micro shoots were transferred to ½ MS media supplemented with various doses of IBA (0.1, 0.25, 0.50, 0.75, 1.0, 1.25, and 1.50 µM) alone and different combinations of optimum concentration of IBA (1.0 µM) with GA_3 (0.1, 0.25, 0.50, 0.75, and 1.0 µM). A 24-hour pre-treatment of IBA of micro shoots activated many root initials at their basal ends that were remarkably elongated to a significant length in ½ MS media supplemented with a combination of IBA and GA_3 (Table 3, Figure 1H-K). Among these combinations, 0.50 µM GA_3 with 1.0 µM of IBA was found to be the most effective for in vitro root formation of D. lutescens. At this level, a maximum number of roots (4.4) with an average root length of 3.8 cmin 88% of cultures were recorded after 8 weeks of sub-culturing at this level.

Table 3. Effect of combination IBA and GA_3 on root induction of 8 weeks old micro shoots *D. lutescens*

PGR		Culture Response (%)	4 weeks	
			No. of Roots Per Culture Vail	Root Length (cm)
IBA	**GA_3**			
0.0	**0.0**	0	0.0±0.00e	0.0±0.00e
0.1	**0.5**	16	2.0±0.48cd	0.8±0.17d
0.25	**0.5**	36	1.4±0.45d	1.0±0.4cd
0.5	**0.5**	88	4.4±0.35a	3.8±0.33a
0.75	**0.5**	56	3.8±0.33b	2.7±0.48b
1.0	**0.5**	84	4.0±0.63ab	3.2±0.33ab
0.5	**0.1**	52	2.8±0.72c	1.3±1.07c
0.5	**0.25**	39	2.2±0.86cd	1.0±0.07cd
0.5	**0.5**	88	4.4±0.35a	3.8±0.33a
0.5	**0.75**	70	1.6±0.45d	1.4±0.04c
0.5	**1.0**	42	1.2±0.33d	0.4±0.48d

Each value represents the mean of ten replicates for every treatment along with standard deviation. Means within a column followed by the dissimilar letter are significantly different at P <0.05.

3.4. Acclimatization of in vitro generated rooted plant

After 8 weeks, rooted plantlets were transferred to thermopane cups filled with a sterilized mixture of sand, peat, and soil (0.5: 1: 1) for 28 days in the glasshouse with artificial irradiance of 50 µmol $m^{-2}s^{-1}$ PPFD followed by artificial irradiance of 100 µmol $m^{-2}s^{-1}$ PPFD for next 28 days and were watered with ¼ MS media without sucrose and organic salts. After 56 days of hardening, survived plantlets were transferred to pots in garden soil with artificial irradiance of 150 µmol $m^{-2}s^{-1}$ PPFD and were watered with tap water in a greenhouse for two weeks. Thereafter, plants were transferred to open fields, where they were grown with a 90.7% of survival rate after 3 months of acclimatization (Figure 1 L-N).

3.5. Appraisal of physio-biochemical traits during acclimatization

Initially, physio-biochemical traits measured in the present study such as chlorophyll contents (Chl a and b), stomatal conductance (*gs*), internal CO_2 concentration (*Ci*), and photosynthetic rate (*Pn*) were decreased during the first fourteen days of acclimatization in a glasshouse under low artificial irradiance of 50 µmol $m^{-2}s^{-1}$ PPFD. After 14 days of acclimatization, all traits under study were remarkably increased up to 90 days despite the increased level of artificial irradiance (150 µmol $m^{-2}s^{-1}$ PPFD) and change in growing media (garden soil) and conditions (open field). The maximum values of photosynthetic parameters such as *P*N [27.7µmol [CO_2] m^{-2} s^{-1}], *gs*[0.69mol (H_2O)$m^{-2}s^{-1}$], *C*i

(322.0 mg/L), *Chl*a (1.91 mg g^{-1} FW) and *Chl*b (0.93 mg g^{-1}FW) were recorded in *in-vitro* raised plants at the end of 90 days (Figure 2 and 3).

Figure 1. A: Excised apical meristem inoculated on MS media; B: shoot formation after 4 weeks: C: shoot formation after 8 weeks; D-F: shoot multiplication after 8 weeks of 1st sub-culturing; G: shoot necrosis in response to a higher dose of TDZ; H-J: root induction in micro shoots; K: normal plantlet after 8 weeks of inoculation in MS media supplemented with a

combination of GA3 and TDZ (0.5 µM each); L-M: acclimatization of plantlets; N: in vitro generated plantlet in an open field after 1 year of acclimatization

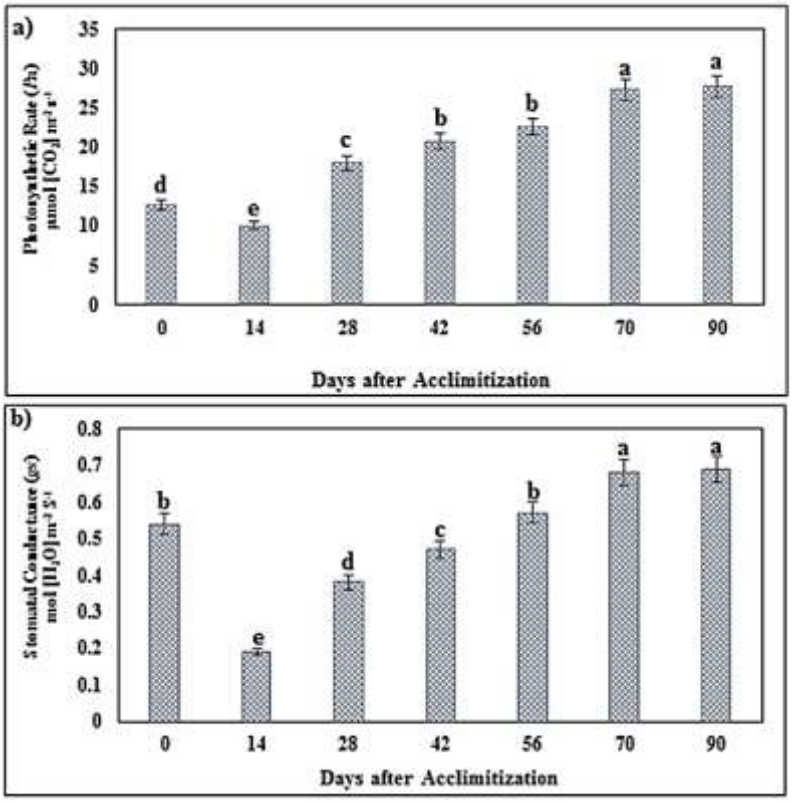

Figure 2. Photosynthetic rate and stomatal conductance during acclimatization of in vitro generated plantlets

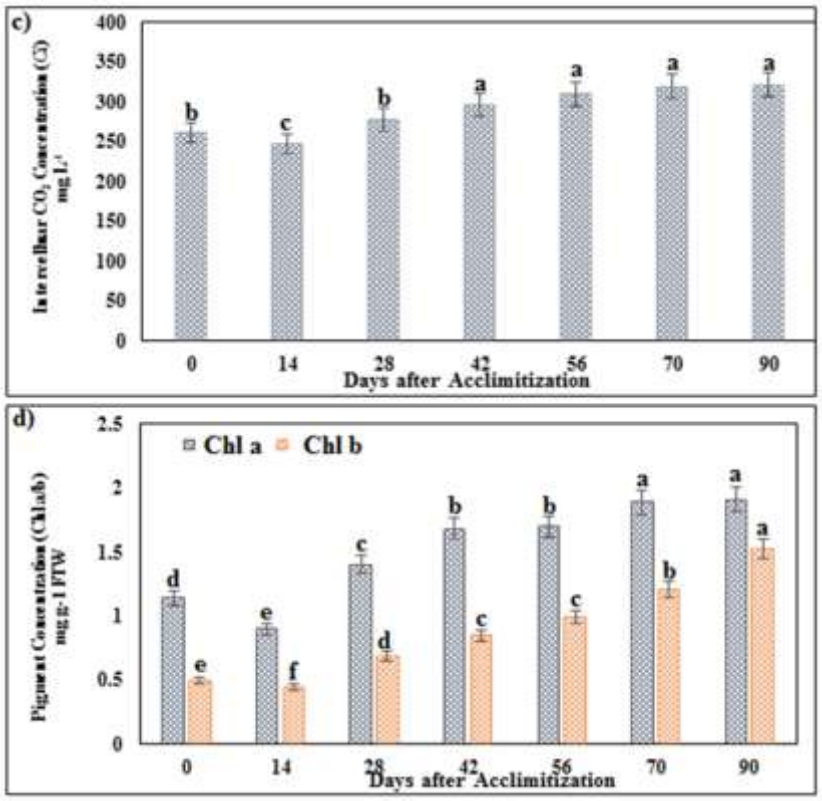

Figure 3. intercellular CO_2 concentration and chlorophyll contents during acclimatization of in vitro generated plantlets

4. Discussion

Different plant species show significant differential shoot and root growth responses during in vitro propagation due to variation in the use of plant growth regulator type, concentration, uptake, and transport as well as endogenous production of phytohormones (Ahmad et al., 2020; Danova et al., 2021). Exogenous application of PGRs modulates the activity of phytohormones and growth-responsive enzymes that ultimately improve the growth and photosynthetic potential (Quamruzzaman et al., 2021). The present study was focused to evaluate the role of PGRs (TDZ and GA_3) in micropropagation of D. lutescens to conserve this endangered palm tree by minimizing the time taken by conventional propagation means. In vitro conservation techniques can be used for rapid production of disease-free, true-to-type planting material for commercial trade of ornamental woody plant species, and also might be useful for forest development and conservation. Previous studies also reported that exogenous use of PGRs induces the rejuvenation of multiple shoots and root initials from different explants during in vitro propagation of various plant species (Phaseolus vulgaris, Murrayakoenigii, Litchi chinensis, Sterculiaurens, and Albizia lebbeck) suggests morphogenic responses are PGRs dependent instead of explant (Ahmad et al., 2021).

In the present study, the shoot regeneration response of apical meristems of D. lutescens was remarkably higher with better shoot elongation and multiplication in an MS medium containing 0.50 μM of TDZ compared to GA3 (Table 1). The present study highlights that a low dose of TDZ was found to be most effective for the propagation of D. lutescens but over-exposure to the same dose of TDZ resulted in shoot and leaf tip necrosis and abnormal shoot and leaf growth (Figure 1). This negative effect of TDZ was minimized by the addition of GA_3 in MS media in the present study as it was already reported in a recent study that GA_3 works antagonistically to cytokinins or TDZ but increases the morphogenic growth (shoot initial stimulation, elongation, and multiplication) responses during in vitro propagation (Cosic et al., 2021). Moreover, maximum shoot proliferation with better shoot elongation in 96% of cultures was recorded in MS media supplemented with optimum concentration (0.5 M) of TDZ in a combination of GA_3 after 8 weeks of sub-culturing (Table 2). Similar findings have been reported in many plant species (Ahmad et al., 2021).

Several previous studies reported the abnormal morphogenic growth responses in shoot/root elongation and multiplication under overexposure to TDZ in various plant species (Kumari et al., 2018). The reason for abnormal morphogenic growth in response to exposure to TDZ is due to auxin transport disruption that could be ameliorated by the addition of GA_3 which works antagonistically to TDZ (Kumari et al., 2021). The findings of the present study support the addition of optimum concentration of GA_3 (0.50 μM) in combination with TDZ (0.50 μM) in MS medium improves the normal morphogenic growth rate during in vitro propagation. Generally, GA_3 regulates the production of α-amylase and brassing steroids enzymes by upregulating the GAMYB transcription factors BRASSINAZOLE-RESISTANT-1 transcription factor which facilitates the seed germination and seedling elongation respectively (Lee et al., 2017). Hence, the combined effect of GA_3 with TDZ is more effective to control the biosynthesis of important growth-promoting elements and signal transduction cascade. Therefore, supplementation of MS medium with a combination of TDZ and GA_3 successfully improved the normal morphogenic growth response (shoot elongation, multiplication and leaf development) of apical meristem of D. lutescens as well as a lower dose (0.5 M) of both PGRs was found to be most effective for in vitro propagation.

Root induction in vitro generated micro shoots is often a major problem and considerably causes economic losses in the micropropagation of woody species (Stevens and Pijut, 2018; Orgec et al., 2021). Thus, in tissue culture, the adventitious root formation is important for healthy plant production by exogenous application of synthetic auxins (Demirci et al., 2021). Indole-3-butyric acid (IBA) is an essential synthetic auxin that successfully induced the adventitious roots in micro

shoots of several plant species due to tolerance against photodegradation and activation of root induction responsive genes (Velada et al., 2020; Lesmes-Vesga et al., 2021). Uptake and transport of IBA are more effective than other auxins (Raspor et al., 2021). In the present study, IBA-pretreated micro shoots were inoculated on ½ MS media supplemented with different regimes of IBA in combination with GA3. A combination of 0.5 M of IBA and 0.5 M of GA3 was found to be most effective for root induction of in vitro generated shoots of D. lutescens was earlier reported in Carica papaya (Zhou et al., 2021). Short roots were observed in GA3 deficient Pterocarpus marsupium cultures in comparison to GA3 treated Pterocarpus marsupium cultures (Ahmad et al., 2021).

In vitro-generated plantlets are adaptive to low relative humidity, high transpiration, and high CO2 conductance and lose their potential to survive in ex vitro conditions due to wilt (Asayesh et al., 2017). This abnormal physiological functioning is a major problem in the acclimatization of in vitro-generated plantlets (Leite et al., 2021). Initial abrupt reduction in physiological traits (Pn, gs, and Ci) of in vitro generated plantlets during the first fourteen days of acclimatization (Figures 2 and 3) was due to inadequate functioning of stomata (Vahdati and Aliniaeifard, 2017). Non-stomatal factors like low concentration of chlorophyll contents, and reduced activity of RUBISCO are also responsible for the low photosynthetic rate of plantlets (Yang et al., 2018). These abrupt changes could also be attributed to somaclonal variations (temporary epigenetic heterogenic changes) of in vitro generated plantlets (Javed et al., 2019). Continuous overexposure to PGRs hampers the genetic fidelity that also affects the physiological activities of in vitro generated plantlets (Javed et al., 2019; Ahmad et al., 2021). A steady change in physiological activities of acclimatized plantlets was recorded after 28 days of acclimatization and plantlets were stabilized and adaptive to ex vitro conditions up to the 90^{th} day of acclimatization.

5. Conclusions

The combined exogenous application of *TDZ* and GA_3 in MS culture media is the most appropriate and efficient dose of PGRs for in vitro propagation and procurement of true-to-type plants from apical meristems to conserve the endangered *D.* lutescens. Both PGRs are involved in the improvement of plantlet regeneration as well as the maintenance of physiological stresses during in vitro propagation. The combination of both PGRs (GA_3 and TDZ) at an optimal concentration (0.5 M) had a synergistic effect on shoot formation, elongation, and proliferation. It was also found that GA_3 in combination with IBA enhanced in vitro root formation in micro shoots. The rooted plantlets successfully acclimatized to soil and did not show any detectable variation in morphology, physiology, and growth of regenerated plantlets during three months. Consequently, this study revealed the high throughput regeneration potential of *D.* lutescens through apical meristem. It could be a useful alternative approach for mass propagation and conservation of woody ornamental trees under appropriate regimes of plant growth regulators.

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