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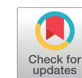
Research article

In vitro propagation and analysis of mixotrophic potential to improve survival rate of *Dolichandra unguis-cati* under ex *vitro* conditions



Vineet Soni [a,*], Kiran Keswani [a,b], Upma Bhatt [a], Deepak Kumar [a], Hanwant Singh [a]

[a] Plant Bioenergetics & Biotechnology Laboratory, Department of Botany, Mohanlal Sukhadia University, Udaipur 313001, Rajasthan, India
[b] Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth University, Pune 411045, Maharashtra, India

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ABSTRACT

An efficient and reproducible protocol for *in vitro* propagation of *Dolichandra unguis-cati* has been established for the first time from nodal segments. In order to enhance survival rate under *ex vitro* conditions, photosynthetic potential of *in vitro* grown plantlets was also studied through JIP test based analysis of polyphasic OJIP chlorophyll *a* fluorescence OJIP transients, density of active reaction centers, light harvesting efficiency, electron transfer rate, dissipation energy, maximum quantum yield of primary PSII photochemistry and photosynthetic performance index. The best morphogenetic in term of explants response (92.2 %), shoot number (3.43 ± 0.07) and shoot length (4.7 ± 0.31 cm) was obtained on Murashige and Skoog medium supplemented with 0.5 mg l^{-1} BAP and 1.0 mg l^{-1} TDZ. The shoots exhibited high frequency rhizogenesis on half strength medium augmented with 2.0 mg l^{-1} IAA. *In vitro* plantlets developed highest rate of photosynthesis on day 18 after the initiation of rhizogenesis. High survival rate (96.16%) under ex vitro conditions was observed when *in vitro* plantlets having high photosynthetic efficiency (Fv/Fm > 0.75) were subjected to hardening and acclimatization process. Plantlets with reduced photosynthetic performance exhibited low survival rate under natural conditions. The developed *in vitro* protocol will be useful for genetic improvement and multiplication of *D. unguis-cati*. The results of this study also show that photosynthetic screening of *in vitro* developed plantlets is highly essential after the rhizogenesis process to achieve higher survival rate under field conditions.

1. Introduction

Dolichandra unguis-cati (L.) Lohmann (Bignoniaceae) commonly known as 'cat's claw' is a perennial rampant liana which is found in diverse regions of the globe like Brazil, South America, Egypt, Mexico to northern Argentina and western India [1, 2]. The plant has been extensively utilized since time immemorial by the ancient Indian system of medicines for combating several ailments like dysentery and stomach bloating [3], flu, arthrosis, bronchitis, splenosis, headache [4], snake bite, diarrhea, fever, inflammatory reactions [5], rheumatism [6], uterus infection and cysts [7] due to the activities of diverse bioactive molecules like corimboside, vicenin-2, O-flavonol, chlorogenic acid, lupeol, vanillinic acid, quercetin, β-sitosterol, isochlorogenic acid, p-coumaric acid, β -sitosterilglycoside, lapachol, allantoin, decaffeoylacteoside, yoniresinol-3α-*O*-β-d-glucopyranside, cirsimarin, cirsimaritin, caffeic acid, ferulic acid, transcinnamic acid, luteolin, apigenin, rosmarinic acid, quercitrin and quercetin ursolic acid [2, 5, 8, 9]. Conventionally, *D. unguis-cati* is propagated by subterranean tubers and seeds [10, 11]. However, seasonal dependency of seed germination makes it an ineffective way for the conservation of this plant species [12]. Furthermore, development of *in vitro* protocol is one of the essential steps of genetic improvement and transformation of this high valued plant species. Therefore, immediate attention is highly required for the development of an efficient and reproducible *in vitro* protocol for mass propagation and genetic improvement of *D. unguis-cati*.

In vitro propagation of plants is an alternative for rapid and large-scale production of plants under control conditions [13]. The ultimate success of *in vitro* protocols relies on the ability to transfer the plantlets from *in vitro* to the *ex vitro* conditions with a high survival rate [14]. Low survival rate during hardening and acclimatization is the major constraint in the large-scale production of micropropagated plantlets [15]. The high concentration of exogenous sucrose in the nutrient medium induces photosynthetic down-regulation in plantlets raised under *in vitro* conditions [16, 17]. Therefore, prior to transfer under *ex vitro* conditions, photosynthetic performance of *in vitro* developed plantlets should be screened.

* Corresponding author.
E-mail address: vineetsonijnu@gmail.com (V. Soni).

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Chlorophyll fluorescence measurements provide valuable information of the physiological condition of photosystem II and components of photosynthetic electron transport chain [18] and have been widely used to screen photosynthetic performance of plantlets growing under *in vitro* conditions [19, 20, 21]. The JIP-test, based on the rise in polyphasic OJIP chlorophyll fluorescence, provides in depth information on the status and function of PSII reaction centers, antenna, as well as on donor and acceptor sides of PSII [22, 23, 24]. The O–J phase indicates the status of PSII, while the J–I step indicates the performance of the QB, plastoquinone, cytochrome b6f, and plastocyanin. The I–P part of the OJIP induction curve is correlated to the reduction of electron transporters of the PSI acceptor side [25].

Present study was aimed (1) to develop a reproducible protocol for *in vitro* regeneration of *D. unguis-cati* and (2) to perform photosynthetic screening of *in vitro* grown plantlets to achieve high survival rate under *ex vitro* conditions.

2. Materials and methods

2.1. Plant material and surface sterilization

Young shoots of *Dolichandra unguis-cati* (L.) L.G.Lohmann (Bignoniaceae) were harvested from Botanical Garden of Department of Botany, Mohanlal Sukhadia University, Udaipur, India. The excised shoots were washed thoroughly under running tap water for 3 min to eliminate dust particles and then treated with 0.1% bavistin and rinsed twice with sterile distilled water. Thereafter, surface sterilization of explants was done under a laminar flow chamber with aqueous solution of 0.1% (w/v) $HgCl_2$ for 3 min. After rinsing with double distilled water, nodal segments were cut into small pieces (2 cm) and used as the explants.

2.2. Culture media and growth conditions

The sterilized nodal explants were placed vertically on solid MS Medium [26] supplemented with 3 % sucrose, 0.8% (w/v) agar (Hi-Media, India) and various combinations/concentrations of plant growth regulators. The pH of the media was adjusted 5.8 before autoclaving at 121 °C for 15 min. All cultures were kept in a growth chamber at 25 ± 2 °C, 65–70 % relative humidity with photoperiod of 16-h using a photosynthetic photon flux density (PPFD) of 40 mmol m^2s^{-1} provided by cool white fluorescent tube lights (Philips, India). After 4 weeks of culture response percentage of the explants, numbers of shoots per explant, length of the shoots were evaluated.

2.3. In vitro rhizogenesis

Well developed shoots were subcultured on MS enriched with various concentrations (0.1–5.0 mg l^{-1}) of auxins viz. IBA, IAA and NAA to induce rhizogenesis *in vitro*. Prior to hardening process, the photosynthetic performance of well rooted plantlets was regularly measured by the analysis of polyphasic chlorophyll *a* fluorescence kinetics.

2.4. Measurement of photosynthetic performance

Plant Efficiency Analyser, PEA (Hansatech Instruments, U.K.) was used to analyze the photosynthetic potential of *in vitro* developed plantlets. Before the measurements, well developed plantlets were kept to darkness for 1 h. Fluorescence transients were induced over a leaf-lamina area of 4 mm diameter by a red light of 3000 μmol$m^{-2}s^{-1}$ provided by a high intensity LED array of three light emitting diodes. A total measuring time of one second was used thought out the experiments. Fluorescence values were used to calculate phenomenological fluxes (ABS/CSm, ETo/CSm and DIo/CSm), RC/CSm, Fv/Fm (ϕPo) and performance index on cross section basis (PIcs) using following equations (see Eqs. (1), (2), (3), (4), (5), and (6)) of JIP test [27, 28] –

$$ABS / CSm = \text{Fluorescence intensity at 50μs (Fo)} \quad (1)$$

$$ETo / CSm = \phi Po \times (ABS / CS \quad (2)$$

$$DIo / CSm = (ABS / CS) - [\phi Po \times (ABS / CS)] \quad (3)$$

$$\phi Po = 1 - (Fo / Fm) \ or \ Fv/Fm \quad (4)$$

$$RC / CSm = \phi Po \cdot (Vj / Mo) \times (ABS / CSm) \quad (5)$$

$$PIcs_m = \frac{ABS}{CS} \cdot \frac{1-(Fo/Fm)}{Mo/Vj} \cdot \frac{Fm-Fo}{Fo} \cdot \frac{1-Vj}{Vj} \quad (6)$$

where Ψ_0 is calculated as 1- V_J (Vj is relative variable fluorescence at the J-step and calculated as (F_{2ms} - F_0)/(F_M - F_0), Fv is variable fluorescence between Fm and Fo) and M_0 (approximated initial slope of the fluorescence transient) is calculated as 4 x ($F_{300μs}$-F_0)/(F_M-F_0).

2.5. Hardening and acclimatization

Only plantlets with functional photosynthetic apparatus and autotrophic potential were transplanted to plastic cups containing autoclaved soil, sand and coco peat (1:2:1) and then kept for 2 weeks in same growth chamber. On the other hand, plantlets with low photosynthetic potential were maintained on nutrient medium until the development of functional photosynthetic apparatus and autotrophic potential. The hardened plantlets were watered once a week. After hardening, the plantlets were subsequently transferred and maintained in the green net house (50% light transparency) with relative humidity 40–50% and 32 ± 2 °C.

2.6. Experimental design and statistical analysis

All tissue culture experiments were conducted with a minimum of 30 replicates per treatment and each experiment was repeated thrice. All data were analyzed statistically using GraphPad Prism 8. Differences were considered significant when the p value was <0.05. Photosynthetic data were analyzed using Biolyzer Software ver. 3.06 [29].

3. Results and discussion

3.1. In vitro establishment and multiplication

BAP at 0.5–5.0 mg l-1 concentrations could not evoke any significant morphogenetic response in nodal explants. After one week of inoculation on MS fortified with 0.5 mg l-1 BAP, explants showed swelling. At elevated concentration of BAP (5.0 mg l-1), formation of single shoot bud followed by the swelling was observed after 11 days of culture (Figure 1a). The shoots emerged on BAP containing medium failed to elongate. Low frequency of shoot bud proliferation was noted on MS augmented with KIN at high concentration (5.0 mg l-1) (Figure 1b). In the present studies, KIN at low 0.5 mg l-1 was found most effective in multiple shoot bud induction from nodal explants.

TDZ, a cytokinin-like compound, promotes a diverse array of morphogenic responses including shoot bud proliferation [30, 31]. Apart from its cytokinin-like activity, TDZ plays important role in modulation of endogenous hormone levels especially auxin/cytokinin ratio [32]. In the present study, our results revealed that the effect of KIN and TDZ combination on multiple shoot proliferation is more compared to KIN and TDZ when used separately. The best morphogenetic response in terms of explants response (92.2 %), shoot length (4.7 cm) and maximum number of shoots (4.33 shoots per node) was observed when 0.5 mg l-1 KIN was incorporated in MS along with 1.0 mg l-1 1 TDZ (Figure 1c), whereas high concentration of TDZ, either singly or in combination with KIN markedly decreased the frequency of shoot bud proliferation

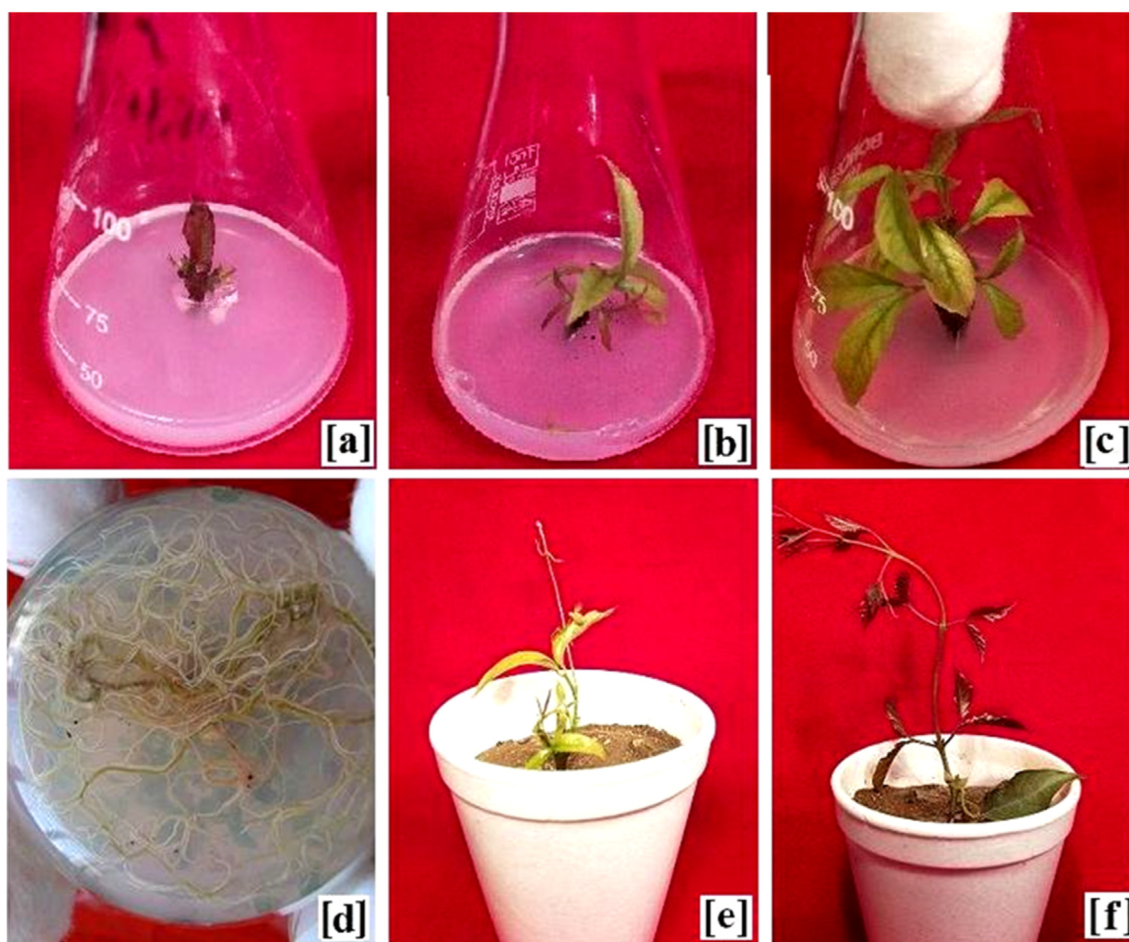

Figure 1. Nodal explants of *D. unguis-cati* showing the development of one shoot/node (a) on MS+5.0 mg l^{-1} BAP, low frequency shoot bud proliferation on MS+5.0 mg l^{-1} KIN (b), multiple shoot induction on MS+0.5 mg l^{-1} KIN and 1.0 mg l^{-1} 1 TDZ (c) rhizogenesis on half-strength MS + 2.0 mg l^{-1} IAA (d), hardened (e) and acclimatized plant (f).

Table 1. Influence of KIN and TDZ on explant response (%) and shoot length (cm) after 3 weeks of culture of nodal explants of *D. unguis-cati*. Values within the columns are highly significant at $p < 0.05$ and represented as mean of 30 replicates ± SD. Different characters indicate significant differences among the results ($p \leq 0.05$).

Plant growth regulators (mg l^{-1})		Explant response (%)	Mean shoot length (cm)	Shoots per node
KIN	TDZ			
Control		0.0^{d}	0.0^{d}	0.0^{c}
0.0	0.5	0.0^{d}	0.0^{d}	0.0^{c}
0.0	1.0	0.0^{d}	0.0^{d}	0.0^{c}
0.0	2.5	0.0^{d}	0.0^{d}	0.0^{c}
0.5	0.0	52.0 ± 3.5^{b}	2.8 ± 0.02^{ab}	2.66 ± 0.52^{ab}
0.5	0.5	51.21 ± 1.7^{b}	2.9 ± 0.06^{bc}	2.63 ± 0.65^{ab}
0.5	1.0	92.2 ± 4.4^{a}	4.7 ± 0.31^{a}	4.33 ± 0.50^{a}
0.5	2.5	13.81 ± 2.3^{c}	0.9 ± 0.06^{cd}	1.41 ± 0.24^{bc}

(Table 1). High cytokinin activity of TDZ inhibits in vitro shoot bud proliferation [33].

3.2. In vitro rhizogenesis

Inadequate rooting is one of the major constraints to the survival rate of plantlets under *ex vitro* conditions [34]. In the present study, Full strength MS singly or in combination with various concentrations of IAA (1.0–3.0 mg l^{-1}) could not evoke significant response in term of rhizogenesis *in vitro*. Half-strength MS supplemented with IAA proved best in term of initiating rhizogenesis *in vitro* as compared to full strength MS (Table 2). Half strength MS supplemented with low concentration of IAA (<1.0 mg l^{-1}) could promote rhizogenesis *in vitro* at low frequency. Half strength MS along with IAA 2.0 mg l^{-1} exhibited highest mean number of roots per shoot (11.40) and increased root length to the maximum (4.03 cm) (Figure 1d), while other concentrations of IAA caused antagonist effect on rhizogenesis *in vitro* in *D. unguis-cati*. Our results are in accordance with the studies carried out on *Cichorium intybus* [35], *Digitalis lanata* [36], *Prosopis laevigata* [37] and *Securidaca longipedunculata* [38]. The superiority of IAA over other auxins for induction of rhizogenesis has also been reported in *Vanda pumila* [39], *Dendrobium chryseum* [40], *Phyllanthus tenellus* [34].

3.3. Chlorophyll a fluorescence analysis

After the visible appearance of root primodia on Half strength MS + IAA 2.0 mg l^{-1}, the polyphasic chlorophyll *a* fluorescence analysis was done prior to hardening process to evaluate the photosynthetic potential of *in vitro* developed plantlets. Fluorescence parameters (F_0, F_m), phenomenological energy fluxes, RC/CSm, ϕP_o and PIcs remarkably altered with increasing days after root primodia formation (DAR). During the initiation days of rhizogenesis (days 0–6), the plantlets were failed to form a complete OJIP curve. On 12 and 18 DAR, chl *a* fluorescence OJIP curve of *in vitro* grown plantlets showed three apparent intermediate phases namely OJ, JI and IP (Figure 2a). Photochemical phase OJ [27]

Table 2. Influence of IAA on rhizogenesis *in vitro* in *D. unguis-cati* after 3 weeks of culture. Values within the columns are highly significant at $p < 0.05$ and represented as mean of 30 replicates ± SD. Different characters indicate significant differences among the results ($p \leq 0.05$).

Media Combinations	% Rooting	Mean no. of root/shoot	Mean root length (cm)
MS full strength	0^{g}	0^{d}	0^{d}
MS full strength + 1.0 mg l^{-1} IAA	13 ± 3.1^{ef}	1.16 ± 0.6^{cd}	1.02 ± 0.4^{cd}
MS full strength + 2.0 mg l^{-1} IAA	26 ± 2.6^{d}	4.16 ± 1.0^{bc}	2.81 ± 0.9^{ab}
MS full strength + 3.0 mg l^{-1} IAA	21 ± 3.2^{de}	4.14 ± 0.8^{bc}	2.87 ± 0.9^{ab}
MS half strength	8 ± 1.8^{fg}	2.82 ± 0.4^{bcd}	1.01 ± 0.4^{cd}
MS half strength + 1.0 mg l^{-1} IAA	57 ± 3.2^{b}	4.73 ± 1.6^{b}	1.42 ± 0.5^{bcd}
MS half strength + 2.0 mg l^{-1} IAA	94 ± 4.8^{a}	11.40 ± 2.4^{a}	4.03 ± 0.8^{a}
MS half strength + 3.0 mg l^{-1} IAA	39 ± 4.1^{c}	4.72 ± 1.8^{b}	1.72 ± 0.6^{abc}

and the thermal phase JI [41] are linked to the accumulation of Q_A and the status of PSII RCs. IP phase displays PQ pool reduction [25, 42]. The minimal (Fo) and maximal fluorescence (Fm) enhanced continuously with increasing the subculture duration on RIM. Sucrose acts as fuel source for growth and development of *in vitro* plantlets. The decreased F_m level and disappearance of O-J and J-I phases indicates the presence of dissociated light harvesting complexes [43] and undeveloped photosynthetic apparatus during the initial days of rhizogenesis. The values of F_m apparently increased with increasing the subculture duration or sucrose consumption rate on RIM. Highest F_m level was noted on 18 DAR (Figure 3a). The concentration of exogenous sucrose progressively reduces with increasing the subculture duration as *in vitro* grown plantlets utilize it as carbon source for their growth and development.

The effects of subculture duration on fluorescence values Fo and Fm, phenomenological fluxes *i.e* ABS/CS, ET/CS, DI/CS, RC/CSm, ϕPo and performance index (PIcs) is represented in radar plot (Figure 3b). The flux of absorption and electron transfer per cross section (CS) of PSII, defined as ABS/CSm and ETo/CSm, respectively, were significantly increased in plantlets with increasing subculture duration on RIM as ES get exhausts over time. ABS/CSm which represents light harvesting efficiency of active PSII RCs, enhanced progressively and reached to highest level on 18 DAR. Low values of ABS/CSm during the emergence of root promidia indicate reduced antenna size and low chl concentration. Similarly, ETo/CSm progressively increased with progression of subculture duration on RIM. Reduced values of ABS/CSm and ETo/CSm during the appearance of root promidia (0 DAR) shows the antagonist effect of higher ES concentration on light harvesting and electron transfer potential in *Dolichandra unguis-cati*. Decline in DIo/CSm with progression of subculture duration on RIM was associated with reduced light harvesting potential (ABS/CSm). The effects of subculture duration on RIM on phenomenological energy fluxes (ABS/CSm, ETo/CSm and DIo/CSm) are diagrammatically represented through the leaf models.

Density of active PSII RCs (RC/CSm) increased as subculture duration increased (inactive PSII RCs are denoted as black dots in leaf models. The quantum yield potential of photosystem II (at t = 0) ($\phi Po = F_v/F_m = TR_0/ABS$) was found lowest (0.603) on 0 DAR, which further increased gradually with increasing subculture duration on RIM (Table 3). Highest Fv/Fm (0.755) was observed on 18 DAR. A reduction in Fv/Fm ratio is associated to photoinhibition or damage of PSII complex [25]. PIcsm, which displays the functionality of active PSII RCs markedly enhanced with increasing the duration on RIM. During the initial days of rhizogenesis, the presence of sugar in the medium lowers the photosynthesis through feedback inhibition. Increment of ABS/CS, ET/CS, DI/CS, RC/CSm, ϕPo and PIcs with increasing the subculture duration is linked to the gradual depletion of exogenous sucrose into the medium. Presents study indicates that *in vitro* plantlets are unable to develop their photosynthetic apparatus until the presence of exogenous sucrose into the medium. *In vitro* plantlets develop their photosynthetic apparatus after the complete consumption of exogenous sucrose from the medium. Exogenous sugars mediated inhibition of photosynthesis has been proven

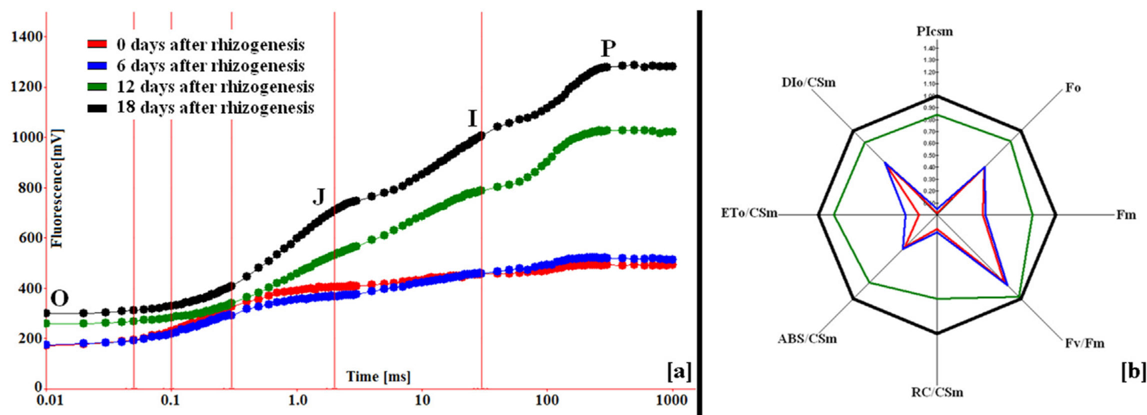

Figure 2. Leaf models showing changes in ABS/CSm, ETo/CSm and DIo/CSm and RC/CSm with increasing days after the emergence of root primodia (DAR) on rooting inducing medium (blacks dots represent inactive PSII RCs).

Table 3. Changes in various photosynthetic parameters with increasing the subculture duration after the initiation of root-primodia (days after rhizogenesis-DAR) on RIM. Values within the columns are highly significant at $p < 0.05$ and represented as mean of 30 replicates ± SD. Different characters indicate significant differences among the results ($p \leq 0.05$).

DAR	Fo	Fm	RC/CSm	ABS/CSm	ETo/CSm	Fv/Fm	PIcsm	Survival rate under *ex vitro* condition (%)
0	164 ± 8.33^{c}	492 ± 14.63^{d}	119.04 ± 5.72^{d}	492 ± 11.76^{d}	86.99 ± 7.14^{d}	0.603 ± 0.05^{d}	750.9 ± 7.14^{d}	0^{d}
6	176 ± 6.56^{c}	521 ± 10.87^{c}	146.43 ± 11.92^{c}	521 ± 14.82^{c}	153.02 ± 8.61^{c}	0.629 ± 0.06^{c}	2176.0 ± 17.34^{c}	4.81 ± 1.03^{c}
12	256 ± 9.24^{b}	1028 ± 11.51^{b}	702.78 ± 12.52^{b}	1028 ± 23.39^{b}	495.98 ± 15.17^{b}	0.738 ± 0.05^{b}	37400.4 ± 92.87^{b}	56.82 ± 3.62^{b}
16	293 ± 6.33^{a}	1278 ± 19.47^{a}	996.17 ± 20.53^{a}	1278 ± 17.90^{a}	570.06 ± 14.89^{a}	0.755 ± 0.07^{a}	44402.4 ± 289.90^{a}	96.16 ± 6.68^{a}

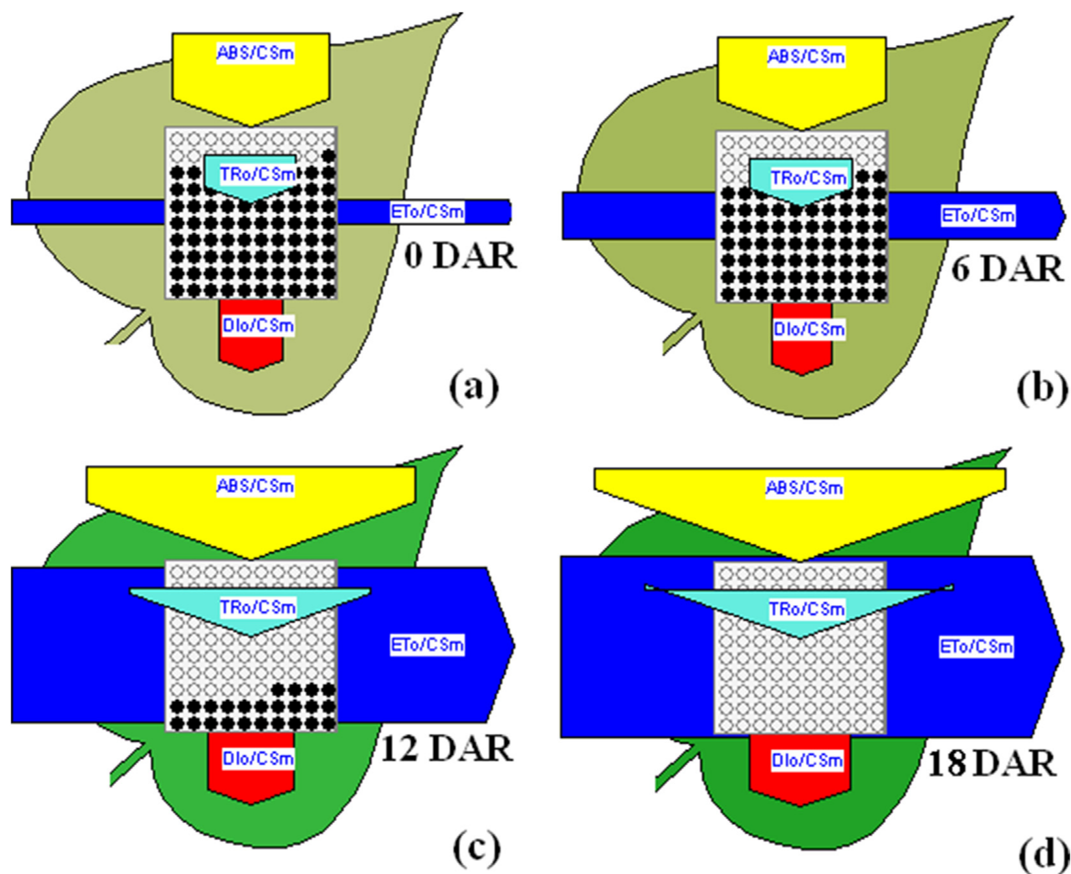

Figure 3. Chl fluorescence OJIP induction curves measured at regular intervals after the emergence of root primodia on rooting inducing medium (a), and radar plot showing the alternations in various photosynthetic parameters with increasing the subculture duration on rooting inducing medium (b).

by many researchers [44, 45, 46, 47], and is consistent with Koch's theory on the inhibitory influence of sugars on photosynthesis [48]. Exogenous sugar inhibits the expression of photosynthetic genes and reduces the activities of enzymes involved in CBB cycle [16]. Therefore, photosynthetic screening of *in vitro* grown plantlets is highly required before transferring plants from growth chamber to *ex vitro* conditions.

3.4. Hardening and acclimatization

Rooted plantlets were having high photosynthesis in term of ABS/CSm, ET0/CSm, DI0/CSm, RC/CSm, ϕPo and PIcsm showed high survival rate (96.16%) during hardening and acclimatization process (Figure 2e, f). *In vitro* grown plantlets with reduced photosynthetic potential showed declined rate of survival under natural conditions. Plants produced through *in vitro* method exhibited similar morphologically to mother plants. The results of this study show that photosynthetic screening of *in vitro* developed plantlets is highly essential prior to hardening process and the fast Chl *a* fluorescence transient measurement with high time resolution provide a non-invasive and rapid method to screen the photosynthetic potential of *in vitro* propagated plantlets to achieve higher survival rate under *ex-vitro* condition. In future, the developed *in vitro* protocol can be used for large scale propagation and genetic improvement of *D. unguis-cati*.

Declarations

Author contribution statement

All authors listed have significantly contributed to the investigation, development and writing of this article.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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