horticulturae

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Micropropagation of *Duboisia* Species via Shoot Tip Meristem

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Abstract: *Duboisia* is an Australian native, commercially valuable for tropane alkaloid extraction. Clonal propagation of elite selections is essential to establish highly productive plantations. The current propagation system using stem cuttings is proven to be inefficient, prompting the industry to seek a more efficient and effective propagation tool. Tissue culture is a cost-effective alternative for mass propagation of true-to-type plants, particularly ideal for propagating elite *Duboisia* selections. In this context, attempts were made to develop a commercially viable high throughput micropropagation system for three *Duboisia* species: *Duboisia myoporoides*, *Duboisia leichhradtii* and *Duboisia hopwoodii*. Various nutrient media, hormone combinations and incubating conditions were tested to optimise each stage of the micropropagation pipeline. The findings revealed that the tissue culture media composition and hormone requirements are species-specific. With the optimised conditions, an efficient tissue culture system was developed, achieving successful meristem induction and multiplication. Species-specific rooting protocol optimisation resulted in 100% rooting for *D. myoporoides* and *D. leichhardtii*, and 70% rooting for *D. hopwoodii*. Furthermore, an optimised acclimatisation protocol supported 100% survival of *D. myoporoides* and *D. leichhardtii* and 80% of *D. hopwoodii* plantlets. This study, for the first time, demonstrated the capacity of successful meristem culture of three *Duboisia* species, establishing the foundation for high throughput micropropagation of *Duboisia* species.

Keywords: *Duboisia myoporoides*; *Duboisia leichhradtii*; *Duboisia hopwoodii*; corkwood; tissue culture; substrate-based rooting; acclimatisation

Citation: Xue, Y.; Hiti-Bandaralage, J.C.A.; Jambuthenne, D.T.; Zhao, Z.; Mitter, N. Micropropagation of *Duboisia* Species via Shoot Tip Meristem. *Horticulturae* **2023**, *9*, 1313. https://doi.org/10.3390/horticulturae9121313

Academic Editors: Vasiliy A. Chokheli and Rupesh Kumar Singh

Received: 14 November 2023
Revised: 2 December 2023
Accepted: 4 December 2023
Published: 6 December 2023

1. Introduction

Duboisia, also known as 'corkwood', is a native Australian shrub that belongs to the Solanaceae family [1]. Primarily, there are four species identified: *Duboisia myoporoides*, *Duboisia leichardtii*, *Duboisia hopwoodii* and a recently identified *Duboisia arenitensis* [2]. It has long been used as a medicinal plant by Australian Aboriginal people, with the earliest records dating back to the 1770s [3,4]. This species has become an economically important crop, commercially grown for two potent anticholinergic (inhibit acetylcholine action) and antispasmodic (relieve spasms or convulsions) alkaloids, scopolamine and hyoscyamine [5]. In humans, these alkaloids exhibit mydriasis, analgesia and sedative properties [6], with scopolamine having fewer adverse effects and greater physiological activity than hyoscyamine [7]. Thus, the global scopolamine demand is expected to be 10 times more than hyoscyamine and its derivative atropine [8]. Scopolamine output falls far below market demand, pushing the price to peak at AUD 15,000 per kilogram, lacking other substitutes [9]. Scopolamine synthesis is possible via yeast [10]; however, the process is complicated and costly. Therefore, several Solanaceae species (*Duboisa*, *Datura*, *Hyoacyamus*, *Atropa* and *Scopolia*) have become the exclusive source for scopolamine production [11].

Duboisia is among the highest producers, recording 6% of the total alkaloids by leaf dry weight, in contrast with 0.2–0.8% that of *Atropa* and *Datura* species [8]. *Duboisia* is a fast-growing plant, takes 10 months from planting to harvest and is less affected by pests and diseases [12,13], making it the best source of commercial crops. Commercial scopolamine production is highly dependent on *Duboisia* cultivation and optimum processes developed for leaf drying and alkaloid extraction. Australia leads the world in *Duboisia* leaf production, exporting 1000 tonnes per year, which accounts for 70% of the global demand [3].

Catering the high plant demand of elite *Duboisia* selections is one of the biggest challenges faced by this industry. *Duboisia* being an outcrossing species, seeds are not true-to-type to the parent material, thus not suitable as a propagule for plantations. Even if compromised, seed germination is difficult and results in poor germination percentages as well as shows poor growth vigour [14]. Cutting propagation is the current, reliable clonal propagation method but is not very efficient owing to poor and inconsistent rooting, especially with ageing mother plants [15]. Further, the volume of material required for cutting propagation is considerably high and economically expensive.

Tissue culture is a well-established plant propagation method worldwide to propagate plants, ensuring genetic stability and in high volumes year-round, utilising a very limited amount of starting material. Among the different techniques available for clonal propagation, meristem culture is considered very effective and efficient for high throughput propagation. Meristem culture is one of the direct regeneration techniques in tissue culture that involves culturing the meristematic dome of either shoot or root tips [16–18]. This technique is well-developed for virus elimination, which plays a significant role in the tissue culture cloning of disease-free plants [19]. For large-scale commercial propagation, meristem culture is also the best in vitro propagation technique due to the fact that it produces enhanced axillary branching and minimizes somaclonal variation [20]. Plants regenerated through meristem culture reportedly have the highest multiplication rate for numerous horticulturally important species such as avocado [18], sugarcane [21] and rose [22]. Despite these advantages, meristem culture is the most challenging technique to be optimised due to the low survival rate and difficulty in the induction of typically less than 0.1 mm meristem explants to grow into individual plants [23]. Especially, woody plants are notoriously recalcitrant to tissue culture conditions [24,25], so developing a meristem culture platform for such species is much more challenging. Several woody plant species, including apple [26], peach [27], pear [28] and avocado [16,18], have been successfully propagated through this technique. The previous attempts to develop a viable micropropagation system for *Duboisia* have proven to be challenging. Most studies relied on indirect organogenesis through callus [29], posing a risk of somaclonal variation. Notably, the work by Kukreja and Mathur [30] stands as one exception, which demonstrated successful direct organogenesis through nodal culture. However, root induction remained recalcitrant, with a maximum rooting rate of 80%. To date, there is no report of commercial propagation of *Duboisia* through tissue culture. In this study, the primary aim was to establish a high throughput meristem culture-based propagation system for three *Duboisia* species, *D. myoporoides*, *D. leichhradtii* and *D. hopwoodii*. Commercially viable selections are hybrids of aforementioned three species, thus, the overarching objective was to use this foundation for high throughput propagation of existing commercial hybrids and faster commercial adoption of new hybrids generated via breeding programs.

2. Materials and Methods

2.1. Sterilisation and In Vitro Culture Establishment

Aseptic cultures were established for the three *Duboisia* species using nodal segments to obtain shoot tips for meristem culture. Mature mother plants (attained flowering) were maintained in the glasshouse at The University of Queensland and used to collect explant material for culture initiation. Branches were carefully selected for no signs of disease or nutrient deficiency to collect 5–8 cm twigs. Leaves were removed, and twigs were washed three times with liquid hand soap in a 500 mL plastic container while continuous

shaking, followed by a washing step under running tap water for 45 min. Next, steps of the sterilisation process were carried out in a laminar flow cabinet, where explants were treated with 70% ethanol for 3 min followed by three times rapid washing with sterile distilled water. Twigs were then soaked with 3% (*v*/*v*) sodium hypochlorite (Ajax Finechem, Sydney, NSW, Australia) solution with one drop of tween-20 (Sigma Aldrich, Bayswater, VIC, Australia) for 3 min. Finally, the twigs were rinsed three times with sterile distilled water. Each twig was cut into smaller nodal segments (2–3 nodes) and cultured on Murashige and Skoog (MS) [31] basal medium supplemented with 2% sucrose and 7 g L^{-1} agar, pH set at 5.7 and autoclaved at 121 °C for 15 min. Cultures were maintained in a growth room at 25 °C with a 16 h/8 h (day/night) photoperiod (Lumilux cool daylight; OSRAM L 36W/865; PPFD 221.417 µmol m^{-2} s^{-1}) for 7 days to select uncontaminated cultures. After 2 weeks of culture, the established aseptic cultures were subjected to subsequent experiments.

2.2. Basal Medium Selection

To select the optimum basal medium for in vitro culture of the three *Duboisia* species, a hybrid between *D. myoporoides* and *D. leichhradtii* was used as a model plant. This approach was chosen to provide a preliminary screening of basal media that can potentially support the growth of both parent species, thus saving time and resources.

Three basal media, including MS, Woody Plant Medium (WPM) [32] and Quoirin and Lepoivre (QL) [33] medium, were evaluated for in vitro culture of hybrid *D. myoporoides* × *D. leichhardtii* (*M* × *L*). Aseptic shoot cultures of *M* × *L* were established using the method described in 2.1. The explants were cut into single nodal segments (0.8–1 cm) with all leaves removed and cultured on individual test tubes containing 15 mL of the selected basal medium (free of hormones) were supplemented with 2% sucrose and 7 g L^{-1} agar, pH set at 5.7 and autoclaved at 121 °C for 15 min. The nodal segments were maintained in the previously mentioned conditions for 6 weeks and subcultured at 3-week intervals. The number of leaves and shoot heights were recorded, and the shoot quality was scored based on morphological and physiological appearance at the time points of 3 weeks and 6 weeks. The scoring system developed in this study consisted of five categories, each indicating a specific level of shoot quality (Table 1). This trial was carried out with 30 single nodal segments per treatment (*n* = 30). Due to the limited plant material, this trial was not repeated. However, the best basal medium evaluated was adapted with the method mentioned above for the three *Duboisia* species through independent trials.

Table 1. Morphological and physiological standard for scoring shoot quality of *Duboisia* in vitro cultures.

Scores	Description of Morphological Features for Scoring
1	No bud breaking, dead shoot, vitrified shoot or callus overgrowth.
2	Unopened green, healthy buds or shoots.
3	Shoots with open leaves between 0–10 mm in length or leaves showing yellowing symptoms.
4	Shoots with at least one fully expanded green, healthy leaf larger than 10 mm and without leaf yellowing.
5	Shoots with more than three green, healthy leaves larger than 25 mm and without leaf yellowing.

2.3. Meristem Induction and Multiplication

To extract the meristems, shoot tips from the aseptic cultures developed in Section 2.1. were excised by removing all outer leaves and visible leaf primordia. During this process, a millimeter grid of graph paper was put under a 60 mm sterile Petri dish to provide a reference for precise positioning and alignment of the meristems. The preliminary experiment revealed that the WPM was better for meristem induction compared to the MS medium. Thus, the meristems were inoculated into a glass test tube containing 15 mL

of half-strength liquid WPM supplemented with different types of cytokinins at various concentrations based on the current *Duboisia* literature [29]. Initially, 6-Benzylaminopurine (BA) in a concentration ranging from 1 to 15 mg L^{-1} was tested for all three species. For *D. hopwoodii*, however, a broader screening on cytokinin types and concentrations was required, including BA (in range of 1–15 mg L^{-1}), kinetin (in range of 1–10 mg L^{-1}), zeatin (in range of 1–10 mg L^{-1}), Thidiazuron (TDZ, in range of 0.05–0.5 mg L^{-1}) and 2-isopentenyladenine (2iP, in range of 0.1–1 mg L^{-1}). All media were supplemented with 1.5% sucrose, pH set at 5.7 and autoclaved for 15 min at 121 °C. The test tubes were put on an orbital shaker (Bioline, Narellan, NSW, Australia) at 50 rpm in the previously described conditions. This experiment was triplicated as an independent experiment, with 10 individual meristem explants used for each treatment.

After 3 weeks, meristem-induced shoot clumps with visible shoot buds were subcultured onto a freshly prepared multiplication medium. The multiplication medium used for *D. myoporoides and D. leichhradtii* was MSK2 medium (amended MS medium); however, *D. hopwoodii* did not initially perform well in this medium. Therefore, a preliminary experiment was carried out supplementing coconut water in the media at different concentrations, leading to identifying MSK2C (modified MSK2 medium with coconut water) as the best media composition for *D. hopwoodii.* Continuous subculturing was carried out every three weeks to maintain shoot quality and growth vigour. The optimum cytokinin treatment for each species was determined by visual observation, and the multiplication rate (MR) was calculated after each subculture (F) for the best treatment. The total multiplication rate was determined at the end of F5. The total MR at the end of F5 indicates the number of shoots multiplied from the initial 10 meristem explants after 5 subculture cycles.

$$\text{Multiplication rate (MR)} = \text{Number of shoots produced/Initial cultures used}$$

2.4. Rooting

2.4.1. General Rooting Methods

For all three species, shoots with at least 4–5 open leaves and 2–3 cm in height were harvested from the 5th subculture cycle onwards. These shoots were then subjected to rooting experiments under sterile conditions using a five-second pulse treatment of Indole-3-butyric acid (IBA) solutions. For individual experiments, shoots with the same subculture cycle were used. All rooting treatments were performed inside a laminar flow cabinet, and IBA solutions were filter sterilised (0.22 µm filter/Milles®-GS). A fresh slant cut was made at the basal end of the shoots, then dipped into IBA solution for 5 s and inoculated into the basal medium/substrate. The basal medium used for agar medium-based rooting was MS medium, supplemented with 2% sucrose and 7 g L^{-1} agar, pH set at 5.7 and autoclaved at 121 °C for 15 min. Independent trials/experiments were carried out for the in vitro agar medium-based rooting and substrate-based rooting of three *Duboisia* species. Root induction was conducted under a controlled environment, using a growth cabinet (Conviron® GEN100) at 25 °C, 16 h/8 h (day/night) photoperiod (Lumilux cool daylight; OSRAM L 36W/865; PPFD 221.417 µmol m^{-2} s^{-1}) and 73% of relative humidity.

2.4.2. Agar Medium-Based Rooting

Initially, agar medium-based rooting of all three *Duboisia* species was trailed by dipping the shoots (10 shoots per treatment, $n = 10$) into an IBA solution (in the range of 1–10 g L^{-1}) for 5 s, then inoculated into MSCh (modified MS medium with activated charcoal) and MS media (control). For *D. hopwoodii*, two extra basal media, MSCo (modified MS medium with coconut water) and MSIr (modified MS medium with iron supplements), were evaluated due to the promoting effects of these additives during the multiplication stage (preliminary experiments). Due to the limited plant material and poor rooting outcome, the above trials were not repeated but provided inputs for subsequent experiments.

2.4.3. Substrate-Based Rooting

To further improve the rooting percentage, substrate-based rooting was attempted for the three *Duboisia* species. Micropropagated shoots of all three *Duboisia* species were treated with the IBA dipping solution (1–10 g L^{-1}) for 5 s, then inoculated into two different substrates, Jiffy cube (Jiffy-7®) and foam rooting cube, for the evaluation of substrate-based rooting. For *D. hopwoodii*, further evaluation on the concentration of IBA dipping solution was required to overcome severe defoliation in the Jiffy cube, with the extra treatment IBA (0.1–5 g L^{-1}) tested. Furthermore, a different rooting substrate rockwool was also tested for *D. hopwoodii* with the optimised IBA concentration (0.1–5 g L^{-1}) for the purpose of improving shoot quality and the rooting percentage. To prepare the rooting substrates, Jiffy cube, foam rooting cube and rockwool were saturated with water in plastic tubs (Sigma Aldrich) and autoclaved at 121 °C for 15 min prior to use. In the case of *D. hopwoodii*, prior to autoclave, 3–5 mL of half-strength liquid MS medium (with no sucrose) was added to each rooting cube/rockwool using a Pasteur pipette (Sigma Aldrich) to provide additional nutrients based on the poor shoot quality observed in preliminary experiments when only water was used. Phenotyping for root induction was carried out 4 weeks after IBA treatment. All substrate-based rooting experiments were triplicated as independent experiments, each with 10 (rockwool treatments, $n = 10$) or 12 (Jiffy and foam rooting cube treatments, $n = 12$) shoots per treatment.

2.5. Acclimatisation

Initial acclimatisation attempts with plants rooted in the agar-based medium were not very promising due to less vigour of the plant, possibly due to a limited number of roots and poor shoot quality. Thus, the subsequent acclimatisation experiments were conducted only with the substrate-based rooted plants. *D. myoporoides* was the first species to come through the micropropagation pipeline with good quality plantlets and an adequate number of rooted plants; therefore, acclimatisation experiments were initially conducted for *D. myoporoides* with two different methods (acclimatisation in Ziploc bag and in seed tray) imposing different acclimatisation climates. This experiment was triplicated with 10 plants for each treatment.

The rooted *Duboisia* plants were gently potted in potting mixture (UQ23) in individual pots (50 mm × 50 mm × 80 mm) without damaging the root system. The potted plants were placed either in sealed Ziploc bags or on seed trays covered with a plastic dome lid. Plants were incubated for 7 days in a growth cabinet (Conviron® GEN100) with the previously mentioned conditions. These plants were misted with water 3 times a day. After a 7-day incubation, the lid of the seed tray was removed, while Ziploc bags were opened and continued to incubate for another 7 days in the growth cabinet until they were transferred to UQ glasshouse, maintained under a 30% shade cloth for 7 more days before scoring for survival.

The best acclimatisation procedure identified for *D. myoporoides* was then adapted with the method mentioned above for the other two species, *D. leichhardtii* and *D. hopwoodii.*

2.6. Data Analysis

All data generated from this study were statistically analysed using GraphPad Prism software (version 9.5.1, GraphPad Software Inc, San Diego, CA, USA). The normality of the data was determined using the Shapiro–Wilk test, and depending on the results, data were analysed using either an independent samples *t*-test or the Mann–Whitney U test for two-group comparisons. For multiple group comparisons, an ANOVA test was conducted, and significant differences among the mean values were calculated using Tukey's HSD test.

3. Results

3.1. Sterilisation and In Vitro Culture Establishment

The sterilisation process successfully eliminated potential contaminants from nodal segments, leading to the establishment of aseptic cultures for all three *Duboisia* species

(Figure 1). The observed rates of aseptic culture establishment were 96.08% for *D. myoporoides*, 96.97% for *D. leichhradtii* and 98.50% for *D. hopwoodii*. These cultures served as the plant material for subsequent experiments involving basal media selection and meristem extraction.

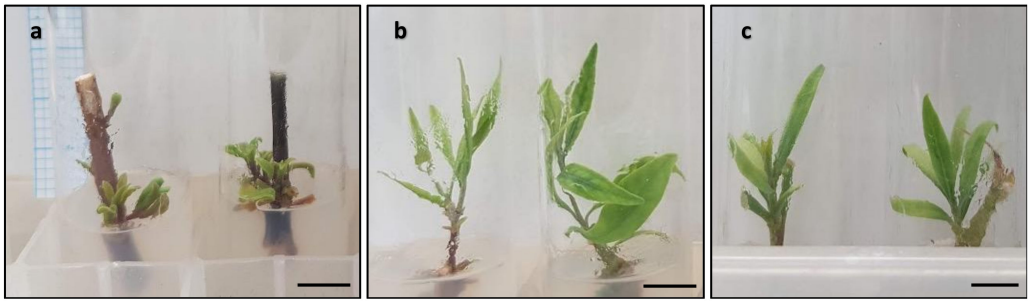

Figure 1. Aseptic culture establishment of (**a**) *D. myoporoides*, (**b**) *D. leichhradtii* and (**c**) *D. hopwoodii* after 2 weeks of culture. Graph paper grid size = 2 mm × 2 mm. Bars = 1 cm.

3.2. Basal Medium Selection

To streamline the screening for basal media that can support the growth of the three *Duboisia* species, a hybrid *D. myoporoides* × *D. leichhardtii* (*M* × *L*), was selected as a model plant for the preliminary screening. At the 3-week time point, the shoots grown on MS medium produced more leaves than those grown on QL medium, while no significant difference was observed in the number of leaves between shoots grown on WPM and QL medium. However, at the 6-week time point, the shoots cultured on MS medium had significantly more leaves compared to those cultured on WPM and QL medium (Figure 2a).

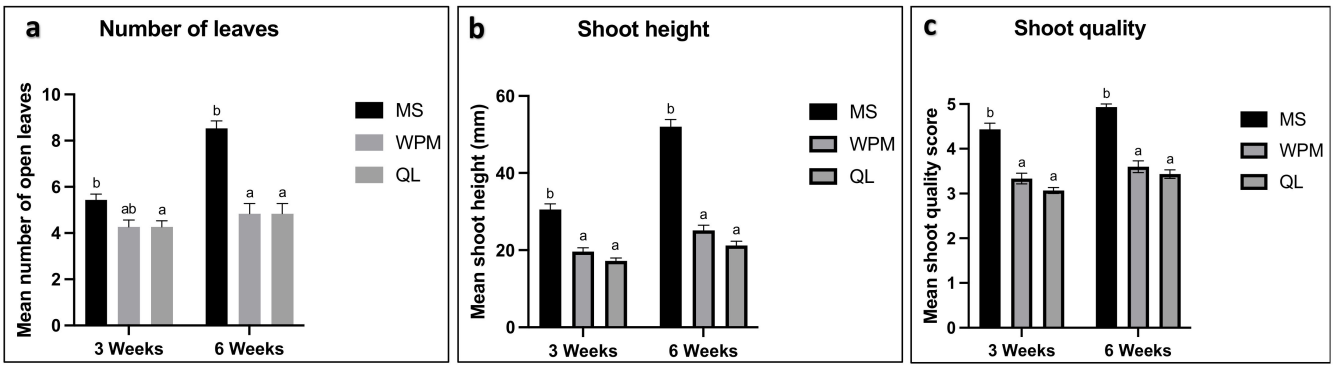

Figure 2. Comparison of the effects of Murashige and Skoog medium (MS), Woody Plant Medium (WPM) and Quoirin and Lepoivre medium (QL) for hybrid *M* × *L* shoot culture at different time points. (**a**) Mean number of open leaves; (**b**) Mean height and (**c**) mean shoot quality score. All data are presented as mean ± standard error of the mean (SEM). Bars within each measured time point with different letters are significantly different ($p < 0.05$).

Shoot height was assessed at both 3-week and 6-week time points to compare the effects of different basal media on the height of the hybrid *M* × *L* shoot culture. Shoots grown on MS medium were significantly taller compared with those grown on WPM and QL media, while the height of the shoots grown in WPM and QL media did not differ significantly. This trend was also observed at the 6-week time point, indicating that MS medium is more effective in promoting shoot height compared with WPM and QL media (Figure 2b).

The effects of different basal media on the shoot quality of the hybrid *M* × *L* were evaluated at the 3-week and 6-week time points. The MS medium was found to be the optimum for maintaining shoot quality, as the shoots grown on MS had a significantly higher shoot quality score compared to those grown on WPM and QL media. However,

there was no significant difference observed between the shoots grown in WPM and QL media. These results were consistent at both 3 and 6 weeks (Figure 2c).

The MS medium was identified as the optimum basal medium for hybrid *M* × *L* shoot culture (Figure 3), supporting the highest mean number of leaves, shoot height and shoot quality score at 6 weeks of culture. Murashige and Skoog's medium was then successfully used for the three *Duboisia* species with similar positive results on shoot quality in independent trials. Therefore, the best-performing basal medium identified for the hybrid *M* × *L* was confirmed to be suitable for the shoot culture of its parent species, *D. myoporoides* and *D. leichhardtii*, as well as *D. hopwoodii*.

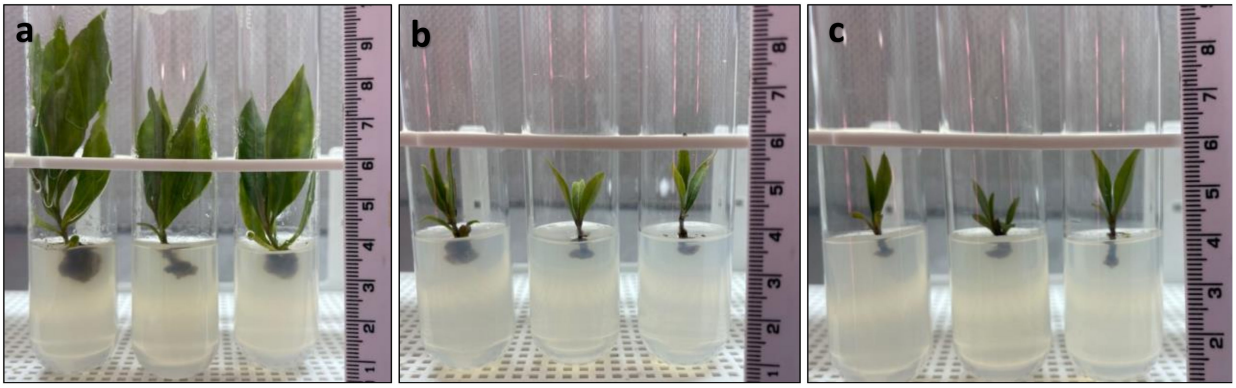

Figure 3. Comparison of different basal media for hybrid *M* × *L* shoots at 6 weeks of culture. (**a**) Shoots in MS medium; (**b**) Shoots in WPM; (**c**) Shoots in QL medium. Bars = 1 cm.

3.3. Meristem Induction and Multiplication

The meristem induction and multiplication of *Duboisia* are markedly affected by cytokinin type and concentration tested viz., 6-Benzylaminopurine (BA, 1–15 mg L^{-1}), kinetin (1–10 mg L^{-1}), zeatin (1–10 mg L^{-1}), Thidiazuron TDZ (0.05–0.5 mg L^{-1}) and 2-isopentenyladenine 2iP (0.1–1 mg L^{-1}). For *D. myoporoides* and *D. leichhradtii*, half-strength WPM supplemented with the optimum cytokinin treatment BA (1–15 mg L^{-1}) resulted in 100% meristem induction and survival. These meristems cultured on MSK2 medium showed successful multiplication (Figure 4). The multiplication rate (MR) at the end of the fifth subculture (F5) reached 1702 and 901 for the two species (Table 2). In the case of *D. hopwoodii*, all tested cytokinins stimulated viable meristem induction and initial shoot bud development (Supplementary Figures S1–S5). However, the meristem-induced shoot clumps treated with all types of cytokinin except kinetin failed to proliferate and developed necrotic browning symptoms, causing callus induction and abnormal growth, leading to 100% mortality at the end of F1 (Supplementary Figures S1–S5). Repeated experiments confirmed that half-strength WPM supplemented with kinetin (1–10 mg L^{-1}) was the optimum treatment for meristem induction of *D. hopwoodii* (Figure 4c). Further multiplication was achieved with MSK2C medium (Figure 4i), MR at the end of F5 reaching 1075 (Table 2).

Table 2. Multiplication rate of *Duboisia* meristem culture under the optimum cytokinin treatment.

Plant Species	Optimum Cytokinin Treatment	Multiplication Rate from F1 to F5	Total Multiplication Rate
Duboisia myoporoides	BA (1–15 mg L^{-1})	2 × 9.33 × 3.39 × 6.85 × 3.93	1702
Duboisia leichhradtii	BA (1–15 mg L^{-1})	3.6 × 3.4 × 5.1 × 3.44 × 4.2	901
Duboisia hopwoodii	Kinetin (1–10 mg L^{-1})	3.4 × 6.8 × 3.8 × 3.4 × 3.6	1075

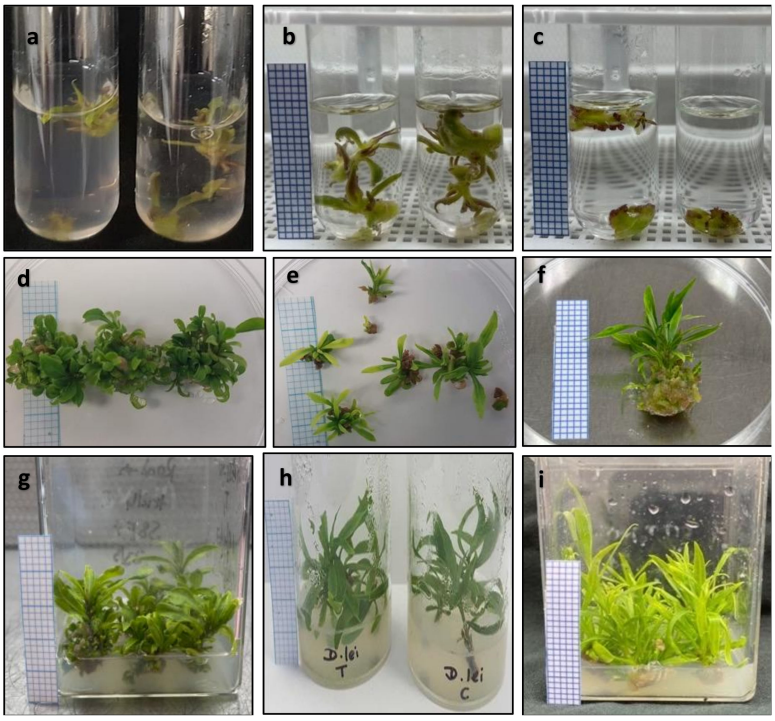

Figure 4. Meristem culture induction of (**a**) *D. myoporoides*; (**b**) *D. leichhradtii*; and (**c**) *D. hopwoodii* after 3 weeks of culture in half-strength liquid WPM; multiplying cultures at second subculture cycle of (**d**) *D. myoporoides* on MSK2 medium; (**e**) *D. leichhradtii* on MSK2 medium; (**f**) *D. hopwoodii* on MSK2C medium; and multiplying cultures at fifth subculture cycle of (**g**) *D. myoporoides* on MSK2 medium; (**h**) *D. leichhradtii* on MSK2 medium; and (**i**) *D. hopwoodii* on MSK2C medium. Graph paper grid size = 2 mm × 2 mm.

3.4. Rooting

3.4.1. Effect of Different Rooting Media on Agar Medium-Based Rooting

For *D. myoporoides*, the rooting medium MSCh along with IBA (1–10 g L^{-1}) is comparatively better than MS medium with the same IBA treatment (Figure 5a), recording a maximum of 50% rooting with good shoot quality (Figure 6c).

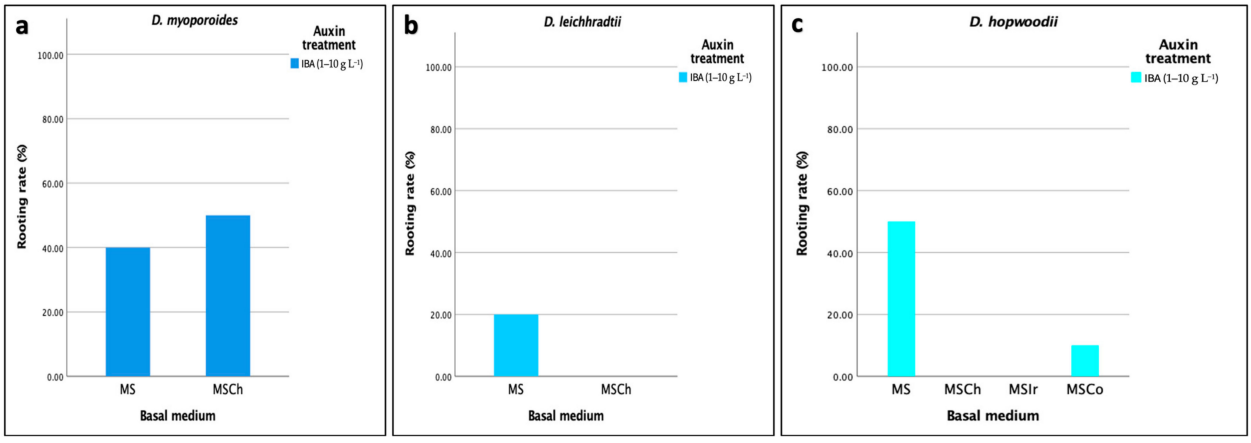

Figure 5. Effects of different rooting media on the rooting rate of three *Duboisia* species under a five-second pulse treatment of IBA (1–10 g L^{-1}): (**a**) *D. myoporoides* with Murashige and Skoog medium (MS) and modified MS medium with activated charcoal (MSCh), (**b**) *D. leichhardti* with MS and MSCh medium and (**c**) *D. hopwoodii* with MS, MSCh, modified MS medium with iron supplements (MSIr) and modified MS medium with coconut water (MSCo) (individual trials conducted separately).

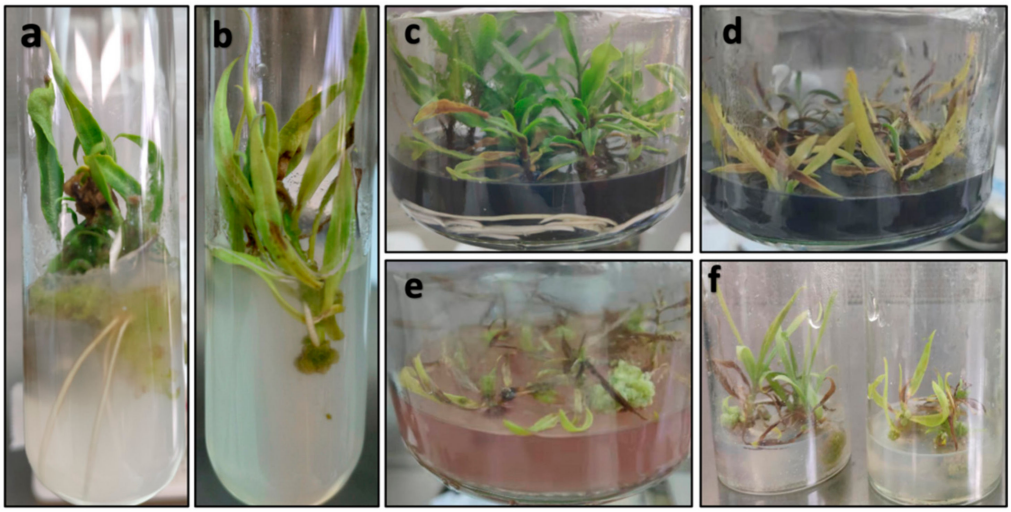

Figure 6. Agar medium-based rooting of *Duboisia* species: (**a**) *D. leichhardti* shoots treated with IBA (1–10 g L^{-1}) in MS medium; (**b**) *D. hopwoodii* shoots treated with IBA (1–10 g L^{-1}) in MS medium; (**c**) *D. myoporoides* shoots treated with IBA (1–10 g L^{-1}) in MSCh medium; (**d**) *D. hopwoodii* treated with IBA (1–10 g L^{-1}) in MSCh medium; (**e**) *D. hopwoodii* shoots treated with IBA (1–10 g L^{-1}) in MSIr medium; and (**f**) *D. hopwoodii* shoots treated with IBA (1–10 g L^{-1}) in MSCo medium (individual trials conducted separately).

A similar trial was conducted with *D. leichhrdtii*, which resulted in only 20% rooting success and also induced large calli (Figure 6a) under the treatment of IBA (1–10 g L^{-1}) when MS medium was used. Other treatments fail to support root induction (Figure 5b).

In the case of *D. hopwoodii*, although a maximum rooting rate of 50% could be obtained in MS medium with IBA (1–10 g L^{-1}) (Figure 5c), the rooted shoots exhibit very poor shoot quality (Figure 6b). *D. hopwoodii* show recalcitrancy to agar medium based rooting, as severe defoliation, heavy callusing and shoot dieback are observed in all tested media (Figure 6b,d–f). In the MSCo medium (modified MS medium with coconut water), the highest rooting rate obtained is 10%, whereas both MSIr (modified MS medium with iron supplements) and MSCh media fail to induce any roots (Figure 5c).

3.4.2. Effects of Different Rooting Substrates and IBA Concentrations on Substrate-Based Rooting

The rooting outcome was found to be species-specific for the three *Duboisia* species, and the rooting percentage was significantly affected by the IBA concentration and rooting substrate. For *D. myoporoides*, the optimum treatment IBA (1–10 g L^{-1}) along with Jiffy cubes resulted in the best rooting rate of 100% (Figure 7a), comparatively better than foam cubes, which achieved 97.33% rooting (Table 3).

Table 3. Effect of tested IBA treatments on *Duboisia* in different rooting substrates.

Plant Species	IBA Treatments	Rooting (%)	
		Jiffy Cube Experiments	Foam Cube Experiments
Duboisia myoporoides	IBA (1–10 g L^{-1})	100 ^{a}	97.33 ^{a}
Duboisia leichhradtii	IBA (1–10 g L^{-1})	100 ^{b}	0 ^{a}
Duboisia hopwoodii	IBA (1–10 g L^{-1})	11.67 ^{b}	6 ^{a}
	IBA (0.1–5 g L^{-1})	44.33 ^{c}	-

Mean values within each species for individual substrates followed by the same letter are not significantly different.

Similarly, IBA (1–10 g L^{-1}) combined with Jiffy cubes was identified as the optimum rooting treatment for *D. leichhradtii*, which resulted in a 100% rooting rate (Figure 7b). None of the examined shoots exhibited root formation in the foam rooting cubes (Table 3).

In the case of *D. hopwoodii*, IBA (1–10 g L^{-1}) resulted in a comparatively low percentage of rooting regardless of the substrate used (Jiffy cubes or foam cubes) (Table 3). The maximum mean rooting rate achieved was 44.33% under the treatment of IBA (0.1–5 g L^{-1}) in Jiffy cube (Figure 7c).

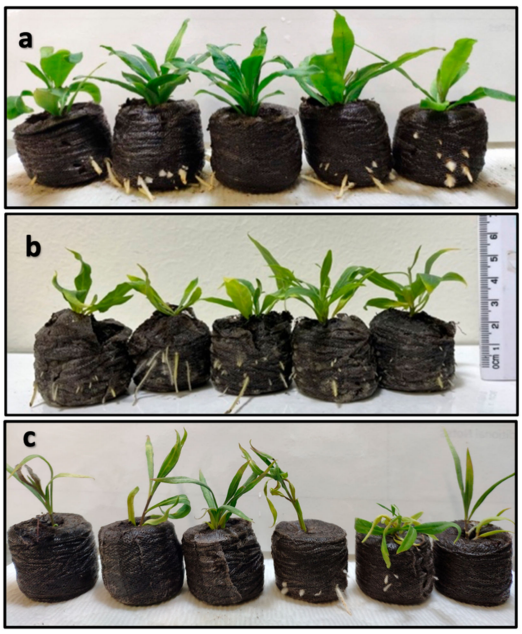

Figure 7. Substrate-based rooting of *Duboisia* species in Jiffy cube: (**a**) *D. myoporoides* shoots treated with IBA (1–10 g L^{-1}); (**b**) *D. leichhradtii* shoots treated with IBA (1–10 g L^{-1}); and (**c**) *D. hopwoodii* shoots treated with IBA (0.1–5 g L^{-1}).

3.4.3. Rockwool as a Rooting Substrate for *D. hopwoodii* Rooting

A different substrate, rockwool, was evaluated along with the best IBA concentration IBA (0.1–5 g L^{-1}) identified in Section 3.4.2 to optimise the substrate-based rooting protocol for *D. hopwoodii*. Inconsistent rooting was observed with *D. hopwoodii*, as the Jiffy cube (control) supported only 10% rooting (Figure 8a), contrasting with the identical treatment in Section 3.4.2. Rockwool as a rooting substrate significantly increased the rooting rate of *D. hopwoodii* to 70%. Compared with the shoots inoculated into Jiffy cubes (10% rooting), rockwool clearly supported better shoot quality and rooting performance (Figure 8b).

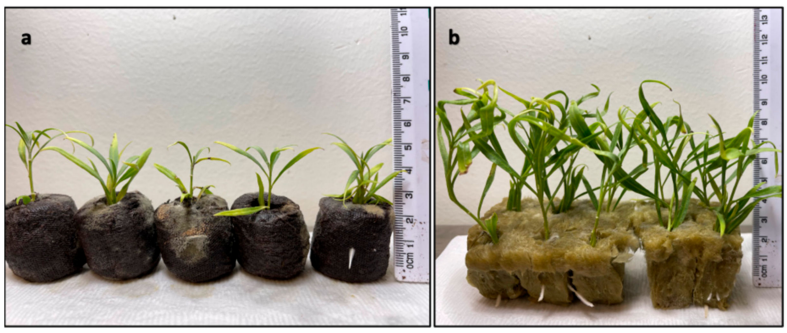

Figure 8. Rooting substrate optimisation for *D. hopwoodii* rooting: (**a**) shoots treated with IBA (0.1–5 g L^{-1}) in Jiffy cube; and (**b**) Shoots treated with IBA (0.1–5 g L^{-1}) in rockwool.

3.4.4. Comparison of Agar Medium-Based Rooting and Substrate-Based Rooting

The substrate-based rooting proved more effective than agar-based rooting across all three *Duboisia* species. The *Duboisia* plants rooted on substrate exhibited a higher rooting rate (Table 4) and overall shoot quality (Figure 7) compared with those rooted on agar medium (Figure 6).

Table 4. The comparison of rooting rates between agar medium-based rooting and substrate-based rooting for three *Duboisia* species.

Plant Species	The Highest Rooting Rate Achieved (%)	
	Agar Medium-Based Rooting	Substrate-Based Rooting
Duboisia myoporoides	50	100
Duboisia leichhradtii	20	100
Duboisia hopwoodii	50	70

The recorded rooting results were obtained from independent experiments.

3.5. Acclimatisation

The rooted *D. myoporoides* plantlets were evaluated with two acclimatisation methods. Plants acclimatised in seed tray have a significantly higher survival rate of 100% (Figure 9a) than those acclimatised in Ziploc bag, which is only 60% acclimatisation success (Table 5). Therefore, this acclimatisation method was trialed for the other two species.

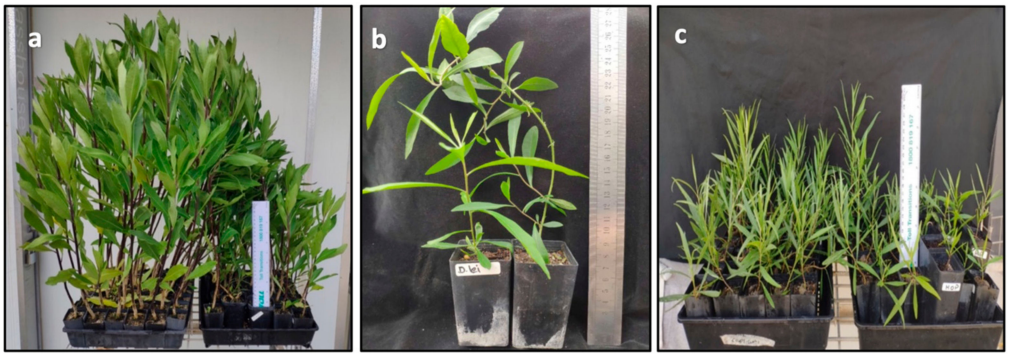

Figure 9. Acclimatised *Duboisia* plants: (**a**) *D. myoporoides*, (**b**) *D. leichhradtii* and (**c**) *D. hopwoodii*.

Table 5. Acclimatisation survival rate of *Duboisia* species.

Plant Species	Acclimatisation Survival Rate (%)	
	Acclimatisation in Seed Tray	Acclimatisation in Ziploc Bag
Duboisia myoporoides	100 [b]	60 [a]
Duboisia leichhradtii	100	-
Duboisia hopwoodii	80	-

Mean values followed by the different alphabet were significantly different. Species were subjected to treatments in individual experiments.

Adaptation of this acclimatisation protocol to *D. leichhradtii* and *D. hopwoodii* was successful (Figure 9b,c), recording a 100% and 80% survival rate (Table 5), respectively.

4. Discussion

Woody species are generally recalcitrant to tissue culture conditions. In the current investigation, it was evident that *Duboisia* is recalcitrant to tissue culture conditions, and careful optimisation of culture media components is critical for success. The majority of research in the field of *Duboisia* tissue culture is confined to plant regeneration through callus [15,34,35]; therefore, it is less preferred for commercial production due to the high risk of variation induced during the regeneration process (somaclonal variation) [36]. On the other hand, it was evident that direct scopolamine production via *Duboisia* callus

yielded no detectable scopolamine [37]. This finding aligns with the study by Hashimoto et al. [38], which revealed that *Duboisia* roots are the main site for scopolamine biosynthesis. Therefore, the callus-based tissue culture system remains restricted in its commercial application. The only nodal culture-based micropropagation protocol developed by Kukreja and Mathur [30] was not conducive to commercial mass propagation due to its ineffective root induction, with a maximum rooting rate of 80%. Prior to the current study, there is no tissue culture protocol for any *Duboisia* species or hybrids that demonstrated potential with commercial viability.

The choice of basal medium can significantly impact the success of a tissue culture protocol. Murashige and Skoog medium, WPM, and QL are widely used for woody plant tissue culture, offering balanced nutrition in different forms. Murashige and Skoog medium has higher macro/micronutrient concentrations compared with WPM and QL, whereas WPM has lower macronutrient content, while QL contains lower macro/micronutrient concentrations compared with both MS and WPM [18]. In the existing literature on *Duboisia* tissue culture, a significant proportion of prior investigations have relied upon the sole use of MS medium for shoot induction and regeneration [29]. However, direct comparisons between MS medium and other basal media are still lacking for *Duboisia* [29]. In this study, MS, WPM and QL were assessed for *Duboisia* shoot culture. Results showed that MS medium outperformed QL and WPM media in promoting shoot growth and quality in *Duboisia* species and hybrid (Figures 2 and 3). As there are few differences among the basal media used, it is quite difficult to identify the exact nutrient that promotes the growth in MS, but this could be the high level of NH_4NO_3 present in MS, which is four times higher than that in QL and WPM. In general, MS is the most widely used medium to support tissue culture across a broad range of plant species [39–42].

Meristem culture is a better platform for higher multiplication of plantlets with less risk of somaclonal variation [20]. A meristem-based tissue culture technique for elite *Duboisia* clonal propagation will allow rapid multiplication of limited explant material. This will dramatically increase the propagation efficiency as an industrially viable tissue culture system can efficiently and effectively increase the volume of plant propagules, which has the potential to improve the speed and throughput of commercial *Duboisia* propagation. Such technology will be a valuable platform for the fast and efficient production of uniform and high-quality plant material, which is critical for the pharmaceutical industry that relies on consistent active ingredient levels.

In the present study, a meristem culture-based high throughput propagation has been explored for *Duboisia* species. The cytokinin type and concentration were found to be specific for *Duboisia* meristem induction and multiplication (Table 2). For both *D. myoporoides* and *D. leichhradtii*, the best rate of meristem induction and multiplication was supported by the treatment of BA as the cytokinin. This result highlighted the superior effect of BA for meristem induction and multiplication for Solanaceae species, which was also reported by Sharmin et al. [43] that BA outperformed kinetin and Gibberellic Acid (GA3) in meristem induction and multiplication of eggplant. BA has also been found to be best for the meristem culture establishment of other woody species, such as apple [28], mulberry [44] and black wattle [45]. For *D. hopwoodii*, out of the tested cytokinins, only kinetin could support viable multiplication of meristem-derived shoots. This result is in agreement with those obtained by Alam et al. [46], which showed kinetin is the optimum cytokinin for sweet potato meristem culture establishment and multiplication, whereas the use of TDZ and BA induced callus. The difference in the hormone requirements for meristem induction and multiplication for three *Duboisia* species could be attributed to genotypic specificity for the type of cytokinins at the shoot proliferation stage [47–49]. The result of the current study is on par, as two *Duboisia* species had a preference for the cytokinin BA while it caused mortality in another.

Appropriate incorporation of auxin and rooting substrate contributes to successful root formation. Application of exogenous auxin in solid agar media for continuous incubation is the most common method for in vitro root induction [50]. In the literature, rooting attempts

of *Duboisia* species in agar-based media, along with constant auxin exposure, have not been successful in supporting effective rooting [29]. This could be due to the suboptimum auxin type, concentration, duration of auxin exposure, and media composition [50]. Pulse treatment using various exposure times with a range of auxin solutions is proven to be effective in agar medium-based root induction of numerous woody species such as almond [51], white pine [52] and cherry [53]. However, in the present study, rooting attempts of *Duboisia* in a solid agar medium with a 5-s IBA pulse treatment did not improve the rooting percentage (Figure 5). Research has demonstrated that the application of activated charcoal into the solid agar medium can effectively enhance root formation and overall shoot quality [54]; however, the extent of this effect may vary depending on the plant species being cultured [55]. In the present study, the rooting results are on par, as the effect of activated charcoal was found to be species-specific (Figure 6c,d). With activated charcoal, the rooting percentage of *D. myoporoides* increased, whereas the opposite trend was observed for *D. leichhrdtii* and *D. hopwoodii*. Supplementing with other additives, such as iron and coconut water, adversely affected the root formation of *D. hopwoodii* (Figure 6e,f), suggesting a possible disruption in the endogenous hormone balance of the microcuttings [56,57]. Further investigation is needed to fully elucidate the mechanisms underlying these observations. The best rooting results reported in the literature previously for *Duboisia* is by Kukreja and Mathur [30], which resulted in 80% rooting for *D. myoporoides* in static liquid MS medium containing 0.5 mg L^{-1} 1-naphthaleneacetic acid (NAA). The higher rooting percentage obtained could be due to the better nutrients/hormones provided by the liquid medium and the specific auxin NAA that was used. Therefore, further optimisation using a liquid, semi-liquid medium or medium with other gelling agents, adopting other auxins could be explored to enhance the rooting outcomes for agar medium-based rooting of *Duboisia*.

Rooting in vitro regenerated shoots in substrates is a common practice for many plant species, including some woody plants such as cherry [58], green ash [59], chestnut [60] and wolfberry [61]. In addition, substrate-based rooting reportedly accelerates the propagation process [62], which is critical for commercial production. In this study, pulse treatment of IBA proved to be highly effective for substrate-based rooting, which resulted in 100% rooting for *D. myoporoides* and *D. leichhradtii* in Jiffy cubes (Table 3). Jiffy cube is a coir product that contains a balanced level of mineral nutrients, which can retain a high level of water and ensure aeration [63]. Similar improvement in rooting success was observed by Newell et al. [64] for several Australian woody species *Grevillea thelemanniana*, *Pimelea physodes* and *Conospermum eatoniae*., when coir-based rooting substrate was used. However, in the case of *D. hopwoodii*, the Jiffy cubes could only support a maximum of 44.33% rooting (Table 3). It was possible to improve the rooting rate to 70% when rockwool was used. In accordance with previous studies such as sweetgum [65] and sweet cherry [66], this study has demonstrated a strong effect of rockwool on rooting recalcitrant woody plants. Rockwool retains a higher level of water and has better oxygen diffusion than Jiffy cube [67], suggesting *D. hopwoodii* requires a higher level of water and oxygen than other *Duboisia* species for root formation.

The success of micropropagation is heavily dependent on the process of acclimatisation. Only plantlets that have survived the acclimatisation and hardening phase from tissue culture may continue to develop under ex vitro environments [68]. Research on acclimatisation for *Duboisia* species has been very restricted, with only investigations carried out by Lin [35] and Luanratana [15], which is presumably due to the lack of in vitro-rooted plantlets. This study reports an optimised protocol for *Duboisia* species that supported 100% acclimatisation survival for *D. myoporoides* and *D. leichhradtii*, and 80% for *D. hopwoodii* (Table 5). Acclimatisation success of tissue culture plants is determined by root quantity and quality. Well-developed roots enable efficient water transport, while an adequate number of roots and abundant root hair provide a larger surface area for nutrient and water uptake [69,70]. In the present study, the success in acclimatisation of *D. myoporoides* and *D. leichhradtii* was attributed to the vigorous root development and ample root hair of the

meristem culture-derived plantlets (Figure 7). This result is in line with the finding obtained by Addae-Frimpomaah et al. [71], which states that improved root quality and root hair abundance better supported the acclimatisation success of meristem culture-derived sweet potato plantlets. Furthermore, the difference in the acclimatisation survival rate among the meristem culture of three *Duboisia* species in this study could be ascribed to the variance in their root quantity and root hairs, suggesting further optimisation in the rooting protocol of *D. hopwoodii* could further improve its acclimatisation success.

5. Conclusions

The three *Duboisia* species investigated in this study showed genotypic specificity for growth hormones/concentration for meristem induction, multiplication and root induction. BA elicited the best response with *D. myoporoides* and *D. leichhradtii* for meristem induction and multiplication, whereas kinetin was best for *D. hopwoodii*. A five-second pulse treatment of IBA is highly effective for *Duboisia* root induction, recording 100% rooting for *D. myoporoides* and *D. leichhradtii* in Jiffy cube and 70% rooting for *D. hopwoodii* in rockwool. The optimised acclimatisation protocol supported a 100% survival rate for *D. myoporoides* and *D. leichhradtii*, and 80% for *D. hopwoodii*. The current study, for the first time, established an effective and efficient meristem culture protocol for three *Duboisia* species, *D. myoporoides*, *D. leichhradtii* and *D. hopwoodii*, providing a feasible platform to commercially adopt tissue culture for clonal propagation of *Duboisia*. Moreover, this development will be critical to facilitate the fast adoption of new breeding lines for commercial plantations, as well as further research in areas such as genetic modification and in vitro breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9121313/s1, Figure S1: Meristem culture induction and multiplication of *D. hopwoodii* under different BA (1–15 mg L^{-1}) concentrations; Figure S2: Meristem culture induction and multiplication of *D. hopwoodii* under different kinetin (1–10 mg L^{-1}) concentrations; Figure S3: Meristem culture induction and multiplication of *D. hopwoodii* under different zeatin (1–10 mg L^{-1}) concentrations; Figure S4: Meristem culture induction and multiplication of *D. hopwoodii* under different TDZ (0.05–0.5 mg L^{-1}) concentrations; Figure S5: Meristem culture induction and multiplication of *D. hopwoodii* under different 2iP (0.1–1 mg L^{-1}) concentrations.

Author Contributions: J.C.A.H.-B.: conceptualization, methodology, writing—review and editing project administration, funding acquisition; Y.X.: investigation, validation, formal analysis, data acquisition, writing—original draft preparation, visualization; D.T.J.: investigation; Z.Z.: investigation and data acquisition; N.M.: resources, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by India Glycol Limited through a collaborative research agreement with The University of Queensland. Y.X. is supported through the Research Training Program scholarship (Ph.D.) by the University of Queensland.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Acknowledgments: We thank Robert Henry for providing access to all the *Duboisia* mother plants and Patrick Mason for maintaining mother plants and acclimatized tissue culture plants.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analysis or interpretation of the data, in the writing of the manuscript or in the decision to publish the results.

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