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Review

Micropropagation of *Duboisia* Species: A Review on Current Status

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Abstract: *Duboisia* is an Australian native woody species of the Solanaceae family, a crucial source of alkaloids, and is naturally extracted for pharmaceuticals. The alkaloid content of the four naturally occurring species of *Duboisia*, i.e., *Duboisia myoporoides* R. Br., *Duboisia leichhardtii* F. Muell., *Duboisia hopwoodii* F. Muell. and *Duboisia arenitensis*, is not conducive for large-scale commercial extraction. High-value hybrids between *D. myoporoides* R. Br. *and D. leichhardtii* F. Muell. have become the commercial crop for the industry. Propagation of these hybrids is key for progression of this industry, especially for the establishment and expansion of plantations and to replenish old plantations. Commercial propagation of *Duboisia* completely depends on cutting propagation to ensure true-to-type propagules. Cutting propagation of this species is associated with several challenges and has been a hurdle for industry expansion for many years. Micropropagation can be an efficient and sustainable alternative for *Duboisia* clonal propagation and is a faster and cleaner propagation avenue for elite propagules. This review compiles the research attempts made in the space of *Duboisia* micropropagation and provides an update on recent advancements to understand the technical capacity, progress and challenges towards a commercial micropropagation platform.

Keywords: *Duboisia*; corkwood; medicinal plant; in vitro culture; biotechnology; scopolamine; secondary metabolite; tropane alkaloid

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1. Introduction

Duboisia is an Australian native, perennial shrub, alternatively called 'corkwood', and which belongs to the family Solanaceae [1]. There are four main species: *D. myoporoides* R. Br., *D. leichhardtii* F. Muell., *D. hopwoodii* F. Muell. and the recently discovered species *D. arenitensis* [2]. The Australian first nations (Aboriginals) have long utilised this plant to aid hunting (sedation) and as a medicinal plant (painkiller). The earliest record of Australians using this species dates back to the 1770s [3,4]. *Duboisia* is now an economically important plant genus commercially grown for extraction of two powerful anticholinergic (inhibiting the action of acetylcholine) and antispasmodic (relieving spasms or convulsions) alkaloids, 'scopolamine' and 'hyoscyamine' [5]. These alkaloids possess mydriasis, analgesic and sedation properties in humans [6]; scopolamine has fewer side effects and a higher physiological activity compared to hyoscyamine [7]. Scopolamine-derived products include Scopoderm®, Isopto® Hyoscine, Spiriva®, Tiova®, Pamine® and Buscopan®, which are available on the market for diverse clinical treatments. Furthermore, there is no substitute for scopolamine with similar effectiveness [8]. As a result, global demand for scopolamine is at an incline.

Scopolamine is naturally produced in several members of the Solanaceae family, such as *Duboisia*, *Hyoacyamus*, *Atropa*, *Datura* and *Scopolia*; however, *Duboisia* is still the sole source of this alkaloid for commercial extraction [9]. Extraction of scopolamine from air-dried *Duboisia* leaves dates back to 1944 [3]. *Duboisia* is among the highest natural producers of scopolamine (2–4% by dry weight of total alkaloids) compared to *Atropa*

spp. or *Datura* spp. (0.2–0.8% by dry weight) [10]. It also has a short cultivation period of 10 months from planting to harvest [11] and is relatively less affected by insect pests [12,13], making this species a perfect candidate for intensive commercial cultivation. With the increasing demand for scopolamine, in the 1950s, large-scale cultivation of *Duboisia* species for leaf production was initiated in Queensland, Australia [3]. Today, Australia is the world leader in scopolamine production, owning 70% of the world supply [3,14]. Since 1992, Australia has produced 994,678 kg of *Duboisia* leaf decennially, with a gross value of AUD 6,604,439, which is exported internationally for alkaloid extraction [3]. Due to the economic value of this species, other regions such as India [15], South America [15], Brazil, and Ecuador [16,17] have also initiated its cultivation.

The industry expansion with the increased demand for scopolamine has directed research scope to increase scopolamine content in this species thorough conventional breeding and modern biotechnology tools. The four *Duboisia* species are not highly rated for their alkaloid contents; therefore, crosses between species to amalgamate beneficial traits for higher biomass, increased total alkaloids, higher scopolamine content, increased rooting ability of cuttings, and enhanced resistance to environmental conditions and pest invasion have been considered [3]. According to Ullrich et al. [14], the broader leaf shape characteristic of *D. myoporoides* is favorable for breeding to provide a larger biomass. In addition, Luanratana [18] reported that the hybrid between *D. myoporoides* R. Br., and *D. leichhardtii* F. Muell.. has higher growth vigour and increased alkaloids content compared to parent species. Most importantly, the alkaloid nicotine is not produced in this hybrid, which in turn increases the biosynthesis and accumulation of scopolamine while reducing the purification cost during extraction [19]. In Australia, a hybrid was selected in the year 2000, derived from over 12,000 crosses between promising *Duboisia* parent species, and was adopted as a commercial hybrid for large-scale cultivations [3]. The current commercial hybrid produced through conventional crossing of *D. myoporoides* and *D. leichhardtii* records a scopolamine content of 6% by dry weight [16], though seasonal variation has been noted.

Characterisation of the site for scopolamine biosynthesis has broadened the horizons of research to improve alkaloid content in *Duboisia*. The biosynthesis of scopolamine is generally believed to occur in the roots, and the synthesised scopolamine is later translocated to the apical parts of the plant [20]. According to Luanratana [21], the accumulation of tropane alkaloids in *Duboisia* leaves is related to the transpiration rate. Increased transpiration during warmer climates leads to higher alkaloid accumulation in leaves compared to cold weather conditions. Thus, seasonal variation in the leaf alkaloid content is evident. This suggests that a controlled environment could be a strategy to achieve year-round, consistent levels of scopolamine in *Duboisia*. Applying conventional biotechnology, scientists have attempted in vitro culture of *Duboisia* and have successfully detected tropane alkaloid in callus cultures in vitro [22] and in shoots [23] and roots [24,25], suggesting another alternative for alkaloid production. However, some literature suggests that *Duboisia* calli are unable to produce both scopolamine and hyoscyamine [26] or no alkaloids are produced in the absence of root [27]. There are reports stating that both callus culture and regenerated shoots are able to produce limited amount of alkaloids [3], but the levels produced are well below the extractable levels for commercial-scale production.

Application of molecular biology has been in the forefront of aiming to increase alkaloid content through genetic transformation. Hairy root cultures produced by genetic transformation of *Duboisia* cells via *Agrobacterium rhizogenes* is one of the strategies found in literature [28]. *A. rhizogenes* causes hairy root disease, which generates extensive proliferation of roots when infected [29]. Since roots are the main site of tropane alkaloid biosynthesis in *Duboisia* species [24], hairy root induction has been intensively studied [30–34]. Nevertheless, the establishment and optimisation of productive root lines are difficult and costly when using this approach as an industrial application [35], which limits this approach from being translated to a commercially viable technology. Moreover, the performance of plants transformed with hairy root genes that have been trialled in field

conditions in comparison to conventional plants has not delivered promising results with regards to growth potential and productivity [36].

Scopolamine production in bioreactors for faster and scalable production has also been a focus. Culturing plant cells, root cultures or engineered microorganisms in controlled bioreactors can eliminate the issues associated with traditional cultivation of pharmaceutical plants, such as external environment stress, pest and disease problems, and non-uniform crop quality [37]. Moreover, scopolamine production in bioreactors reduces the inputs of land and management resources as well as the downstream cost of extraction and purification [38]. Numerous studies have been carried out on scopolamine production via root cultures of Solanaceae species in bioreactors, including *Duboisia* [32], *Atropa* [39], *Brugmansia candida* [9], *Scopolia parviflora* [40], *Hyoscyamus niger* [41] and *Datura innoxia* [42]. More recently, production of scopolamine in yeast has been successful [37]. However, for large-scale production, the bioreactor systems require complex optimisation of the bioreactor operation parameters based on the interactions among cell biology/microbiology, kinetics, thermodynamics and transport phenomena [38]. Thus, the cost, time and efficiency factors involved in these bioreactor systems are unfavourable for a commercially feasible product [5].

The best commercial variety used by the industry is a *Duboisia* hybrid, *D. myoporoides* × *D. leichhardtii*. Propagation of such elite selections while preserving the genetic stability is one of the important aspects for the *Duboisia* industry, as well as a challenge. *Duboisia* seeds are not a preferred material for propagation of elite selections as the seeds are genetically different to parents due to the outcrossing nature of this genus [43]. Since the value of *Duboisia* is associated with its high tropane alkaloid content, genetic diversity is disadvantageous in the context of propagation. However, even if we do so, seed propagation itself has several challenges associated with it, such as poor germination rates (about 8%) and slow growth of seedlings [18]. Together with the transient seed viability, the production of *Duboisia* seedlings is grievously limited [44]. As an alternative, the conventional propagation of *Duboisia* hybrids relies on vegetative propagation methods, mainly through stem cuttings [18]. This ensures the genetic uniformity of the propagules, but limitations still exist, such as low rooting rate, inconsistent rooting and long time for root initiations [18,44]. In addition to the problems with rooting efficiency, cutting propagation requires a steady supply and high volume of cuttings in a rhizogenic state, which can constrain production volumes and requires extra plantation maintenance to collect suitable cuttings.

Propagation of high volume of genetically uniform plants through micropropagation for conventional cultivation seems to be the most viable and economically feasible alternative [45]. Micropropagation is a tissue culture tool based on the phenomenon called 'totipotency' or 'total potential' of plant cells, which is described as the ability of individual cells to grow into complete plants [46]. Through this process, a large number of individual plants are generated starting from somatic cells, plant tissues or organs (leaves, buds, shoot tips and nodal segments) utilising a species/cultivar specific nutritive medium under sterile in vitro conditions maintained in controlled environments [47]. This method allows for rapid and high-volume production of clones that are genetically identical to the elite mother plant, provided that the tissue culture media and growth conditions are favourable and carefully optimised.

A robust micropropagation protocol has a capacity for rapid multiplication with limited explant sources in a sterile in vitro environment independent from external dynamic natural pressure [45]. It is an effective and resource-efficient method of true-to-type propagation of plants, that can efficiently, effectively, and more sustainably increase the volume of plant propagules to ensure a smooth supply to boost horticultural and agricultural production. Nowadays, tissue culture has evolved to a well-established propagation technique for mass propagation of many commercially important species, such as strawberry [48], raspberry [49], cymbidium [50], sugarcane [51], banana [52], apple [53], pear [54] and avocado [55,56]. Apart from propagation, tissue culture is a prerequisite for genetic modifi-

cation of plants, germplasm preservation, plant breeding and the production of secondary metabolites [57–59].

To date there are no reports on commercially viable tissue-culture-based mass propagation systems for *Duboisia*. This review intends to capture and summarise the current knowledge on *Duboisia* relating to tissue culture research with greater emphasis on the micropropagation aspect. All the available literature on *Duboisia* micropropagation to date was reviewed to capture as many details as possible. The studies were categorised under different micropropagation techniques with the intention of presenting a concise and informative summary. Unpublished advancements relating to *Duboisia* micropropagation, specifically on new technological advancements and challenges, has also been included to make this review informative not only for researchers but also for broader industry stakeholders to provide directions for future advancement in the tissue culture research space for this species.

2. In Vitro Culture Techniques for *Duboisia* Micropropagation

There are three basic techniques available for propagation through tissue culture: meristem culture, nodal culture and regeneration through callus [19,60]. Both meristem and nodal culture are direct organogenesis methods where the alteration of parental genotypes is at a very low risk during the process [61,62]. Callus culture is an indirect organogenesis method, which requires a callogenesis stage before regeneration. It is a two-step process where differentiated cells are first induced to produce undifferentiated cell masses (de-differentiate), then induced to form shoots leading to acquire complete plants (re-differentiate) [63,64]. This method involves rapid cell division, thus has a high risk of mutation induced through the process. Over the past three decades, all three techniques have been attempted for micropropagation of *Duboisia* species with limited success.

2.1. Meristem Culture

The cultivation of an extreme meristematic dome (<0.1 mm) of either shoot or root tips is referred as meristem culture [65]. However, the size of the explant that responds to meristem culture largely depends on the plant species. Large explant sizes used with more leaf primordia are termed 'shoot tip culture' [55,66]. This is an essential technique coupled with cryo/thermo therapy for virus elimination of plant species to produce disease-free clonal plants [67]. In addition, plants regenerated through meristem culture reportedly have the highest multiplication rate, a key consideration for commercial micropropagation [68]. Shoot tip culture has been previously attempted by Lin [19] using hybrid *D. myoporoides* × *D. leichhardtii* (*M* × *L*). The shoot tips (2–4 mm) which were cultured in MS medium supplemented with 5 mgL^{-1} BA did not survive, due to browning of shoot tips leading to complete mortality of explants. Despite the advantages of meristem culture, this provides evidence that meristem or shoot tip culture is the most difficult technique [69], especially in the context of woody plants of which the recalcitrancy is very high [70,71]; it thus poses a huge challenge [72]. In recent years, this technique has been widely used for several woody crops such as apple [53], peach [73], pear [54] and avocado [55]. With respect to *Duboisia*, to date, application of meristem culture has not been reported.

2.2. Nodal Culture

Nodal culture is a method where nodal sections are cultured to activate the axillary bud growth, followed by shoot elongation to produce more nodal sections to achieve multiple nodal sections through several cycles of subcultures [74]. Most woody plants are amenable to nodal culture conditions [72,75]. For *Duboisia* species, the only reported study on nodal culture was by Kukreja and Mathur [44], who managed to regenerate *D. myoporoides* R. Br. by directly inoculating nodal segments into Murashige and SkooG (MS) medium [76] supplemented with 3 mgL^{-1} 6-benzyleaminopurine (BA) and 1 mgL^{-1} indole-3-acetic acid (IAA). Within 6 weeks, an impressive level of multiplication was achieved by producing 50 shoots per initiated nodal explant.

2.3. Direct Organogenesis from Somatic Cells

Some species show capacity in direct organogenesis from somatic tissues. Plants from the Solanaceae family are a good example of that, such as potato [77], eggplant [78] and tobacco [79]. Direct organogenesis of *Duboisia* has been attempted by Lin [19] using several explants of hybrid *D. myoporoides* × *D. leichhardtii* (*M* × *L*). According to this study, adventitious shoots as explant for shoot formation and multiplication were not successful, even though shoot buds were formed within 6 weeks of inoculation to MS medium supplemented with 5 mgL^{-1} BA. However, successful shoot induction could be achieved within 4 weeks by culturing shoots and leaf segments (2–4 mm) in the same medium. Subculture of these shoots in MS medium supplemented with 2 mgL^{-1} kinetin and 0.1 mgL^{-1} 1-naphthalene acetic acid (NAA) resulted in 15 times multiplication per explant inoculated. Luanratana [18] also achieved successful results in direct organogenesis of *D. myoporoides* leaves on revised-tobacco medium supplemented with 4 mgL^{-1} NAA and 4 mgL^{-1} BA.

2.4. Indirect Organogenesis through Callus

In callus culture, callogenesis is firstly achieved by application of different plant growth regulators (PGR) to various explants such as stem, leaf, flower and cotyledon [80]. Calli are undifferentiated cells, which can be then induced to regenerate shoots/plants, or for the production of secondary metabolites through suspension culture [19,81]. Callus culture relies on indirect organogenesis, imposing a high risk of induced variation due to high level of cell division [82], and thus is less preferred for clonal propagation. The majority of the research in *Duboisia* callus culture has been focused on in vitro production of tropane alkaloids [23,83–89]. Therefore, the developed tissue culture protocols were not aimed at intact plant regeneration, and, even if regenerated, the frequency was low. In the reported literature, callogenesis has been the common practice for *Duboisia* micropropagation by applying PGR combination of auxin and cytokinin. Luanratana [18] successfully induced calli from *D. myoporoides* by inoculating either the leaf or stem into revised-tobacco medium supplemented with 1 mgL^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mgL^{-1} IAA, and 0.06 mgL^{-1} BA. For hybrid *M* × *L*, Luanratana [18] obtained 90% callus induction frequency by application of 1 mgL^{-1} NAA and 2 mgL^{-1} BAP to leaf explants cultured on MS medium, while Lin [19] achieved 95% callus induction frequency by inoculating 2 mm seedling segments onto MS medium supplement with 10 mgL^{-1} NAA and 0.2 mgL^{-1} BA. After callus formation, vigorous callus lines were selected and subcultured in differentiation medium for shoot induction and multiplication. Luanratana [18] employed revised-tobacco medium supplemented with 4 mgL^{-1} NAA and 4 mgL^{-1} BA for *D. myoporoides* and MS media containing 0.5 mgL^{-1} NAA and 1 mgL^{-1} BA for *M* × *L* hybrid, to successfully induce shoots for both species. Lin [19] subcultured calli of *M* × *L* hybrid on MS medium supplemented with 5 mgL^{-1} BA and 2.5 mgL^{-1} 6-(γ,γ-dimethylallylamino) Purine (2-iP), to obtain 70% shoot induction.

3. Optimisation of Micropropagation Process for *Duboisia* Species

The process of micropropagation generally comprises four stages irrespective of the technique used: (1) establishment of aseptic culture to remove microorganisms, (2) shoot induction and multiplication to obtain larger number of shoots, (3) root induction to obtain a complete rooted plantlet and (4) acclimatisation of rooted plants to train them to withstand the harsh outside environment [19,55,69]. Intact plant regeneration of *Duboisia* was first achieved in 1980 [90] and there have been several advances in micropropagation over the last four decades.

3.1. Sterilisation of Explants and Aseptic Culture Establishment

The first step in any micropropagation protocol is to treat the explant material using various chemical treatments and a series of washing steps to remove any microbial contamination to establish an aseptic culture. In addition to the chemicals, the type of

explant, material collection season/time and health of the mother plant are important factors to consider for successful clean culture establishment [91]. Generally, the mother plants are maintained in containment under a glasshouse or controlled condition with strict pest disease management strategies in place, such as application of antibiotics and fungicides [62]. In establishing clean cultures of *Duboisia*, different sterilisation protocols have been reported previously (Table 1). Comparing the contamination level of different *Duboisia* explant sources, Lin [19] showed that shoot tips are comparatively difficult to disinfect compared to young leaves, seeds or seedlings. They recorded 100%, 97%, 52% and 31% clean culture establishment with seedlings, seeds, young leaves and shoot tips, respectively. In the same study, explants collected during the later spring season were found to be more difficult to sterilise (clean culture 26%) compared to winter material (clean culture 40.7%). This was attributed to the microorganism suppression effect due to the low temperature during winter periods compared to warmer and more humid summer months.

Table 1. Summary of sterilisation protocols for *Duboisia* culture initiation.

Sterilisation Protocol	Explant Type	Clean Culture Initiated (%)	Reference
Treat with 20% (*v*/*v*) Milton antibacterial solution (1% chlorine as sodium hypochlorite and 16.5% sodium chloride) for 20 min and rinse 3–4 times with sterile water.	Shoot tip, leaf, seed and seedling	Shoot tip—31% Leaf—52% Seed—97% Seedling—100%	[19]
Wash the petiolar base with distilled water containing Tween 20, surface sterilised with 0.1% $HgCl_2$ for 1 min, and rinse several times with double-distilled water.	Nodal segments	Data not available	[44]
Treat with 20% (*v*/*v*) Milton antibacterial solution for 20 min and rinse 3–4 times with sterile water.	Shoot tip	Data not available	[84]
Treat with 70% *v/v* ethanol for 30 s followed by 10 min treatment of 1% hypochlorite solution and wash 3 times with sterile water.	Leaf	Data not available	[85]
Wash under running water for 1 h, treat with 20% (*v*/*v*) Milton antibacterial solution for 20 min and rinse 3–4 times with sterile water.	Shoot tip and seed	Data not available	[89]
Treat with 2% sodium hypochlorite solution for 15 min and rinse 3 times with sterile water.	Nodal segments	Data not available	[90]
Dip the leaf explants into 75% ethanol for 10 s, rinse with sterilised water, surface sterilise in 2% sodium hypochlorite containing Tween 20 (1 drop per 40 mL) for 10 min and then wash 3 times with sterilised water.	Leaf	Data not available	[92]

3.2. Shoot Regeneration

Shoot regeneration under in vitro conditions is determined by several factors. This includes the quality and the type of explant, culture media composition and culture incubation conditions [93,94]. Most often, different species of plants have specific requirements with respect to the factors mentioned above, but in the case of most woody perennials, there are cultivar-specific requirements for culture media composition, which have been well documented for peach [95], apple [96] and avocado [55]. Physiological maturity is

also an important determinant for shoot regeneration in culture. Juvenile tissues (before flowering stage) are reported to have a higher potential for in vitro regeneration compared to mature material (after flowering) [97], particularly for woody species [98,99]. With respect to *Duboisia* species, young seed and seedling explants exhibit a faster response and higher shoot induction frequency than shoot tips and young leaves, which have been shown to produce calli with better quality, benefitting indirect shoot regeneration [19].

It can be shown that appropriate culture media promote healthy and quality growth of the culture, while unsuitable media adversely inhibit culture development and even cause mortality [69]. The selection of appropriate media depends on several factors: type of basal media, strength of salts, vitamins, plant growth regulators, pH, gelling agents and other additives [100]. Due to the fact that the requirements of the nutrient composition vary by plant species and even cultivars [101], the medium often needs to be specifically optimised for optimum micropropagation performance measured in plant quality and multiplication rate. The nutrient composition may vary depend on the culture stage, such as induction, shoot regeneration, multiplication and rooting. In *Duboisia* micropropagation, limited studies are available on the testing suitability of basal nutrient formulations (Table 2). It is evident that all studies utilised MS medium except Luanratana [18], who used revised-tobacco medium for *D. myoporoides* and Yamada and Endo [27] who used B5 (Gamborg B5) medium [102] for *D. leichhardtii* F. Muell. There have been no studies conducted to compare the effect of basal nutrients on shoot regeneration, multiplication and rooting of this species. However, the effect of vitamins and salt strength on the culture development of *Duboisia* species has been discussed, but only confined to the callus growth stage. Lin [19] employed different strengths of MS salts (1/2, 1/4 and double strength) and various vitamins including MS vitamin, Griffin vitamin [88], Linsmaier and Skoog vitamin, and B5 vitamin to compare the callus growth of an $M \times L$ hybrid. Among various combinations of different MS salt strength and vitamins, the highest callus growth rate (0.164/tube/day) was obtained in the media containing full-strength MS salts and B5 vitamin. Likewise, Luanratana [18] applied 1/2, 1/4, 1/8 and full-strength MS salts into medium to compare the callus growth of $M \times L$ hybrid. The best callus growth was achieved on MS medium containing full-strength MS salts.

Table 2. Summary of basal media used in *Duboisia* studies.

Duboisia **Species/Hybrid**	**Basal Media**	**Culture Stage**	**Reference**
Duboisia myoporoides × *Duboisia leichhardtii*	MS	Callogenesis, shoot induction, multiplication and root induction	[18,19,84,89]
Duboisia myoporoides	MS	Callogenesis, shoot induction, multiplication and root induction	[23,44,85,87,90]
Duboisia myoporoides	Revised-tobacco	Callogenesis, shoot induction, shoot elongation and root induction	[18]
Duboisia leichhardtii F. Muell.	B5	Callogenesis, shoot induction, multiplication and root induction	[24]

Research in basal media comparison is not available for *Duboisia*; the reviewed studies included in the table did not provide results for culture performance with the selected medium.

Plant growth regulators (PGRs) are chemical substances, either naturally occurring or synthetic, that influence the growth and development of plants by acting at cellular

levels to elicit specific growth responses. PGRs play a crucial role in a wide range of physiological processes, such as cell division and elongation, organogenesis, and responses to biotic and abiotic stresses. The main plant hormones categorised under PGRs include auxins, gibberellins, cytokinins, ethylene and abscisic acid, and there have been other growth promotors and inhibitors/retardants discovered in recent years [103]. In tissue culture, incorporation of one or more PGRs in culture medium to ignite cellular responses to achieve shoot growth or root induction is a common practice [91]. The specific PGR for a specific function at optimum concentration is essential. Cytokinin as a PGR promotes cell division and stimulates axillary bud outgrowth, thus it is the main hormone used for shoot proliferation [104]. However, the plant physiology is complex and physiological changes are often governed by the interaction of more than one PGR. Hence, a low concentration of auxin and gibberellin are applied in conjugation with cytokinin to maintain the optimum plant health and vigour [72,105].

The reported work on *Duboisia* shoot regeneration highlights BA as the most frequently used cytokinin within a concentration range of 2–15 mgL^{-1} (Table 3). Application of other cytokinins has also been evaluated; however, the two cytokinins kinetin and zeatin have been found to be more effective for callus induction [44]. The BA concentration of 3–4 mgL^{-1} incorporated with 4 mgL^{-1} NAA, or 1 mgL^{-1} IAA has shown successful shoot induction of *D. myoporoides* [18,44]. The highest shoot induction frequency of 70% in $M \times L$ hybrid was obtained using dual cytokinins 5 mgL^{-1} BA and 2.5 mgL^{-1} 2-iP [19]. Most studies apply the same medium for both shoot induction and shoot multiplication phase, while a few authors have employed different medium compositions for shoot multiplication. Lin [19] subcultured shoot buds of $M \times L$ hybrid on MS medium containing 2 mgL^{-1} kinetin and 0.1 mgL^{-1} NAA for shoot multiplication. For *D. myoporoides* R. Br., Kukreja and Mathur [44] multiplied shoots by culturing the shoots on MS medium containing 3 mgL^{-1} BA and 1 mgL^{-1} IAA.

Table 3. Summary of PGRs used for shoot induction in *Duboisia* micropropagation.

Duboisia Species/Hybrid	Explant Origin	PGRs	Shoot Induction Outcome	Reference
Duboisia myoporoides	Callus	4 mgL^{-1} BA + 4 mgL^{-1} NAA	Successful induction	[18]
	Nodal segments	3 mgL^{-1} BA + 1 mgL^{-1} IAA	Successful induction	[44]
Duboisia leichhardtii	Not reported for micropropagation			
Duboisia hopwoodii	Not reported for micropropagation			
Duboisia myoporoides × *Duboisia leichhardtii*	Shoot tip	5 mgL^{-1} BA	Failed induction	[19]
	Adventitious shoots	5 mgL^{-1} BA	Successful induction	
	Shoot and leaf segments	5 mgL^{-1} BA	Successful induction	
	Callus	5 mgL^{-1} BA + 2.5 mgL^{-1} 2-ip	70% induction	
	Callus	15 mgL^{-1} BA + 0.1 mgL^{-1} NAA	18% induction	[18]
		2.5 mgL^{-1} BA + 12.5 mgL^{-1} 2-ip	22% induction	

3.3. Root Induction

Root induction is one of the most critical and difficult steps in woody plant tissue culture; however, it may vary depending on the species or cultivar. According to Klerk et al. [106], major losses in plant micropropagation occur at the rooting stage due to poor and slow rooting. Several factors influence the root formation: shoot quality, nutrients, hormone composition and culture incubation condition. Good shoot quality supports root initiation and development [107]. Successful root induction requires optimum growth conditions and a balanced auxin–cytokinin interaction [108]. Therefore, during an in vitro rooting process, the regenerated shoots from a shoot regeneration medium are transferred

into a root-promoting medium containing different PGRs with other suitable conditions for root induction and extension. Auxin is the primary PGR for root induction in plants [109]. There are different types of auxins available; indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and NAA are the most common auxins used in the horticulture industry. IBA and NAA are stable auxins compared to the naturally occurring IAA, which is prone to faster degradation upon radiation and high temperature [110]. Application of auxin in rooting media for continuous incubation is the most common method to induce roots during micropropagation [108].

Under in vitro conditions, *Duboisia* species have been shown to have limited rooting capacity without the application of exogenous auxin [44]. The literature supports IBA as the most commonly used PGR for the rooting of *Duboisia* species [18,19,23,27,84,85,87,89]. Application of 4 mgL^{-1} IBA in MS basal medium has resulted in a maximum 65% rooting for $M \times L$ hybrid and 68% for *D. myoporoides* [18]. However, the hormone requirements for the rooting of *Duboisia* species show a variance depending on the cultivar and culture types (Table 4). In rooting studies, NAA was reported to be superior to IBA and IAA for *D. myoporoides* R. Br. A maximum of 70–80% rooting has been achieved in static liquid MS medium containing 0.5 mgL^{-1} NAA [44]. To date, rooting percentages have not exceeded 80% for any *Duboisia* species in the published literature.

Table 4. Summary of PGRs used in shoot induction in *Duboisia* micropropagation.

***Duboisia* Species/Hybrid**	**Explant Origin**	**Basal Media**	**PGRs**	**Rooting Success**	**Reference**
Duboisia myoporoides	Callus	MS	4 mgL^{-1} IBA	68%	[18]
	Nodal segments	Static liquid MS	0.5 mgL^{-1} NAA	70–80%	[44]
Duboisia leichhardtii			Not reported		
Duboisia hopwoodii			Not reported		
Duboisia myoporoides × *Duboisia leichhardtii*	Callus	MS	5 mgL^{-1} IBA + 0.1 mgL^{-1} NAA + 0.1 mgL^{-1} kinetin	53.4%	[19]
	Callus		4 mgL^{-1} IBA	65%	[18]

3.4. External Incubation Condition for In Vitro Cultures

Another essential factor for plant micropropagation is incubation conditions. Plant health in vitro is highly influenced by temperature and light regimes [111]. These conditions need to be set according to plant species and culture stages for micropropagation systems [112]. Cool white fluorescent light is frequently used for illumination [113] and the incubation temperature is maintained at 25 °C consistently, while a lower temperature (18 °C) is suggested for bulbous species and a higher temperature (28–29 °C) for tropical species [114]. For *Duboisia* species, the majority of studies reported incubation temperatures of 25 ± 2 °C with a photoperiod ranging from 12 to 16 h light using cool white fluorescent light and a corresponding dark period of 12 to 8 h respectively, or with a continuous dim light (Table 5).

Table 5. Summary of external incubation conditions for *Duboisia* in vitro regeneration.

Duboisia Species/Hybrid	Temperature	Light and Photoperiod	Media pH	Reference
Duboisia myoporoides × *Duboisia leichhardtii* and *Duboisia myoporoides*	26–28 °C	Light condition	N.A.	[18]
Duboisia myoporoides × *Duboisia leichhardtii*	27 °C	Day-light fluorescence with 31.22 µmol s^{-1} m^{-2} intensity	5.7	[19]
Duboisia myoporoides R. Br.	25 ± 3 °C	16-h photoperiod with 40.48 µmol s^{-1} m^{-2} intensity	5.8 ± 0.1	[44]
Duboisia myoporoides × *Duboisia leichhardtii*	27 °C	Continuous light with 31.22 µmol s^{-1} m^{-2} intensity	N.A.	[84]
Duboisia myoporoides R. Br.	25 ± 2 °C	14-h photoperiod of cool-white light with 15.2 µmol s^{-1} m^{-2} intensity	5.7	[85]
Duboisia myoporoides R. Br.	26 ± 2 °C	Continuous light with 20.24 µmol s^{-1} m^{-2} intensity or dark/light cycle (12/12 h)	6	[90,115]
Duboisia myoporoides R. Br. × *Duboisia leichhardtii* F. Muell.	27 °C	N.A.	5.7	[89]

3.5. Acclimatisation

Acclimatisation is a critical process which determines the success of any micropropagation process [116]. The in vitro regenerated plantlets have several structural differences due to long-term exposure to luxurious high humidity and low light conditions in vitro. In vitro plants have fewer stomates, lack a cuticle, possess non-functional roots, and have a poorly performing vascular system [117]. Therefore, direct transplantation would result in plant wilting, desiccation and susceptibility to bacterial and fungal infection [105]. To ensure the success of acclimatisation, great care is required to gradually train the in vitro generated plants to develop structural features to withstand dynamic external environment conditions.

For *Duboisia* species, studies on acclimatisation have been very limited, possibly due to the limited success in generating rooted plantlets through tissue culture. The acclimatisation protocols summarised from the work of Lin [19] and Luanratana [18] are listed in Table 6. So far, the maximum acclimatisation survival reported in literature is 88% for *D. myoporoides*, and 68% for $M \times L$ hybrid by Luanratana [18].

Table 6. Acclimatisation protocol summarised from the work by Lin and Luanratana for *Duboisia*.

Steps	Acclimatisation Protocol
1	Wash out agar medium without damaging the root system during the transplantation to avoid the risk of fungal infection.
2	Pot the rooted shoots in a mixture of sand and rice ash (50:50) or moss with sand; the optimum temperature during transplantation period is 26–28 °C.
3	Contain the potted plants in a Ziploc plastic bag or a tray with lid to maintain humidity.
4	Maintain 100% humidity with gradual exposure to the external environment with frequent sprays of water to maintain high moisture levels.
5	Maintain under shade house for the hardening process with daily watering for the first two weeks.

3.6. Challenges in Duboisia Micropropagation

Starting from explant sterilisation, there are several problems encountered in *Duboisia* tissue culture at every culture stage. Browning is one of the main problems of in vitro culture [18]. *Duboisia* is a woody species that produces polyphenol exudates under in vitro

conditions, which can inhibit cell growth and cause mortality [118,119]. According to Luanratana [18], the reduction of salt strength in the medium can reduce browning. In addition, incorporation of ascorbic acid or activated charcoal at a concentration of 0.1% has been shown to reduce extra exudates. However, application of ascorbic acid has led to other complications, such as retarded growth.

Previous work conducted by Hiti Bandaralage et al. at the University of Queensland (unpublished data) has identified several challenges pertaining to *Duboisia* micropropagation. Vigorous callusing is one of the prominent problems in *Duboisia* micropropagation. Callus formation is characteristic of plants as a response to stress such as wounding and application of exogenous auxin and cytokinin [120]. Callogenesis is closely related to cell dedifferentiation and redifferentiation, which is important for organogenesis [121]. However, over callusing inhibits outgrowth of shoots/roots in plant tissue culture [120]. The balance of auxin to cytokinin ratio is essential for callus regulation, while silver nitrate is also effective to reduce callus formation in woody species [122,123]. Defoliation is another major problem for *Duboisia* micropropagation; the severity of this is especially high during continuous culture and root induction stage. Frequent subculturing is proven to be effective to overcome defoliation caused by continuous culturing. For auxin-induced rapid defoliation during the root induction period, Hiti-Bandaralage et al. [72] used silver nitrate or silver thiosulphate in the culture media for avocado. Thus, effect of silver nitrate and silver thiosulphate on auxin-induced defoliation is suggested to be evaluated for *Duboisia* species.

A commercially viable micropropagation system generally requires high rooting percentages and good functional roots to assist acclimatisation. In vitro root induction of *Duboisia* has been proven to be difficult and mostly produced non functional roots causing problems during acclimatisation stage. This could be the reason for lack of commercially viable tissue culture technology for the species.

4. Assessment of Somaclonal Variation for In Vitro Regenerated Plants

Evaluation of somaclonal variation is an important step in determining the genetic stability of in vitro regenerated plants for any micropropagation platform, and is necessary for their successful use in commercial production and further experimentation. Somaclonal variation refers to the genetic alterations that may occur during micropropagation, including mutations, chromosomal aberrations, and epigenetic modifications [124]. Various techniques are available to assess somaclonal variation in in vitro regenerated plants, including morphological, cytogenetic, molecular, and biochemical analyses. These approaches have been used to detect genetic alterations such as altered leaf morphology, chromosomal number and structural changes, and molecular-level genetic variation [125].

In the case of *Duboisia*, a limited number of studies have evaluated somaclonal variation. According to Lin [19], somaclonal variation has been observed in in vitro culture of hybrid $M \times L$, as evidenced by the varying scopolamine content in the plants regenerated from different types of explants. In addition, regenerated plants have exhibited variation in leaf shape, with some plants having broader or narrower leaves than the donor plant. In a separate study, Mano et al. [31] observed considerable variation in growth rate, alkaloid content, and productivity among 45 hairy root clones of *Duboisia leichhardii* F.v.M. generated from individual root meristems. The authors emphasised the great potential of somaclonal variation as a tool for crop improvement in *Duboisia* species. However, further studies are required to fully understand the extent of somaclonal variation in *Duboisia* and to develop effective strategies for its practical application. On the other hand, somaclonal variation is a critical problem in micropropagation [126]. Despite its potential for crop improvement, somaclonal variation is often unpredictable and can lead to undesirable traits such as genetic instability, morphological abnormalities, reduced viability, and altered physiology [127]. The production of high quality and uniform plants through tissue culture is essential in crops, particularly in *Duboisia*, which is valued for its secondary metabolites. Therefore, in-depth research is required to employ more approaches such as cytogenetic

techniques and molecular markers to validate the developed micropropagation protocols and facilitate a reliable and sustainable micropropagation system for *Duboisia*.

5. Recent Advances in Meristem Culture for Commercial Micropropagation of *Duboisia*

The *Duboisia* industry is desperate for a better propagation system to clone large volume of disease-free plants from limited explant material. At the University of Queensland, a high-throughput meristem-culture-based micropropagation system has been developed for *Duboisia* spp. Species-specific optimisations of the micropropagation processes have been completed for three *Duboisia* parent species *D. myoporoides*, *D. leichhardtii*, *D. hopwoodii* and two elite hybrids $M \times L$ and $M \times H$ (*D. myoporoides* × *D. hopwoodii*). The system developed has the capacity of an overall 1379 multiplication rate (number of shoots produced at the end of the fifth subculture/initial cultures used) with 70–100% rooting potential and 80–100% survival after acclimatisation for *D. myoporoides*, *D. leichhardtii*, *D. hopwoodii*, $M \times L$ hybrid and $M \times H$ hybrid shoots obtained through meristem culture (unpublished data). This for the first time demonstrates the capacity of a *Duboisia* meristem culture platform to produce an average of 500–800 (depends on the species/hybrid) field-ready plants from one single shoot tip meristem within a short period of 6 months (Figure 1), solving a burning propagation problem faced by the industry [128].

Figure 1. Meristem culture micropropagation platform for $M \times L$ hybrid. Adapted from Mason et al. [128].

Field evaluation of the growth rate, leaf production and leaf scopolamine content of meristem-culture-derived *Duboisia* plants in comparison to cutting-propagated plants has been conducted in Queensland, Australia [128]. No significant difference was observed with the scopolamine content and production between meristem-culture-derived plants and cutting-propagated plants, suggesting that the meristem culture micropropagation system is feasible for high-throughput commercial propagation of *Duboisia* spp.

6. Conclusions

Taken together, this review supports the notion that *Duboisia* has been an extensively studied medicinal plant over the past four decades. Conventional farming of *Duboisia* is still the most commercially feasible source of the powerful alkaloid scopolamine, but propagation is a major bottleneck in the industry to produce large volumes of elite clones. Micropropagation is a viable and economical solution but, to date, reports poor efficiency in multiplication, rooting, and acclimatisation, requiring species/selection-specific optimisation for improvement. New advances have been made in the space of micropropagation

through meristem-culture-based high-throughput clonal propagation platforms. This technology exemplifies a propagation solution for the *Duboisia* industry with great potential for large-scale, year-round commercial propagation of true-to-type elite *Duboisia* selections.

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