Development of a robust method for propagation of *Leptospermum scoparium* J.R.Forst. & G.Forst. from semi-hardwood cuttings

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Methodology

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Abstract

Background

Leptospermum scoparium J.R.Forst. & G.Forst. is highly valued for the production of antimicrobial honey. Both researchers and the Australian honey industry are interested in the clonal propagation of these plants to be used for experiments and plantation establishment. However, little information is available on clonal propagation by cuttings. Here we sought to develop a detailed method for *L. scoparium* propagation by semi-hardwood cuttings by testing the influence of genotype and auxin (indole butyric acid (IBA) and IBA + naphthalene acetic acid (NAA)) concentration on plants originating from four natural populations in Victoria, Australia.

Results

There was no significant difference in root induction, which ranged from 50.9 – 68.0%, in the four clonal source plants selected. The application of 3 g/L IBA increased root induction from 46.6% of the control cuttings to 77.6% of treated cuttings and stimulated longer roots. The application of 8 g/L IBA increased leaf fall, the presence of necrosis, from 22.5% on the controls to 82.8% on dipped cuttings, and cutting death.

Although there was no significant difference in root induction, cuttings that were ‘watered-in’ after planting dropped significantly fewer leaves and had significantly lower rates of necrosis than cuttings watered in after 2-3 days. It was also found that the maturity stage of semi-hardwood cuttings did not significantly affect any of the variables measured.

Transplant survival ranged from 78.1 – 89.0% for cuttings treated with auxin and 77.3 to 92.2% for the four clonal source plants.

Conclusion

This study found that the clonal source plants selected did not affect the success of propagation. Overall for maximum success, semi-hardwood cuttings can be collected from the current or previous seasons growth, be treated with 3 g/L IBA gel, ‘watered in’ and placed into a propagation tent for approximately six weeks. Cuttings that form roots will transplant easily with high transplant survival.

Background

Leptospermum scoparium J.R.Forst. & G.Forst. is native to Australia and New Zealand where local industries are establishing plantations for the production of bioactive honey (1–4). *Leptospermum scoparium* (Manuka) honey has non-hydroxide antibacterial properties largely due to the presence of methylglyoxal (MGO) (5). The precursor to MGO is dihydroxyacetone (DHA) and is found in the nectar of *L. scoparium* plants (6–9). Plants with high DHA to total sugars (DHA:Tsugar), in the nectar, are believed to produce honeys with the highest MGO concentration (6, 7).

To ensure economical production of high quality bioactive honey, plantations should focus on using plant selections with high levels of DHA:Tsugar, excellent flower production and high nectar flow (2, 8, 10). Williams, King (9) and Williams, Pappalardo (8) found the production of DHA to total sugars to be variable within and across both wild and cultivated populations of *L. scoparium* in New Zealand and Australia. This suggests seedling-grown populations are likely to have variable DHA to total sugars. This variability is problematic when plantations are

established from seed, however through selection of elite genotypes from the wild or cultivation of existing germplasm, advances can be made quickly. Elite genotypes can be clonally propagated to ensure genetic integrity and reliability in the production of high-quality honey.

Clonal propagation of plants is used to maintain desirable features from one plant to another; there are many cultivars of *L. scoparium* that are clonally propagated by cuttings (11–14). Research into propagation of *Leptospermum* species by tissue culture (micropropagation and micro cuttings) has also been reported (15–17). Tissue culture can be used where species are difficult to propagate using conventional cutting propagation, as is the case for a number of other Myrtaceous genera (18), including species of *Leptospermum*. Compared to cuttings, propagation by tissue culture can often be labour intensive (19), but is a useful option when more traditional methods of propagation are not successful, as in the case of the genus *Eucalyptus* (20, 21). However, to meet the growing needs for the Australian bioactive honey industry, where low-cost high-quality plant material for large plantations is required, a reliable and economical method of clonal propagation needs to be developed.

Propagation by cuttings is often a cost-effective alternative to seedlings or tissue culture; benefits include being able to keep track of known genotypes, minimal handling using simple and straightforward techniques, and only readily available equipment is required. Cutting propagation can be scaled to meet demand, from small scale research projects to large scale plantations, provided adequate propagation methodologies are established (22). Currently, there is little information available on successful methods of propagating *L. scoparium* from cuttings (15) and only brief mentions of methods for related species are available (16, 23). Seelye, Hofmann (16) suggest *Leptospermum* need rooting hormone, aerated media and misting in a glasshouse to form roots but does not provide a clear method or report the rate of success. Woodgyer (23) suggest propagation of *L. lanigerum* from semi-hardwood tip cuttings in July (northern hemisphere) with bottom heat of 21°C using a 2500 ppm IBA rooting hormone dip but also did not report on success rates.

Successful methods for cutting propagation have been developed for *Kunzea pomifera*, a closely related genus in the Myrtaceae family. Page (24) discusses influences of cutting length and diameter, age of material, media, bottom heat, irrigation and propagation tent setup for *Kunzea pomifera* propagation. These methods were found to be very similar to the anecdotal methods collected from the Horticultural Curator of Plant Propagation for the Botanic Gardens of South Australia (personal communications, Coulter, 2019). These methods were also compared to anecdotal methods received from the ERA Nurseries, Hamilton, Victoria who initially propagated the plants, from wild collection, used for this study. The method developed by Page (24) and these anecdotal methods formed the basis of the variables, factors and conditions investigated here.

The aim of this study was to investigate the effect of hormones and clonal source plants on cutting propagation of *L. scoparium* and how they influenced root initiation and subsequent cutting survival. By comparing different concentrations and types of plant hormones, on four different clonal source plants, using conventional nursery practices, an economical and reliable method of cutting propagation has been developed.

Results

Effect of auxin application on propagation

Compared to the control, the application of 3 g/L of IBA increased the percentage of cuttings with root induction (Table 1) to 77.6%. Whilst increases in root induction were seen (on average) for the other treatments, at 56.9–

66.8%, these were not significantly different to the control or 3 g/L IBA (Table 1). It was found that 8 g/L IBA produced more cuttings with roots that originate above the media, at 12.9%, than any other treatment (Table 1, Fig. 1).

Table 1
Effect of hormone application on the number of alive cuttings and root induction.

	Control		1.5 g/L IBA		3 g/L IBA		8 g/L IBA		1.6 g/L IBA + 1.6 g/L NAA	
Total cuttings	800	%*	800	%*	800	%*	800	%*	800	%*
Dead/alive cuttings	36/	(4.5/	87/	(10.9/	75/	(9.4/	156/	(19.5/	180/620	(22.5/
	764	95.5) a	731	89.1) ab	725	90.6) ab	644	80.5) b		77.5) ^b
Root induction	365	45.6 ^a	534	66.8 ^{ab}	621	77.6 ^b	507	63.4 ^{ab}	455	56.9 ^{ab}
Origin of roots (below media)	364	45.5 ^{ab}	507	63.4 ^{bc}	574	71.75 ^c	267	33.4 ^a	412	51.5 ^{abc}
Origin of roots (above media)	1	0.1 ^a	9	1.1 ^a	20	2.5 ^a	103	12.9 ^b	13	1.6 ^a
*percent of 800 cuttings planted										
Significant differences ($p < 0.05$) in treatments are indicated by different superscript letters.										

Figure 1. *Leptospermum scoparium* cuttings with roots. Cuttings with roots below the propagation media but without aerial roots (a) were transplanted to the same depth as they were buried in the propagation media. Cuttings with roots below the soil and aerial roots (b) were transplanted to cover approximately half to two thirds of the aerial roots. Cuttings with aerial roots but no roots below the propagation media (c) were transplanted to cover half to two thirds of the aerial roots. When cuttings with aerial roots were transplanted, the leaves that would be located below the 'soil' were removed (b, c).

The control category (no treatment) resulted in the highest number of cuttings still alive (Fig. 2), at 95.5%, after six weeks in the propagating tent (Table 1). Compared to the control, the application of 8 g/L IBA or 1.6 g/L IBA + 1.6 g/L NAA significantly reduced the percentage of cuttings still alive by 15–18% respectively (Table 1).

Figure 2: Dead (a) and live (b) cuttings of *L. scoparium* cuttings with callus formation occurring on cuttings of 'C' from the control.

Compared to the control, cuttings treated with hormone gel showed increased rates of necrotic tissue at the base of the cuttings and the application of 8 g/L IBA resulted in the highest rate of necrosis at 82.8% (Table 2). Callus formation (Fig. 2) and the presence of new growth (Fig. 1) decreased significantly with the application of 8 g/L IBA and 1.6 g/L IBA + 1.6 g/L NAA compared to the control (Table 2).

Table 2
Effect of hormone application on necrosis, callus formation, new growth and leaf drop.

	Control		1.5 g/L IBA		3 g/L IBA		8 g/L IBA		1.6 g/L IBA + 1.6 g/L NAA	
Total cuttings	800	%	800	%	800	%	800	%	800	%
Necrosis	180	22.5 ^a	401	50.1 ^b	452	56.5 ^b	662	82.8 ^d	551	68.9 ^c
Callus formation	628	78.5 ^c	570	72.5 ^{bc}	635	79.4 ^c	394	49.3 ^a	440	55.0 ^{ab}
New shoot/s present	399	49.9 ^b	366	45.8 ^b	402	50.3 ^b	105	13.1 ^a	269	33.6 ^{ab}
Leaf Drop	136	17 ^a	196	24.5 ^{ab}	184	23 ^a	315	39.4 ^{bc}	360	45 ^c
*percent of 800 cuttings planted										
Significant differences ($p < 0.05$) in treatments are indicated by different superscript letters.										

Overall, the application of any hormone, at any concentration, increased the number of roots on a cutting compared to the control (Table 3, Fig. 3). Average root counts ranged from five to ten roots per cutting for treated cuttings compared to two roots for the control.

Table 3
Effect of hormone application on the number of roots initiated and the longest root on *L. scoparium* cuttings.

	Control	1.5 g/L IBA	3 g/L IBA	8 g/L IBA	1.6 g/L IBA + 1.6 g/L NAA
Roots initiated per cutting	2 ^a	5 ^b	8 ^{bc}	10 ^c	6 ^b
Longest Root (mm)	31 ^{ab}	42 ^{bc}	48 ^c	25 ^a	30 ^{ab}
Significant differences ($p < 0.05$) in treatments are indicated by different superscript letters.					

Cuttings treated with 3 g/L IBA produced significantly longer roots than the control (Table 3). There was no significant difference in root length between the control and the remaining hormone treatments.

Figure 3. Root abundance on cuttings of *Leptospermum scoparium* 'C' treated with 0 g/L IBA (a), 3 g/L IBA (b) and 8 g/L IBA (c).

The control cuttings and those receiving 3 g/L IBA application retained more leaves than those treated with 8 g/L and 1.6 g/L IBA + 1.6 g/L NAA (Table 3).

Figure 4: *Leptospermum scoparium* flower buds (fb) on cutting of 'M' with roots (a) and in the field (b).

There was no significant difference in the percentage of cuttings with flower buds (Fig. 4) or cuttings with secondary root formation (Fig. 5, data not shown).

Figure 5. Primary (1^o) and secondary (2^o), or lateral, root formation in *L. scoparium* 'S' cuttings with no hormone application.

Effect of auxin application on transplant survival

After measurements were taken, cuttings with intact roots were potted up. If roots were compromised in any way, the cuttings were discarded (i.e. where roots were damaged or rotted, or the cuttings had desiccated during the hardening off period [Figure 6]).

Figure 6. Examples of cuttings of *L. scoparium* that were damaged as a result of desiccation (a) or rot (b). Cuttings with aerial roots that had not penetrated the media (a) were not able to take up water during the hardening off period.

The survival rate of plants 8 weeks after transplanting ranged from 78.1–88.8%. The highest survival rates were for cuttings treated with 1.5 g/L IBA or 3 g/L IBA at 88.8% and 87.8% respectively, and the lowest survival rate was 78.1% for cuttings exposed to 8 g/L IBA (Table 4).

Table 4
Effect of hormone application on the transplant survival of *L. scoparium* cuttings.

	Control		1.5 g/L IBA		3 g/L IBA		8 g/L IBA		1.6 g/L IBA + 1.6 g/L NAA	
	800	%**	800	%**	800	%**	800	%**	800	%**
With Roots/ Planted	365/		534/		621/		507/		455/	
	333		473		559		383		398	
Planted (Dead/Alive)	52/	15.6/	53/	11.2/	68/	12.2/	84/	21.9/	67/	16.8/
	281	84.4	420	88.8	491	87.8	299	78.1	331	83.2
**as a percent of transplanted cuttings										

Effect of clonal source plants on propagation

There was no significant difference in root induction between clonal source plants (Table 5).

There was also no significant difference in the percent of cuttings that had begun to grow (Table 5), were flowering (data not shown) or produced secondary roots (data not shown) between clonal source plants. The percent of flowing cuttings was significantly less than vegetative cuttings, at 0–4.6% and 95.4–100% respectively.

Table 5
Effect of clonal source plants on propagation and transplant survival.

	'C'		'J'		'M'		'S'	
Total	1000	%*	1000	%*	1000	%*	1000	%*
Dead/alive cuttings	74/ 926	(7.4/ 92.6) ^a	238/ 762	(23.8/ 76.2) ^b	165/ 835	(16.5/ 83.5) ^{ab}	57/ 943	(5.7/ 94.3) ^a
Root induction	675	67.5 ^a	509	50.9 ^a	618	61.8 ^a	680	68.0 ^a
Origin of roots (below media)	631	63.1 ^b	372	37.2 ^a	511	51.1 ^{ab}	610	61.0 ^b
Necrosis	373	37.3 ^a	796	79.6 ^b	690	69.0 ^b	387	38.7 ^a
Callus formation	833	83.3 ^c	526	52.6 ^a	599	59.9 ^{ab}	719	71.9 ^{bc}
New shoot present	472	47.2 ^a	362	36.2 ^a	372	37.2 ^a	335	33.5 ^a
Leaf Drop	140	14 ^a	464	46.4 ^b	416	41.6 ^b	171	17.1 ^a
Roots initiated per cutting	5 ^a		5 ^a		5 ^a		8 ^b	
Longest Root (mm)	45 ^b		19 ^a		35 ^b		41 ^b	
With Roots/Planted	675/ 575		509/ 415		618/ 523		680/ 633	
Planted (Dead/Alive)	46/ 529	8.0/ 92.0 ^{**}	94/ 321	22.7/ 77.3 ^{**}	107/ 416	20.5/ 79.5 ^{**}	77/ 556	11.6/ 83.9 ^{**}
*percent of 1000 cuttings planted								
**as a percent of transplanted plantlets								
Significant differences (p < 0.05) in treatments are indicated by different superscript letters.								

Effect of clonal source plants on transplant survival

The survival rates of transplanted cuttings ranged from 77.3–92.0% (Table 5).

Effect of 'watering-in' on propagation

Clonal source plants 'J' and 'M' were first hand watered 2–3 days after the cuttings were planted whereas 'C' and 'S' were 'watered-in' by hand immediately after the cuttings were planted.

We found that 'watering-in' immediately after planting had a significant effect on the number of viable cuttings, extent of leaf drop, callus formation, necrosis and aerial root formation (Table 6) after six to seven weeks. Cuttings that were not 'watered-in' immediately after planting had a significant increase in aerial root formation, necrosis and leaf drop as well as a significant decrease in callus formation and the survival rate of cuttings (Table 6).

Table 6
Variables significantly affected ($P < 0.05$) by watering regime of *L. scoparium* cuttings after planting.

	Dry		Watered	
	2000	%*	2000	%*
Alive cuttings	1597	79.9	1869	93.5
Necrosis	1486	74.3	760	38.0
Callus	1125	56.3	1542	77.1
Leaf Drop	880	44.0	311	15.6
Aerial root formation**	244	12.2	114	5.7
*as a percent of 2000 cuttings per watering regime				
** cuttings with aerial roots and/or cuttings with aerial roots and roots below the media				

Effect of material age on propagation

It was found that there was no significant influence of the age of the material harvested (current seasons semi-hardwood, with softwood tips removed, or previous seasons semi-hardwood [Figure 7]) for cuttings on any of the variables measured here (data not shown).

Figure 7. Previous season (a) and current season (b) semi-hardwood cuttings of *L. scoparium* 'S' and 'J' after planting.

Effect of flowering on propagation

Although flowering occurred during propagation (Fig. 4), this had no significant effect on the variables measured.

Discussion

Vegetative propagation is often needed to multiply elite plants coming from breeding and selection programs (22, 25). The propagation methodology must be reliable, as economic losses occur when cuttings do not form roots or form poor quality root systems (25). The quality of plants produced from cutting propagation can be influenced by genotype (24, 26, 27), hormone application (25, 28, 29), the age of material at harvest (20, 30, 31), whether wood is flowering or vegetative, as well as water loss from the cuttings at vulnerable stages (22, 32). In this study, however, it was found that the rooting potential of *L. scoparium* cuttings was only significantly influenced by the application of IBA hormone gel, although water stress after cuttings were first planted reduced the quality and quantity of the surviving cuttings by 20.2% (Table 6).

This was a somewhat surprising result since genotype can have a significant impact on the ability of a particular species to form adventitious roots, especially in members of the Myrtaceae family (27, 28, 33, 34). Doran, Baker (26) attempted propagation of 50 genotypes of *Melaleuca alternifolia* and found that 21 genotypes were readily propagated, 22 were more difficult to propagate and seven did not produce roots. Page (24) successfully propagated four genotypes of *Kunzea pomifera* and found that genotype influenced the percent of rooted cuttings. We expected that *L. scoparium* might perform similarly to other members of the Myrtaceae family, however, it was

found that source genotype did not significantly influence the outcomes for the cuttings taken from them in this study. Therefore, the effect of hormones on the initiation and subsequent growth of adventitious roots, as well as the survival of the cuttings, could be investigated without the need to pay attention to the influence of genotype.

The application of exogenous hormone to stimulate adventitious root formation is a practice that was developed in 1936 (35), which followed the discovery of the rhizogenic activity of auxins in 1934 (25, 36). When cuttings are dipped in 'rooting' gels, the auxins enter the cutting mainly through the cut surface (ends or leaf scars; (37, 38) by pH trapping (39), influx carriers (25, 40) and suction (29). Upon entering the cutting, IBA may be oxidised, an irreversible change, or conjugated, which can be reversible, leaving very little in the free form (25) to stimulate the early stages of rooting (de-differentiation and induction; (25, 28). When *L. scoparium* was treated with a range of concentrations of IBA or IBA + NAA in this study, and measured at six weeks, the number of cuttings that formed roots increased over those not treated. It was greatest for those treated with 3 g/L IBA gel which also increased the number of roots and root length compared to cuttings not treated with hormone. This likely occurred as there was sufficient IBA to stimulate de-differentiation into meristematic tissue, followed by induction of root initials (25, 28). However, with time, it is possible that the freely available IBA became depleted, as discussed by de Klerk, van der Krieken (25), allowing the root primordium to form, from the root initials, initiate growth and emerge from the stem (28).

Auxin application can speed up root initiation (22, 29). This potentially explains why cuttings treated with 3 g/L IBA produced longer roots than cuttings that were not treated with hormone. Cuttings treated with 3 g/L IBA likely produced roots earlier than the control, and therefore grew longer because they were growing for longer. However, the difference observed in root length between 3 g/L IBA and the control did not influence the transplant survival rates. This suggests that cuttings treated with 3 g/L IBA could be removed earlier (i.e. when roots are shorter) from the propagation tents without transplant survival being affected, freeing up propagation space and, potentially, reducing plant residency in the nursery (22).

High concentrations of auxin applied to cuttings can have negative effects on their health and survival (25, 28, 29). Here *L. scoparium* treated with 8 g/L IBA showed an increase in visible necrosis, necrosis severity, leaf senescence, as well as a reduction in new growth, and, in some cases, cutting death. Research has shown that cuttings that have been exposed to high levels of auxin/s can show growth abnormalities (29, 41), as well as inhibition of root primordia emergence, root growth and foliar growth (22, 25, 28, 42, 43). In addition, high IBA can reduce stomatal aperture and transpiration rates as well as cause increased foliar senescence (25, 29), chloroplast damage, necrosis and, eventually, plant death (29).

There is contradictory information on when to water newly planted cuttings. Hartmann, Kester (22) suggest cuttings be watered after planting to settle the rooting medium, while the instructions for application of the hormone products provided by the manufacturer suggest misting commence immediately after gel application (44, 45). This method is used at the Botanic Gardens of South Australia with success (46). Therefore, the decision was made to follow the manufacturer's instructions. This was revised when it was found that the intermittent misting was insufficient to prevent 'J' and 'M' from dehydrating. Supplementary water was applied, to prevent complete dehydration of 'J' and 'M', and rehydration occurred in most of the cuttings. Hartmann, Kester (22) suggest that the dehydration and rehydration prevents cuttings from rooting. In this experiment, however, there was no significant difference in root induction or number of roots per cutting between the rehydrated and 'watered in cuttings, but rather, the quality and quantity of the material was reduced. Murthy and Goldfarb (47) also found similar results in

Pinus taeda cuttings. Therefore, cuttings of *L. scoparium* clonal source plants tested here appeared to benefit from 'watering-in' after being planted into the media.

Other factors that have been reported to influence the success of cuttings are the time of year (in relation to flowering) the material is harvested and the overall age of the material. Day and Loveys (32) found that adventitious rooting of *Hypocalymma angustifolium* was greater prior to flowering, whereas in *Boronia megastigma* adventitious rooting was greatest after flowering. In this experiment, it was found that flowering (during the experiment), and/or the collection of material prior to flowering, had no significant effect on any of the variables measured. *Leptospermum scoparium* flowers from October to February (1), with these particular genotypes flowering sporadically outside of this period. These experiments also found no influence of material age on adventitious rooting. This was unexpected as many authors have shown that maturation stage and aging effects in Myrtaceae can negatively affect the production of adventitious root formation (21, 30, 31, 48). Day and Loveys (32) also showed that the removal of the tip of cuttings in some species, as was done for 'C' and 'J' to utilize the semi-hardwood of the new growth, can affect adventitious root formation. Therefore, these results suggest that, at least for these clonal source plants, this method can be used on a broad range of material ages and over a greater period of time to produce successful cuttings.

In addition, the economics of cutting propagation for the establishment of bioactive honey plantations must be considered. This depends on the ease of root induction and plant survival, as well as the superiority and reliability of plants produced (22). This work shows that *L. scoparium* can be easily propagated from cuttings using 3 g/L IBA gel and transplant survival is high at this hormone level. However, to determine if the qualities of elite lines are reliably maintained beyond cutting propagation, further field testing is necessary. Research on the species suggests that DHA:Tsugar levels in the nectar are maintained through clonal propagation (2) but slight variations in concentrations occur depending on the environment, soil and year (2, 9).

Conclusion

With interest in establishing plantations of *L. scoparium* for bioactive honey production increasing, it is important to develop reliable and cost-effective methods to propagate elite lines. In this experiment, *L. scoparium* propagation and survival of transplanted cuttings was not dependent on clonal source plant. It was found that application of 3 g/L IBA gel to cuttings produced the greatest number of healthy plants. This method provides a good foundation for further improvements in *L. scoparium* propagation. However, further research is required to determine the economics of this method compared to seedling production or tissue culture, and also to clarify the role of genotype versus environment and soil type on DHA:Tsugar levels in the nectar of the successfully propagated plants

Methods

Plant Material

In 2016, 160 plants from five different wild grown populations of *L. scoparium* in the southern Grampians of Victoria, Australia, were sampled and tested for the nectar DHA:Tsugar ratio (following the method of Williams, King (9)). From that assessment, 20 individuals with the highest DHA concentrations were selected, propagated by cuttings in Victoria (ERA Nurseries, Hamilton, Victoria), sent to the University of Adelaide and grown in an open nursery on the Waite Campus, Urrbrae, South Australia. Subsequently, four clonal source plants 'C', 'J', 'M' and 'S',

were selected for this experiment (representing four of the five wild grown populations) (Table 7). These were selected based on the concentration of DHA initial nectar results, amount of available material in the nursery, the uniformity of the material available and the health of the material as well as the initial plants sent to us in 2016.

Table 7
Origin, DHA levels and plant material details for *L. scoparium* used in this study.

Clonal source plant	Origin of Material	DHA levels of Original Material (DHA:Tsugar)	Material age	Number of clonal source plants
'C'	Victoria Point Road, Victoria Valley, Victoria	3,180	Current season	10
'J'	Grampians Road, Dunkeld, Victoria	2,996	Current season	7
'M'	Mallah Park, Mooralla, Victoria	2,531	Previous season	19
'S'	Carlsens Rd, Cavendish, Victoria	3,237	Previous season	22

Clones of 'C' and 'J' were grown in 10 L pots and had begun to produce new growth, (approximately 20–30 cm) with the lower proportion of this growth hardening off. Clones of 'M' and 'S' were grown in 4.5 L pots and still 'dormant' with no new growth. All plants were moved to a controlled environment glasshouse on 4th October 2019. At the time of harvest, 'C' and 'J' were approximately 1-1.4 m tall while 'M' and 'S' were approximately 0.8-1 m tall.

Preparation of semi-hardwood cuttings

Cutting material was collected from the clonal source plants 'C', 'J', 'M' and 'S' between the 8th and 11th of October 2019. Cuttings were 5–10 cm, cut approximately 0.5-2 mm below a bud, from semi-hardwood growth, and 1–2 mm in diameter. Semi-hardwood growth was identified by the presence of extensive secondary thickening and stiffening of the stem (33), and in the change in colour of the stem from green to brown and the hardening of the leaves. To utilize the semi-hardwood of the current flush of new growth for 'C' and 'J' the softwood tip of the cuttings was removed (Fig. 7). This was not necessary for 'M' and 'S' as the material was in a 'dormant' state from the previous flush of growth (Fig. 7).

The number of buds/leaves on a semi-hardwood cutting ranged from 6–20 depending on the vigour (i.e. internode length) of the previous season's growth. Leaves were removed from the lower half of the stem by grasping the centre of the stem and pulling down to remove the leaves.

Propagation media

The propagation media comprised 80% medium grade perlite (Medium grade perlite, Chillagoe Perlite, Mareeba Queensland, Australia), 15% hydrated coco peat (Cair Pith Blocks 4.5 kg, Galuku Exports India Pvt. Ltd., Pollachi, Coimbatore, Dt. Tamilnadu, India) and 5% coarse sand (Builders Sand, Berri, South Australia, Australia); placed into MK9 square punnets (95 × 115 × 120 mm, approximately one litre in volume; Quality Plastic Products Pty Ltd, Clontarf, Queensland, Australia) and watered before cuttings were inserted.

Auxin application

Cuttings were dipped for 1–2 seconds to a depth of 15–25 mm in gelatinous formulations of IBA or IBA + NAA and then immediately inserted into the propagation media. Cuttings were treated with three concentrations of IBA rooting hormone gel at 1.5 g/L IBA, 3 g/L IBA and 8 g/L IBA (Clonex Rooting Hormone Gel Green, Clonex Rooting Hormone Gel Purple, Clonex Rooting Hormone Gel Red; Growth Technology Pty Ltd, O'Connor, Western Australia, Australia) (44) and one with 1.6 g/L IBA + 1.6 g/L NAA (Ezi-Root Rooting Gel; Esi-Root and Ezi-Root Propagation, Brookvale, New South Wales). A procedural control was included where cuttings were dipped in reverse osmosis water but no hormone treatment was applied.

Watering-in

Initially 'watering-in' should have occurred one week after the cuttings were planted. However, after a two to three days, cuttings of 'J' and 'M' began to show signs of water stress (colour change; Fig. 8). Therefore, to prevent further damage, the cuttings of the affected clonal source plants were watered with Adelaide, South Australia, tap water until field capacity of the propagation media. The remaining clonal source plants, 'C' and 'S', were 'watered-in' by hand using Adelaide, South Australia, tap water immediately after the cuttings were planted to reduce water stress.

Randomisation and Replication

All cuttings for one clonal source plant and treatment were taken at the same time. Cuttings were arranged in pots in four rows of five cuttings. Ten replicates were prepared for each clonal source plant by treatment in the crossed design (200 cuttings per clonal source plant x treatment, 1000 cuttings per clonal source plant, 800 cuttings per treatment, resulting in 4000 cuttings in total). Each pot was randomly allocated to a location within each propagation tent so that each tent contained the same number of replicates of each clonal source plant and treatment. Eight pots, filled with the propagation media alone (blanks), were randomly placed within each side to determine if any contamination of the media occurred.

Environment

Propagation tent information

Cuttings were placed in two adjacent, naturally illuminated, commercially purchased (10+ years ago), propagation tents (2.0 × 0.9 × 1.5 m) within a controlled environment glasshouse (Fig. 9, Supplementary Figure I). The glasshouse is UV opaque, maintained at 25 degrees Celsius (+/- 5 °C) and shaded with 50% shade cloth. The propagation tent was covered in an opaque white plastic cover and a layer of white cloth to reduce the light intensity and minimise heat build-up (Fig. 9). Bottom heat was provided continuously by a Hotbox Heatwave bench heating system (Hotbox International Ltd, Newport, United Kingdom), at 25 ± 3 °C under capillary matting (Table 8). Misting of the tent, (using tap water), occurred for two seconds every 20 minutes during the day and was turned off during the night. Supplementary hand watering with tap water occurred twice a week.

Figure 9. Diagrammatic representation of propagation tent (not to scale) used for *L. scoparium* propagation that is housed within a controlled environment glasshouse. The relative positioning of pots (po), trays (t), capillary matting (c), water proof membrane (wp), Hotbox Heatwave bench heating system (h), Styrofoam base (s), bench (b), opaque white plastic covering (pc), white cloth covering (wc), thermostat (th), thermostat probe (tp) and misting jets (mj) are shown.

Hardening off

Approximately one week prior to data collection the sides of the propagation tent were incrementally raised and the duration between misting events increased (Table 8). This was to 'harden off'/acclimatise the cuttings prior to transplanting and maximise transplant survival.

Table 8
Schedule for hardening off *L. scoparium* cuttings ready for transplanting.

Date	Equipment	Setting
< 14/11/19	Misting system	2 seconds every 20 minutes Turned off over night
	Bench heating system	25 ± 3 °C
	Tent sides	Closed
14/11/2019	Misting system	2 seconds every 20 minutes Turned off over night
	Bench heating system	Off
	Tent sides	1/4 open
15/11/2019	Misting system	2 seconds every 30 minutes Turned off over night
	Bench heating system	Off
	Tent sides	1/2 open
16/11/2019	Misting system	2 seconds every 30 minutes Turned off over night
	Bench heating system	Off
	Tent sides	Open
	Additional watering	Provided

Transplant survival

Cuttings were removed from pots (Supplementary Figure II) and those with roots were transplanted into square 50 × 50 × 120 mm tube-stock pots containing Bio Gro Premium Potting Mix (Van Schaik's Bio Gro, Mount Gambier, South Australia, Australia). They were planted to the same depth as they were in the propagation media unless they had formed roots above the media. If roots had formed above the media, they were planted to cover at least half of these roots (Fig. 1, Supplementary Figure III).

The newly transplanted cuttings with roots were then placed randomly on a large bench in the glasshouse and watered. After the initial watering, watering occurred on Monday, Wednesday and Friday for another six weeks.

Data collection

Data were collected on the effect of auxin application, clonal source plant, 'watering-in' and material age over a 10-day period from the 19th of November 2019 to 28th of November 2019. This was approximately 6–7 weeks after the experiment commenced. Data were collected on the number of cuttings that were dead or alive; the number of living cuttings that formed roots (root induction); the origin of the roots (above, above and below, below media); the presence of necrosis, callus formation, and new shoots on cuttings; the number of roots initiated per cutting and the length of the longest root (mm); the number of cuttings with leaf drop (%), the number of cuttings with flower buds present and those that were vegetative; the presence of secondary roots, and the number of cuttings planted (potted up).

Transplant survival data were collected 6–8 weeks (8/1/20 to 21/1/20) after transplanting. Data were collected on plant survival, plant height (cm), cutting height above soil (cm before new growth), and presence of flowering (Supplementary Figure IV).

Statistical Analysis

Effect of auxin application and clonal source plant on propagation

Statistical analysis was performed using GenStat Version 19 (49). For each variable related to the propagation success, analysis of variance (ANOVA) was conducted to determine if there was a significant influence ($P < 0.05$) of clonal source plant, treatment or an interaction of the two factors. If significant differences were found, Tukey's multiple comparison tests were conducted.

The frequency of the occurrence per pot was collected for the measured variables of presence of alive and dead cuttings, origin of the roots, necrosis, callus formation, new shoot presence on cuttings, the number of cuttings with leaf drop, the number of cuttings with flower buds present and those that were vegetative; and the presence of secondary roots. Therefore, blocking of these variables took the form of which propagation tent they were located in, the clonal source plant of the material and auxin treatment.

For longest root and the number of roots initiated by cuttings, measurements were taken on each cutting. Therefore, blocking of these variables took the form of which propagation tent they were located in, the clonal source plant of the material and auxin treatment.

Effect of watering and material age on propagation

ANOVA was also conducted to determine if there was a significant difference in 'watered-in' cuttings, material age or an interaction between the two factors. If significant differences were found, Tukey's multiple comparison tests were conducted. Variables were blocked by propagation tent, watering time and material age.

Abbreviations

IBA - indole butyric acid

NAA - naphthalene acetic acid

MGO - methylglyoxal

DHA - dihydroxyacetone

DHA:Tsugar – ratio of dihydroxyacetone to total sugars in the nectar

ANOVA – Analysis of variance

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Not applicable

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Authors' contributions

TJJH designed and executed the experiment as well as collected, analysed and interpreted data. TJJH was also responsible for the production of the manuscript.

RAB significantly assisted with the drafting of the manuscript and accompanying figures. RAB was the co-supervisor of TJJH.

KLD supervised the experimental design and data interpretation, and had a major role in drafting the manuscript as well as being the Principal supervisor of TJJH.

All authors read and approved the final manuscript.

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Figures

Figure 1

Leptospermum scoparium cuttings with roots. Cuttings with roots below the propagation media but without aerial roots (a) were transplanted to the same depth as they were buried in the propagation media. Cuttings with roots below the soil and aerial roots (b) were transplanted to cover approximately half to two thirds of the aerial roots. Cuttings with aerial roots but no roots below the propagation media (c) were transplanted to cover half to two thirds of the aerial roots. When cuttings with aerial roots were transplanted, the leaves that would be located below the 'soil' were removed (b,c).

Figure 2

Dead (a) and alive (b) cuttings with callus formation of *L. scoparium* cuttings. Callus formation (b) occurring on cuttings of 'C' from the control.

Figure 3

Root abundance on cuttings of *Leptospermum scoparium* 'C' treated with 0 g/L IBA (a), 3 g/L IBA (b) and 8 g/L IBA (c).

Figure 4

Leptospermum scoparium flower buds (fb) on cutting of 'M' with roots (a) and in the field (b).

Figure 5

Primary (1o) and secondary (2o), or lateral, root formation in *L. scoparium* 'S' cuttings with no hormone application.

Figure 6

Examples of cuttings of *L. scoparium* that were damaged as a result of desiccation (a) or rot (b). Cuttings with aerial roots that had not penetrated the media (a) were not able to uptake water during the hardening off period.

Figure 7

Previous season (a) and current season (b) semi-hardwood cuttings of *L. scoparium* 'S' and 'J' after planting.

Figure 8

Leptospermum scoparium 'J', treated with 3 g/L IBA, two days after planting. The colour change is pronounced between cuttings that are healthy (h) and those that are water stressed (s).

Figure 9

Diagrammatic representation of propagation tent (not to scale) used in the set up used for *L. scoparium* propagation that is housed within a controlled environment glasshouse. The relative positioning of pots (po), trays (t), capillary matting (c), water proof membrane (wp), Hotbox Heatwave bench heating system (h), Styrofoam base (s), bench (b), opaque white plastic covering (pc), white cloth covering (wc), thermostat (th), thermostat probe (tp) and misting jets (mj) are shown.