

Article

Stem Anatomy and Adventitious Root Formation in Cuttings of *Angophora*, *Corymbia* and *Eucalyptus*

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Abstract: Many plantation eucalypts are difficult to propagate from cuttings, and their rooted cuttings often possess very few adventitious roots. We microscopically examined the stem anatomy of cuttings from 12 species of eucalypts and we determined whether adventitious root formation in auxin-treated cuttings of four species was limited to particular positions around the vascular tissue. Most species contained a central pith that was arranged in a four-pointed stellate pattern. The surrounding vascular tissue was also arranged in a stellate pattern near the shoot apex but it developed a more rectangular shape at the outer phloem as the stems enlarged radially. Adventitious roots formed at, or slightly peripheral to, the vascular cambium, and they formed at both the corners and the sides of the rectangular-shaped vascular tissue. The study highlighted that auxin-treated eucalypt cuttings can produce roots at multiple positions around the vascular tissue and so propagation methods can aim to produce more than four adventitious roots per rooted cutting. Higher numbers of adventitious roots could improve the root system symmetry, stability, survival and growth rate of clonal eucalypt trees.

Keywords: auxin; clonal forestry; eucalypts; morphology; nursery; propagation; rooting; tissue culture

1. Introduction

The eucalypts, *Angophora*, *Corymbia*, and *Eucalyptus*, are the world's most widely planted hardwood trees because of their diversity of species, adaptability to marginal production environments, and wide variety of commercial uses [1,2]. Productivity of eucalypt plantations has been limited by the low amenability of many species to vegetative propagation. Eucalypts are commonly grown from seed although several breeding programs have incorporated cutting propagation systems to supply superior eucalypt clones for plantations [3–9]. Vegetative propagation methods have been suitable for some species, such as *Eucalyptus grandis* and *E. camaldulensis*, from high-rainfall or riparian habitats, but vegetative propagation has proven difficult for species, such as *Corymbia citriodora* and *E. cloeziana*, from lower-rainfall environments [3,6–11].

One of the main problems encountered during clonal propagation of eucalypts has been the low number of adventitious roots produced per cutting, which affects root system symmetry and tree stability [12]. Other aspects of tree growth can also be affected by the number of adventitious roots; e.g., raising the number of adventitious roots from one to five in *Pinus* cuttings increases subsequent tree survival, height and trunk diameter [13–15]. Adventitious root number in eucalypts can be elevated by auxin rooting hormones [16–20], with many studies finding that the most effective treatments induce approximately four adventitious roots per eucalypt shoot [3,17,19–25]. Adventitious roots of some easy-to-propagate woody plants are formed in longitudinal rows along the stem, with the positions being related to the anatomy of the underlying vascular tissue [26]. The anatomy of young shoots has not been studied across the taxonomic range of eucalypts, although a rectangular arrangement of vascular tissue is evident from transverse sections of the shoots of *C. ficifolia*, *C. torelliana* × *C. citriodora*, *E. benthamii*, *E. camaldulensis*, *E. grandis*, *E. preissiana*, *E. grandis* × *E. urophylla* and *E. pellita* × *E. grandis* [5,18,19,27–30].

In this study, we hypothesized that the number of adventitious roots produced by auxin-treated eucalypt shoots is related to the arrangement of vascular tissue in the stem. We examined the anatomy of young stems of 12 species across the taxonomic range of eucalypts, and we observed whether adventitious root formation in four of these species was limited to particular positions such as corners or sides of the vascular tissue.

2. Experimental Section

2.1. Stock Plants and Cuttings

Stock plants of *Angophora costata*, *Corymbia torelliana*, *C. citriodora*, *Eucalyptus tetradonta*, *E. microcorys*, *E. cloeziana*, *E. pilularis*, *E. marginata*, *E. grandis*, *E. camaldulensis*, *E. globulus* and *E. nitens* were raised from commercial seeds (Nindethana Seed Service, Albany, Australia). The seedlings were grown in 2.8-L pots containing the eucalypt seedling mixture described previously [10,11]. The plants were maintained in an Adaptis A1000 growth chamber (Conviron, Winnipeg, Canada) with a photoperiod of 14 h ($\sim 500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ irradiance) at 25 °C and darkness for 10 h at 15 °C. The seedlings were managed as stock plants with multiple orthotropic shoots by pruning at regular intervals to a height of 15–30 cm and a canopy diameter of ~ 20 cm [10,11,31,32].

Shoots from all twelve species were used to examine stem anatomy at the base of cuttings. Shoots from two of the species, *C. citriodora* and *E. grandis*, were harvested and dissected into six consecutive single-node cuttings, with each cutting dissected at 0.5–1.0 cm above a node. The apical cutting was termed “N1”, the next cutting was termed “N2”, and so on, until the most-basal of the six cuttings, *i.e.*, “N6”. Shoots from the remaining ten species were also harvested and dissected into single-node cuttings, but retaining only the fourth cutting from the apex; *i.e.*, “N4”. A sample from the base of each cutting was fixed in 3% glutaraldehyde (in 0.1 M sodium phosphate buffer) under vacuum.

Additional cuttings from two of the species, *C. torelliana* and *E. camaldulensis*, were used to identify sites of adventitious root formation and examine adventitious root development. These species were selected because they are easier to root than many other species in their respective genera [9,33,34]. A small number of *C. citriodora* and *E. grandis* cuttings was also examined to identify sites of root formation. Cuttings from all four species were dipped 0.5 cm into powder containing 8 g·kg⁻¹ indole-3-butyric acid (IBA) for 3 sec, and placed 1 cm deep into a 70 mL propagation tube containing the eucalypt cutting mix described previously [10,11]. Cuttings were harvested after 7, 14, 21, and 28 days, and a sample from the base of each cutting was fixed in 3% glutaraldehyde (as above).

2.2. Microscopy

Fixed samples were rinsed three times in phosphate buffer and processed in a Shandon Excelsior ES Tissue Processor (Thermo Electron Corp., Marietta, OH, USA). The samples were rinsed three times in deionized water, each for 30 min, before being dehydrated through graded solutions of alcohol, cleared in xylene, and infiltrated with wax. Embedded sections were transverse sectioned at 8 µm thickness and transferred onto glass slides. The slides were dipped twice in xylene for 5 min each and in ethanol for 5 min. The slides were then air dried and dipped in 1% safranin for 15 min, rinsed three times in deionized water to remove excess stain, dipped in 0.75% malachite green solution (with 5% acetic acid and 8.5% glycerol) for 15 s, and again rinsed three times in deionized water. The slides were then dried and mounted using Permount™ mounting medium (ProSciTech, Thuringowa, Australia). All sections were examined using an Eclipse E200 microscope (Nikon, Lidcombe, Australia).

3. Results

3.1. Stem Anatomy

The shoots of both *C. citriodora* (Figure 1) and *E. grandis* (Figure 2) contained a central pith surrounded by vascular tissue, cortex and epidermis. The pith was arranged in a four-pointed stellate pattern, which retained its shape as the stems increased in diameter from the apical node (N1) to the most-basal node (N6). The vascular tissue was also arranged in a stellate pattern near the apical node (N1) but it developed a rectangular shape towards its outer perimeter as the stems enlarged radially. Therefore, the stems of *C. citriodora* (Figure 1) and *E. grandis* (Figure 2) became rectangular, or even slightly circular, in transverse section as they became more lignified.

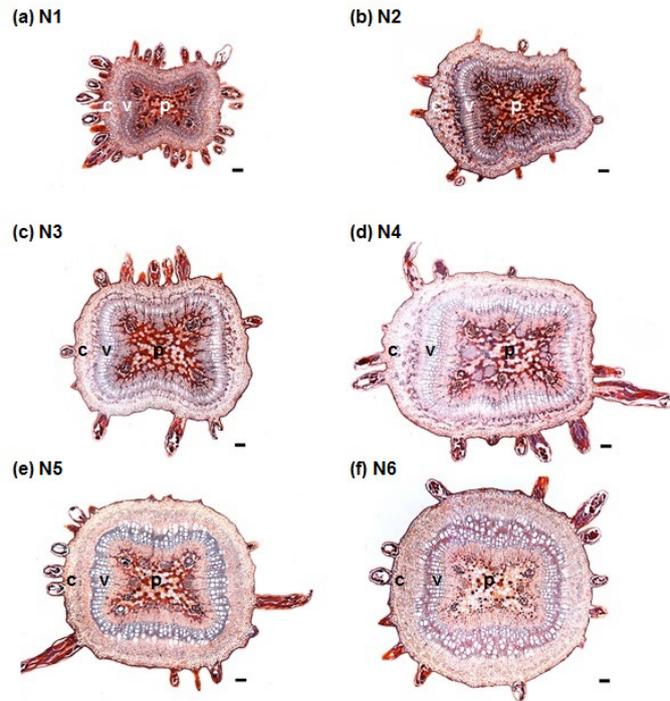


Figure 1. Transverse sections from the stem of cuttings from near node 1 (“N1”: the apical cutting) to node 6 (“N6”: the most basal cutting) of *Corymbia citriodora* stock plants (a–f). c = cortex; v = vascular tissue; p = pith. Scale bars = 100 μ m.

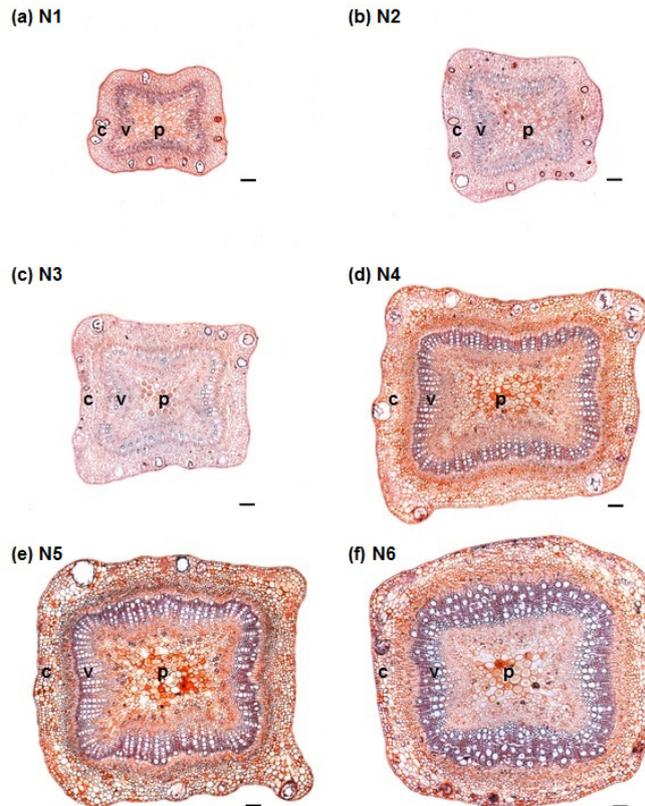


Figure 2. Transverse sections from the stem of cuttings from near node 1 (“N1”: the apical cutting) to node 6 (“N6”: the most basal cutting) of *Eucalyptus grandis* stock plants (a–f). c = cortex; v = vascular tissue; p = pith. Scale bars = 100 μ m.

Stems from across the 12 species of *Angophora*, *Corymbia* and *Eucalyptus* shared a similar arrangement of pith surrounded by vascular tissue, cortex and epidermis, although there were differences in the shape of the individual tissues among species (Figures 3 and 4). The pith near the fourth-most apical node was clearly arranged in a stellate pattern in *A. costata*, *C. torelliana*, *C. citriodora*, *E. cloeziana*, *E. grandis*, *E. camaldulensis*, *E. globulus* and *E. nitens* and it was also arranged in a stellate pattern, though less clearly, in *E. microcorys*, *E. pilularis* and *E. marginata*. The pith of *E. tetradonta* had a rectangular shape in transverse section. The shape of the outer perimeter of the vascular tissue near the fourth node varied from rectangular/slightly stellate in *C. citriodora*, *E. camaldulensis*, *E. globulus* and *E. nitens*, to rectangular in *C. torelliana*, *E. microcorys*, *E. cloeziana*, *E. marginata*, and *E. grandis*, to rectangular/circular in *A. costata*, *E. tetradonta*, and *E. pilularis* (Figures 3 and 4). As a result, the stems of most species were rectangular or circular in transverse section near the fourth-most apical node. However, prominent outgrowth of the cortex, peripheral to the outer points of the pith, meant that the stems of *E. globulus*, *E. nitens* and, to a lesser extent, *E. microcorys* and *E. camaldulensis*, were stellate in cross-section near this node (Figures 3 and 4). Formation of a dark-staining ring of sclerenchyma cells at the periphery of the vascular tissue was evident in *C. citriodora* (Figures 1d and 3c), *C. torelliana* (Figure 3b), *E. tetradonta* (Figure 3d), *E. pilularis* (Figure 4a), and *E. nitens* (Figure 4f).

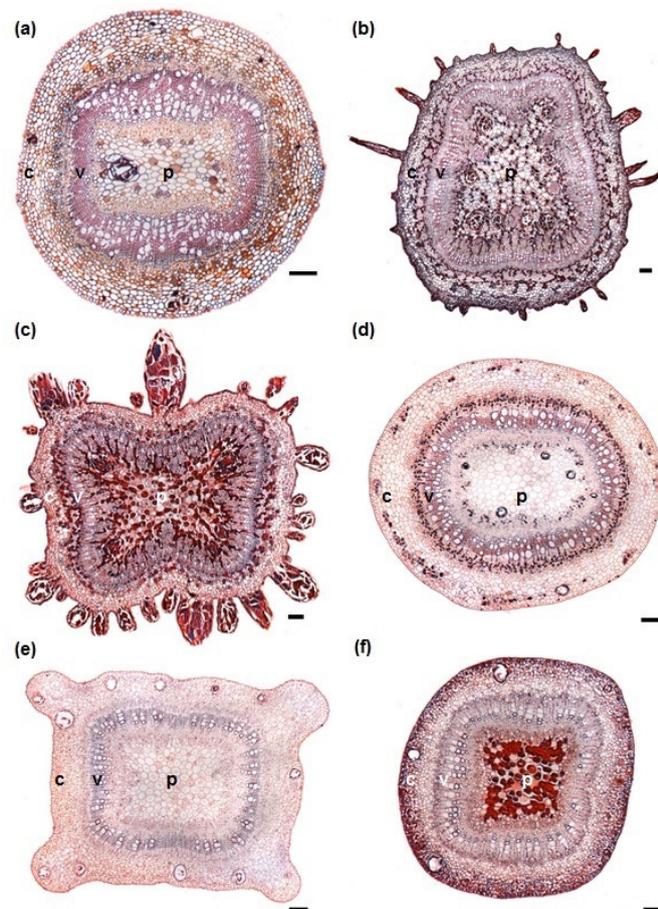


Figure 3. Transverse sections from the stem of cuttings from near the fourth-most apical node (N4) of stock plants of (a) *Angophora costata*, (b) *Corymbia torelliana*, (c) *C. citriodora*, (d) *Eucalyptus tetradonta*, (e) *E. microcorys*, and (f) *E. cloeziana*. c = cortex; v = vascular tissue; p = pith. Scale bars = 100 μ m.

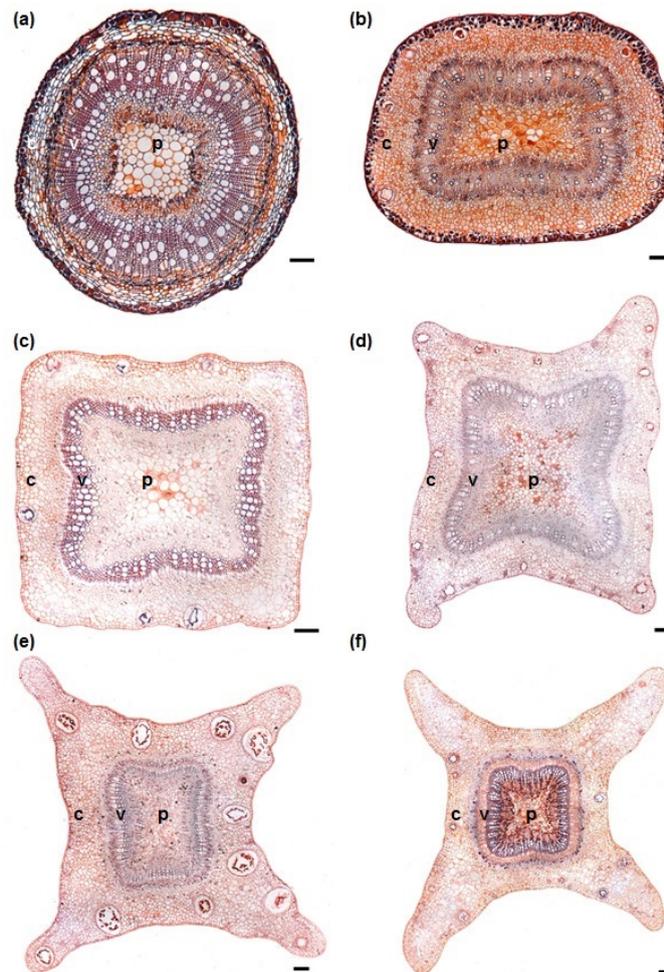


Figure 4. Transverse sections from the stem of cuttings from near the fourth-most apical node (N4) of stock plants of (a) *Eucalyptus pilularis*, (b) *E. marginata*, (c) *E. grandis*, (d) *E. camaldulensis*, (e) *E. globulus*, and (f) *E. nitens*. c = cortex; v = vascular tissue; p = pith. Scale bars = 100 μ m.

3.2. Adventitious Root Formation

Adventitious roots arose within the phloem or vascular cambium in both *C. torelliana* (Figure 5a) and *E. camaldulensis* (Figure 5b). Root formation was direct; *i.e.* in an organized pattern without an intervening period of callus formation [26]. The roots grew through the cortex (Figure 5c,d) and often penetrated the epidermis (Figure 5e,f) during the 28-d period after inserting the cuttings in potting mix. Adventitious roots arose from both the corners (Figure 5a–d,f) and the sides (Figure 5b,d–f) of the rectangular-shaped vascular tissue in auxin-treated cuttings of *C. torelliana*, *C. citriodora*, *E. camaldulensis* and *E. grandis* (Table 1). Of those that arose from the sides, some arose from the middle of the sides (Figure 5b,f) and some arose from positions that were closer to the corners (Figure 5d,f). Roots arose from both corner and side positions within the same stem section of *E. camaldulensis* (Figure 5b,d,f).

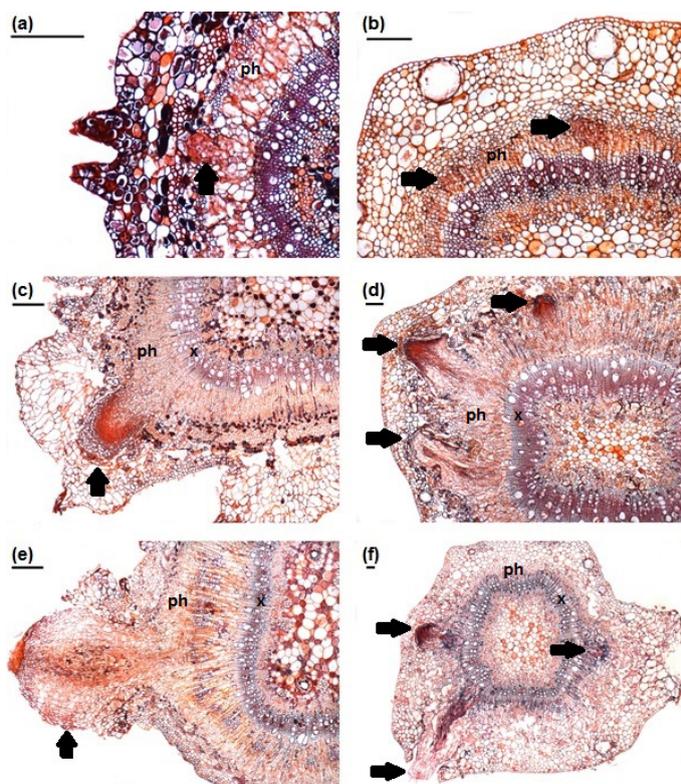


Figure 5. Adventitious root formation and elongation in the base of auxin-treated cuttings from (a,c,e) *Corymbia torelliana* at 21, 14 and 21 days after insertion, respectively, and (b,d,f) *Eucalyptus camaldulensis* at 14, 21 and 28 days after insertion, respectively. Arrows indicate (a,b) root primordia in vascular tissue, (c,d) roots elongating through the cortex, and (e,f) roots elongating through the cortex or emerging through the epidermis. ph = phloem; x = xylem. Scale bars = 100 μ m.

Table 1. Sites of adventitious root formation (corners or sides of the vascular tissue) identified from transverse sections of the stems of auxin-treated eucalypt cuttings.

Species	Total Number of Adventitious Roots	
	Corners	Sides
<i>Corymbia torelliana</i>	5	5
<i>Corymbia citriodora</i>	2	2
<i>Eucalyptus camaldulensis</i>	46	71
<i>Eucalyptus grandis</i>	3	4

4. Discussion

The stems from both *C. citriodora* and *E. grandis* cuttings were stellate in cross-section near the shoot apex but they developed a more rectangular or circular shape as the vascular tissue developed and the stem enlarged radially. This rectangular or circular shape was also evident in *A. costata*, *C. torelliana*, *E. tetradonta*, *E. cloeziana*, *E. pilularis* and *E. marginata* stems at the fourth node below the shoot apex. This shape is similar to previous specimens from the stems of *C. ficifolia* [27], *E. preissiana* [29], *E. grandis* [18], *E. benthamii* [5], *E. pellita* \times *E. grandis* [19], *E. grandis* \times *E. urophylla* [30], and *C. torelliana* \times *C. citriodora* [35]. These species cover the full taxonomic range of the eucalypts from

Angophora and *Corymbia* to the widely diverse *Eucalyptus* subgenera, *Eudesmia* (*E. tetradonta*), *Idiogenes* (*E. cloeziana*), *Eucalyptus* (*E. marginata*, *E. pilularis*, and *E. preissiana*), and *Symphyomyrtus* (*E. benthamii*, *E. grandis*, *E. pellita*, and *E. urophylla*) [36]. A highly-stellate or slightly-stellate stem shape, however, was evident in the fourth-node stems of *E. microcorys*, *E. camaldulensis*, *E. globulus* and *E. nitens*, due to outgrowth of the cortical tissue opposite the outer points of the pith. *E. microcorys* is the sole species in subgenus *Alveolata*, while *E. camaldulensis*, *E. globulus* and *E. nitens* belong to the large subgenus, *Symphyomyrtus* [36]. Slight outgrowth of the cortex is also evident in a previous transverse section from *E. camaldulensis* stems [28].

Eucalypt cuttings are often harvested from either apical or sub-apical positions, or both, on the shoots of stock plants, with the position depending on the stock plant system, the frequency of cutting collection, the species, and the developmental phase of the shoot tips [3,5,7,9,17,37–41]. Eucalypt cuttings, therefore, may have either a four-pointed stellate or rectangular shape in cross-section. Rooting capacity can vary because of maturation- or morphologically-related topographic effects; *i.e.*, positional effects within shoots on the rooting and growth of cuttings [42,43]. These topographic effects can occur at very fine scales in eucalypts. For example, the rooting capacity of cuttings declines greatly from the cotyledonary node to the fifteenth node of *E. grandis* seedlings [18] and rooting capacity of *in vitro* shoots declines greatly from the cotyledonary node to the fifth node of *C. torelliana* × *C. citriodora* seedlings [44]. The rooting capacity of *C. torelliana* × *C. citriodora* cuttings also varies with their position from the shoot apex although, in this case, rooting is correlated inversely with the degree of vascular lignification and sclerification, and so apical cuttings have the highest rooting capacity [35]. These different gradients in rooting capacity of *C. torelliana* × *C. citriodora* seedling nodes may reflect lower levels of lignification and sclerification under *in vitro* conditions than under outdoor nursery conditions [35,44]. Interestingly, we observed a ring of sclerenchyma forming by the fourth node below the shoot apex in a taxonomically diverse range of the eucalypt species—*C. citriodora*, *C. torelliana*, *E. tetradonta*, *E. pilularis*, and *E. nitens*. This level of sclerification might affect the capacity of cuttings from this node to produce adventitious roots.

The precise site of adventitious root initiation was difficult to identify in *C. torelliana* and *E. camaldulensis* cuttings but the location at, or peripheral to, the vascular cambium was consistent with the sites observed recently in *E. benthamii* [5], *E. grandis* [45] and *E. grandis* × *E. urophylla* [30] stems. Importantly, adventitious roots arose from both corner and side positions within the same stem section of auxin-treated *E. camaldulensis* cuttings, as demonstrated recently in cuttings from *E. grandis* × *E. urophylla* that were not treated with auxin [30]. Adventitious roots also appear to form in both the corner and side positions of auxin-treated *C. ficifolia* shoots [27], and in both the corner and side positions of *E. pellita* × *E. grandis* cuttings that were not treated with auxin [19]. Therefore, we reject the study hypothesis that adventitious root formation in eucalypt cuttings is limited to one particular position—corners or sides—of the vascular tissue.

Many untreated eucalypt shoots produce just one or two adventitious roots [16,18–20,37,44–46]. Optimally-treated eucalypt shoots often produce a mean of about four adventitious roots but individual shoots can produce higher root numbers [3,4,9,17,19–24,47,48]. These roots could have emerged along more than four radii at the same level within the shoot. It is possible that adventitious root formation in untreated eucalypt shoots could be more-confined to particular positions of the vascular tissue, with auxin treatment, to some extent, overriding this positional regulation. None the less, this study has

highlighted that auxin-treated eucalypt shoots are capable of forming adventitious roots at many positions around the rectangular vascular tissue, and so stock plant management and propagation treatments can aim to produce at least four, and preferably more, adventitious roots per rooted cutting. Higher numbers of adventitious roots could contribute greatly to the root system symmetry, wind-firmness, survival and trunk volume of growing eucalypt trees.

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Author Contributions

Philippa H. Bryant raised the stock plants, propagated cuttings, performed the microscopy, and contributed to the study design and writing. Stephen J. Trueman designed the study, assisted with microscopy, and wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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