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Optimizing the size of root cutting in *Melia volkensii* Gürke for improving clonal propagation and production of quality planting stock

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Melia volkensii is a tree species growing in semi-arid regions of East Africa, and is recognized as one of the most valuable forestry species in such regions of Kenya. The use of root cutting is an easy method for its clonal propagation, but the most appropriate conditions have not been systematically examined. In this study, the relationship between diameter of root cut edge or fresh weight and formation frequency of adventitious bud were assessed. In addition, roots were divided into two fragments, and formation frequency of adventitious bud was compared between cross-sections roots without root tip and roots with root tip. Both the diameter of the cut edge and fresh weight of roots forming adventitious buds were significantly higher than in those not forming them (unpaired _t_-test, p<0.01). Formation frequency of adventitious bud was 77.0% in roots satisfying the criteria of cut edges diameter >15 mm and fresh weight >20 g, but it decreased (37.2%) in roots that did not meet this criteria. Based on anatomical analysis, an effect of the developmental stage of root fragments on adventitious bud formation was suggested, and root size is thought to reflect the stage of root development. The formation frequency of adventitious buds was 72% even in cross-sectioned roots, and the proportion was not significantly different from that of normal root tip (p>0.05). Therefore, it is concluded that root cutting using cross-sectioned roots are also available for clonal propagation.

Key words: Clonal forestry, anatomical analysis of root, availability of cross-sectioned roots.

INTRODUCTION

Melia volkensii Gürke is a deciduous, broad-leaved monoecious tree species distributed in semi-arid regions

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of east Africa (Orwa et al., 2009). The tree is recognized as one of the most valuable species for agroforestry purposes in the area because of features such as its drought tolerance, fast growth, high wood quality and ant resistance (Kariuki et al., 1990; Mulanda et al., 2015). Plus trees have been selected in Kenya, and improvement for drought tolerance and growth rate are being attempted through a breeding program (Muchiri and Mulatya, 2004; Luvanda et al., 2015). Superior varieties will be developed in the near futures, and the availability of clonal propagation protocol has important implications in the distribution of these varieties. This is because cloned trees maintain the genetic makeup of their mother trees, and the use of clones with homogeneous and desirable traits will improve productivity in forestry and agroforestry (Park et al., 1998; McKey et al., 2009). It is known that succession rate of stem cutting is not necessarily high (up to 33%) in *M. volkensii* although the reason is unclear (Indieka and Odee, 2004). Grafting is generally used as the clonal propagation method in *M. volkensii*. However, the method requires an investment in time and effort to prepare the plant materials, and a practiced technique and dedicated tools are also required. Therefore, other easy and cost-effective method for clonal propagation is needed for *M. volkensii*.

Root cutting is one of the general clonal propagation method, and it's use have been reported in various species such as *Populus tremuloides* (Schier, 1974; Snedden et al., 2010), *Populus tremula*×*Populus tremuloides* (Stenvall et al., 2004), *Paulownia tomentosa*, *Paulownia fortunei* (Ede et al., 1997), *Prunus avium* (Ghani and Cahalan, 1991) and *Detarium microcarpum* (Ky-Dembele et al., 2010). These studies have identified the effects of some factors on the succession of root cutting. For example, Ede et al. (1997) reported the importance of root size (whether > 5 mm of diameter of root cut edge or not) and the position where the root was obtained. Ky-Dembele et al. (2010) also reported significant effect of root size (> 10 cm of length and 15 mm of diameter) and no effect of root alignment on succession of root cutting. These studies indicate that optimum condition for root cutting should be considered in accordance with root configuration or other attributes of the target species.

A high ability to produce root suckers in *M. volkensii* is associated with mechanical damage to the root (Stewart and Blomley, 1994), and viability of root cutting was also reported by Mulatya et al. (2002). In addition, *M. volkensii* develop thick, deep roots (Mulatya et al., 2002) and therefore, clonal propagation using the abundant root resources is feasible. However, essential conditions for successful root cutting have not been systematically tested in *M. volkensii*, but very important knowledge for efficient clonal propagation. The present study evaluated: 1) the relationship between root size and formation of adventitious buds, 2) the anatomical differences between roots on which adventitious buds formed or did not form, to understand the anatomical factor needed for successful root cutting, and 3) the usefulness of cross-sectioned roots without root tips for enhancing the clonal propagation efficiency of *M. volkensii*.

MATERIALS AND METHODS

Experiment 1: Effect of root size on efficiency of adventitious bud formation

The relationship between root size (diameter of cut edge and fresh weight) on the efficiency of adventitious bud production was examined. Plant materials were potted one-year-old juvenile trees of *M. volkensii*, derived from open-pollinated seeds collected in Kenya. The plants were grown in pots in temperature-controlled greenhouse with natural light in Hitachi, Japan (36.69N, 140.69E). One to four root tips of about 8 to 16 cm length were cut from each of the 48 juvenile trees with tree height of about 40 cm (Figure 1a). The diameter of the cut edge (average of two sides) and fresh weight were measured by caliper and electronic balance, respectively. Root length was not measured in this study because it was difficult to measure curved root fragments (Figure 1a). In total, 94 roots were randomly selected, and these roots were planted in polypots (diameter and height are 10 and 21 cm, respectively) with mixed soil (Yabashi et al., 1992; Kanuma soil: leaf mold = 1:2 volume ratio). Note that, about 0 to 5 mm of proximal end was exposed above ground (Figure 1b). They were kept in the above mentioned greenhouse, watered every two to three days until the 60th day after planting, and then the presence of adventitious buds was observed. Temperature and humidity in greenhouse during the experiment period were 24.9±3.5°C (mean±SD) and 75.2±17%, respectively. The difference in root size for roots with and without adventitious buds was tested by unpaired *t*-test using the *t test* function in R software (R Development Core Team 2015).

Experiment 2: Attempt at promotion of propagation

The suitability of cross-sectioned roots for improving the efficiency of propagation was tested. Roots were cut from four *M. volkensii* trees with diameter at breast height of about 3 cm that had been grown in the greenhouse (potted 3-year-old trees with height controlled at about 2 m). The roots were subjected to two kinds of treatments, that is, cross-section of roots without root tips (hereafter called C-S roots) and roots with root tips (hereafter called RT roots) were used for the experiments. The length of both kinds of roots was about 5 to 10 cm. In total, 22 C-S and 17 RT roots were used for the experiment. Environmental condition and subsequent procedures until observation of adventitious buds were the same as experiment 1. When C-S roots were planted, distal end was always buried in soil. If proximal end was buried in soil, adventitious bud would be formed in the soil as was found in the preliminary studies (Figure 2).

Testing for the difference in the proportion of roots forming adventitious bud in C-S and RT roots was carried out by *prop.test* function in R software (R Development Core Team, 2015).

Anatomical observation of adventitious bud formation

To reveal difference between adventitious buds formed and not formed roots, anatomical observation was carried out by frozen

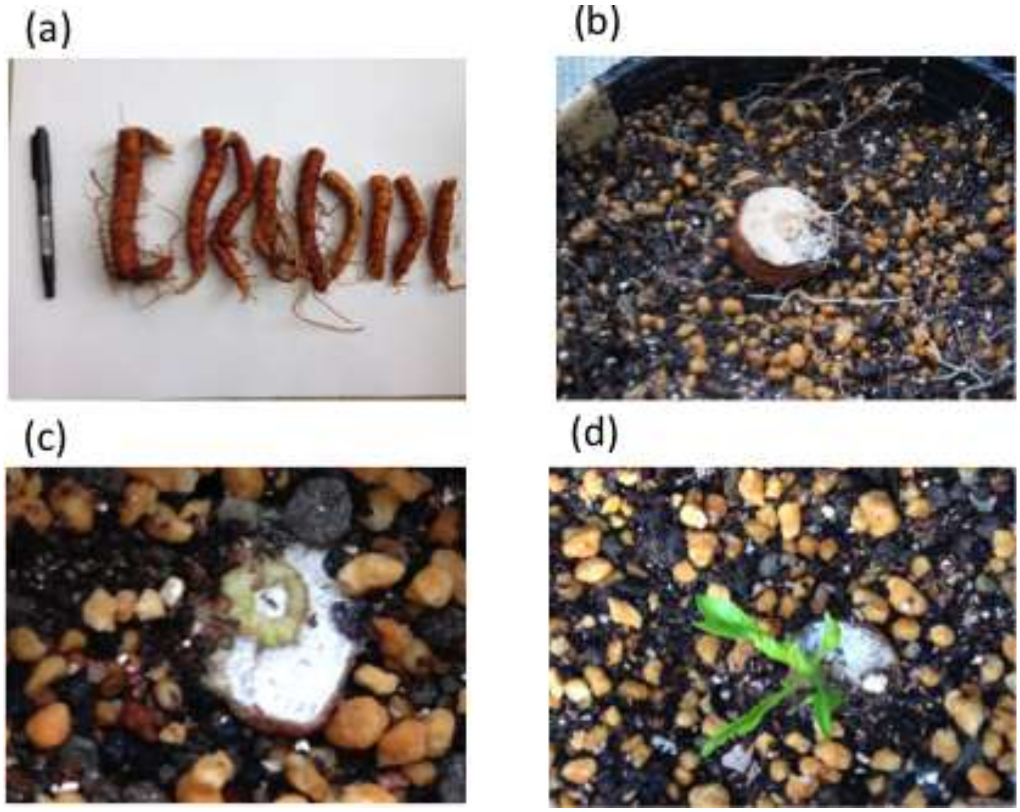

Figure 1. Representative root fragments used for experiment 1 (a), planted root fragments; (b), callus on root cut edge; (c) adventitious buds on callus (d).

section technique (Kawamoto, 2003). Three samples were selected from roots both freshly forming and not forming adventitious buds. The roots were placed in a stainless steel container filled with SCEM-L1 embedding gel (Section-lab Co. Ltd., Japan) and frozen in cooled n-hexane by liquid nitrogen. The frozen blocks were removed from the container, and sectioned at a thickness of 10 µm using a CM3050S cryomicrotome (Leica Microsystems, Germany). The sections were stained with 0.5% toluidine blue or iodine-potassium iodide solution. The stained sections were observed under a DMRXE light microscope (Leica Microsystems, Germany) and photographed by a digital camera. The diameters of cut edges and secondary xylems were measured by a digital caliper. Regarding projected area of starch in secondary xylem or secondary phloem and cortex, two to five photos (size: 2048 × 1536 µm) of the sections stained with iodine-potassium iodide solution were randomly taken in each sample, and the photos converted to binary images to cover areas of starch grains using the software ImageJ 1.48v. The rates of starch (black) area were also calculated by the measure command of ImageJ software.

RESULTS

Effect of root size on efficiency of adventitious bud formation

Green, nodular and compact callus formation was observed within two to four weeks on most roots eventually forming adventitious buds (Figure 1c); adventitious buds developed from the callus (Figure 1d). The relationship among diameter of root cut edge, fresh weight and existence of adventitious buds is shown in Figure 3. Adventitious buds were observed in 50 out of 94 roots (53.2%).

Both the diameter of the cut edge and fresh weight were significantly different between roots that formed adventitious buds and those that did not form them ($p < 0.01$), and roots that formed adventitious buds were larger than those that did not form them (Figure 4).

Effect of sectioning of root cutting on adventitious bud formation

Adventitious buds were observed in 16 out of 22 (72.7%) C-S roots and 16 out of 17 (94.1%) RT roots. The relationship between the diameter of root cut edge, fresh weight and existence of adventitious buds is shown in Figure 5. The proportion of roots forming adventitious buds was not significantly different between C-S and RT roots ($p > 0.05$).

Figure 2. An example of root with adventitious bud produced in soil and grown.

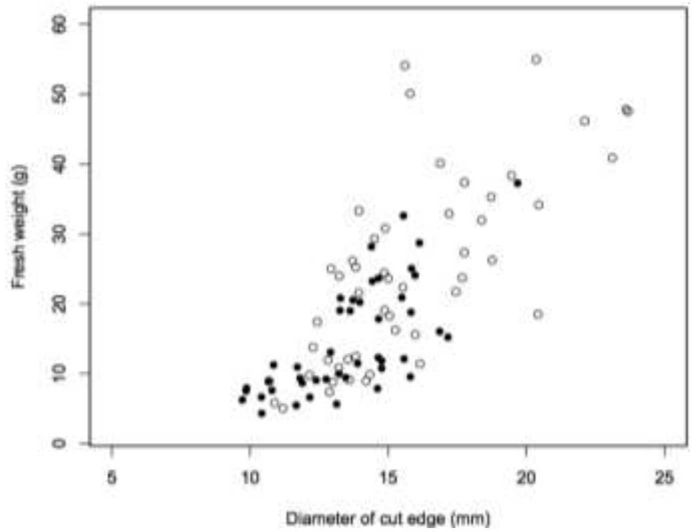

Figure 3. Relationships between diameter of root cut edge, fresh weight and adventitious bud formation in experiment 1. Open and filled circles indicate roots forming and not forming adventitious buds, respectively.

Anatomical observation of adventitious buds

Secondary xylem was observed in the core of roots (Figure 6a). The diameter of secondary xylem was larger in roots forming adventitious buds (4.0, 7.3, and 7.7 mm) than in those that did not form adventitious buds (2.6, 2.8 and 3.5 mm) (Table 1). Secondary xylem and vascular cambium developed in all cut roots forming adventitious buds (Figure 6b). On the other hand, interfascicular cambium and vascular cambium were not detected in roots that did not form adventitious buds (Figure 6c). Starch grains were more abundant in the cortex, secondary phloem (Figure 6d) and secondary xylem (Figure 6f) of roots that formed adventitious buds (Figure 6d, f and Table 1). Most vessels were occluded in roots that did not form buds (comparison between Figure 6h and i).

DISCUSSION

Effect of root size on efficiency of adventitious bud formation

In experiment 1, the frequency of formation of adventitious buds was higher in larger roots; both the diameter of the cut edge and fresh weight of roots forming adventitious buds were significantly higher than in those that did not form them (Figure 4). Therefore, root size (volume) is thought to be important for the success of root cutting in *M. volkensii*. For practical purposes, a cut edge larger than 15 mm and fresh weight greater than 20 g will be criteria to follow because the frequency of formation of adventitious buds was 77.0% under these conditions. On the other hand, it decreased to 37.2% in roots that did not meet these criteria.

Possible factors affecting adventitious bud formation in root cutting of *M. voleksnii*

The effect of root size on the formation frequency of adventitious buds has been reported in some species [for example, *Actinidia chinen*sis (Lawes and Sim 1980), *Prunus avium* (Ghani and Cahalan, 1991), *Detarium microcarpum* (Ky-Dembele et al., 2010)], and the importance of nutritive conditions correspond to root size in some of them. In *M. volkensii*, starch content tended to be high in roots forming adventitious buds (Figure 6d-g and Table 1). However, it varied in roots that did not form adventitious buds. In populus, it is reported that

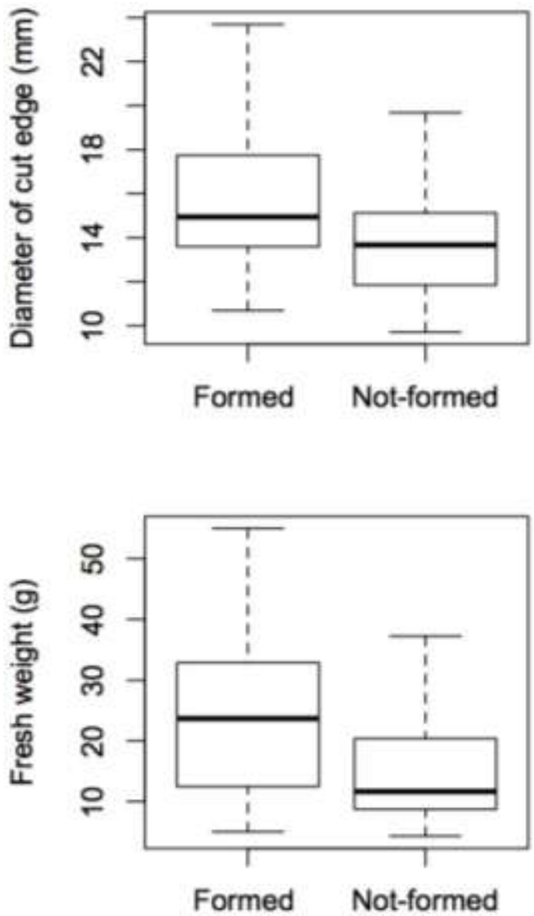

Figure 4. Differences in size of root cut edge (a) and fresh weight (b) between roots forming and not forming adventitious buds.

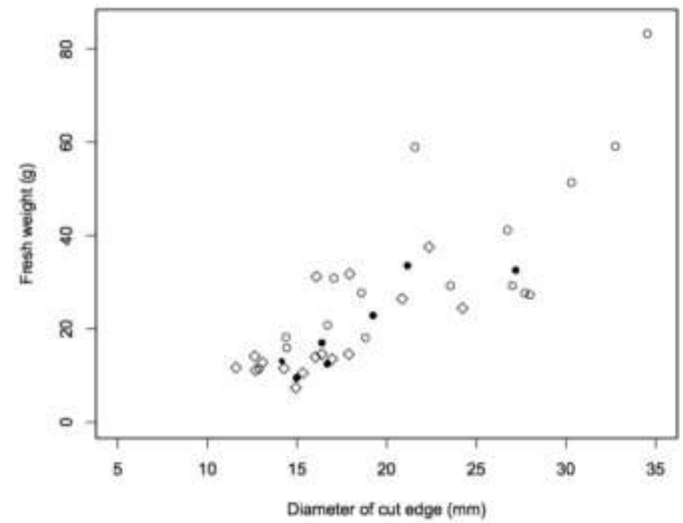

Figure 5. Relationship between diameter of root cut edge, fresh weight and adventitious bud formation in experiment 2. Open and filled circles indicate C-S roots forming and not forming adventitious buds, respectively. Open and filled diamonds indicate RT roots forming and not forming adventitious buds, respectively.

carbohydrate is not necessarily a determining factor for successful root cutting, and the importance of hormones or unknown substances in roots was reported (Snedden et al., 2010). A similar phenomenon is thought to be applicable to *M. volkensii*, and starch accumulation may be a factor, though not a major one, for successful root cutting.

Three samples were selected for anatomical observation of roots both forming and not forming adventitious buds. Callus and adventitious buds usually formed around the cambium between the secondary xylem and cortical layer in *M. volkensii,* and the vascular cambium could be clearly distinguished in roots forming adventitious buds (Figure 6b). This means that mature roots in secondary growth stage might be easy to form adventitious bud. On the other hand, the vascular cambium was indistinct in roots not forming adventitious buds (Figure 6c). The average diameter of the secondary xylem was 2.4 mm in roots that did not form buds (n=3), which was smaller than that of roots that formed adventitious buds (5.7 mm, n=3). Therefore, roots that did not form adventitious buds are thought to be transition stage from primary growth to secondary growth, which might affect the efficiency of adventitious bud formation. This study findings on more bud induction in larger roots indicated that root size might reflect the effect of the developmental stage of the root. In addition, it was observed that the vessels in one of the roots that did not form adventitious buds appeared to be filled by tylose or a similar substance, an indication of infection, water stress and/or senescence (Phillips et al., 1987; Neuhaus et al., 2007). However, only a limited number of samples could be observed in this study, and therefore, the relationship between root size, developmental stage and the resulting efficiency of formation of adventitious buds should be observed concurrently using more abundant samples in future studies.

The effect of some factors other than root size has also been reported in other species. For example, the effect of clonal variation (Stenvall et al., 2004) or temporal variation on efficiency of root cutting has also been reported (Snedden et al., 2010). In this study, many unknown tree families were used in experiment 1, and therefore, clonal variation could not be considered as part of the experiment. On the other hand, the efficiency of adventitious bud formation was confirmed in each family in experiment 2, and all families showed a high rate of formation of adventitious buds (80.0, 66.7, 80.0 and100% for the four mothers). Therefore, clonal variation was not observed, but a high tendency for root suckering in *M. volkensii* was confirmed. There may also be phenological

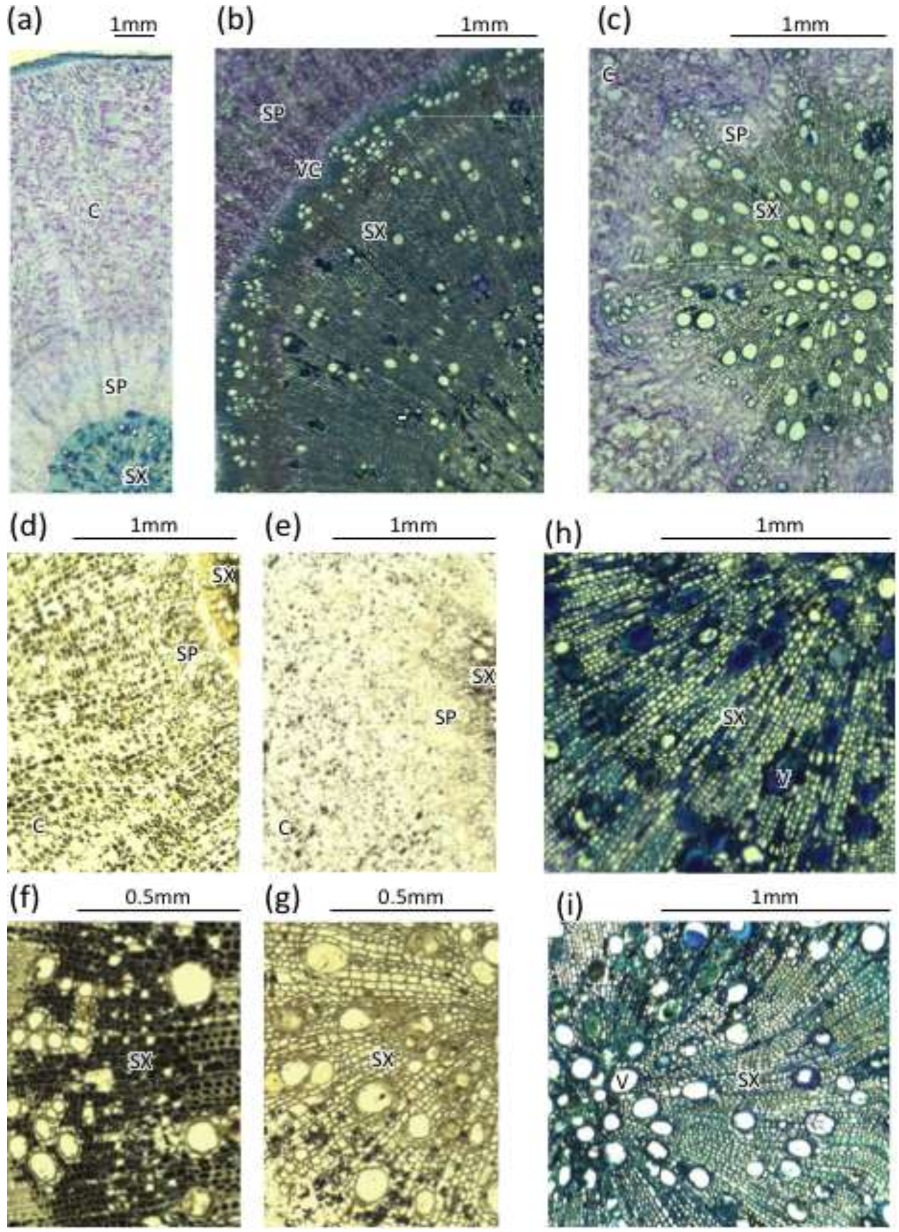

Figure 6. Representative cross-section of roots. (a) Vascular cambium in a root forming adventitious buds; (b), undeveloped vascular cambium from a root not forming adventitious buds; (c), starch (dark granules) distribution in secondary phloem and cortex of a root forming adventitious buds; (d), secondary phloem and cortex of a root not forming adventitious buds (e); secondary xylem of a root forming adventitious buds (f); (g), secondary xylem of a root not forming adventitious bud; (h), vessel embolism in a root not forming adventitious buds; (i), open vessels in a root forming adventitious buds. (C) cortex; (SP) secondary phloem; (SX) secondary xylem; (VC) vascular cambium; (V) vessel are also shown in each figure.

effects: *M. volkensii* grows in east Africa, and clear rainy and dry seasons alternate in the region. Therefore, hormones and metabolic resources in roots will be different in different seasons of root collection. Unfortunately, source trees were grown in a greenhouse with controlled temperatures and watering conditions, and therefore, other conditions could not be tested in this study.

Table 1. Results of anatomical observation

Sample	Formation of *adventitious* bud	Diameter of cut edge (mm)	Diameter of secondary xylem (mm)	Projected area of starch in secondary phloem and cortical layer (%)	Projected area of secondary xylem (%)
1	Yes	22.7	7.7	32.1	30.6
2	Yes	21.2	4.0	37.7	47.0
3	Yes	19.2	7.3	15.7	35.7
4	No	16.5	2.6	27.2	13.9
5	No	14.3	3.5	14.9	5.0
6	No	13.7	2.8	8.5	20.5

Viability of cross-sectioned root for root cutting

In experiment 2, most roots used in this second experiment satisfied the criteria for success root cuttings, that is, >15 mm cut edge and >20 g fresh weight (Figure 5), and formation frequency of adventitious bud were 74.4% in C-S root and 94.1% in RT root. Although the frequency was slightly lower in C-S roots, the values were not significantly different ($p > 0.05$). Therefore, efficient propagation will be achieved by using both C-S and RT roots.

Conclusion

From this study, the importance of root size for successful root cuttings in *M. volkensii* is concluded. Anatomical analysis indicated that root size roughly reflects the developing stage of root, and larger root (> 15 mm of diameter and > 20 g of fresh weight) are recommended for root cutting. Both parameters will be effective in inferring the developing stage of roots because significant correlation between root cut edges and fresh weight was observed (experiment 1, $r^2 = 0.58$, $p < 0.01$). However, diameter of cut edge could be more convenient visual parameter, and therefore, root cut edge will be more convenient parameter for practical utility.

Conflict of Interests

The authors have not declared any conflict of interests.

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