**Title**:

**Propagation of an *Eucalyptus globulus* genotype by rooted cuttings**

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rooting ability, seasonal variation, auxin treatment, d, ***N ,N′-bis-(3,4-methylenedioxyphenyl)urea (3,4-MDPU),***  within shoot position,

**Abstract**

*Eucalyptus spp* is a genus regrouping a large number of diverse species planted for ornamental and landscaping uses as well as for timber and pulpwood production mainly from clonal plantations. However, in contrast to other species, some like *E. globulus* are difficult to mass propagate clonally by rooted cuttings due to a low capacity for adventitious rooting .

The influence of cutting within shoot position, rooting period, as well as the type and the concentration of exogenous auxin used on rooting ability of cuttings was assessed on an *E. globulus* genotype .

The experimental results showed that higher rooting rate ( ) was observed for cuttings derived from the seventh internode and during e summer season (mid-July) . The lower rooting scores capacities observed during winter (mid-November and February) can be improved by heating the soil with an electric cable. 0.8 % of indole butyric acid (IBA) was the more rooting effective of the various auxin treatments tested , while combined application of IBA and of diphenylurea derivative, *N,N′*-*bis*-(3,4-methylenedioxyphenyl)urea, increased rooting efficiency (63%) and survival rate (83%) of the cuttings compared to the application of IBA alone (47% and 53%, respectively).

**Introduction**

The genus *Eucalyptus*, which belongs to the family of Myrtaceae, is native to Australia and includes more than 700 species. *Eucalyptus* species are one of the most widely planted dicotyledonous trees in the tropical and subtropical regions including South America, Oceania, and South East Asia because of its superior growth rate and wood characteristics (. The wood of *Eucalyptus* trees is used for firewood, charcoal, flooring, furniture, and pulp, and the essential oil extracted from the leaves is used as a source of perfume, medicines, and detergents . They also have been used as ornamental and landscaping plants . *Eucalyptus* trees are currently planted in many regions of the world,

In addition to natural propagation by seeds,, *Eucalyptus* can also be e propagated by rooted cuttings . Efficiency of propagation of *Eucalyptus* clones by cutting differs between and within species according to genetic and physiological influences ( . Thus, the rooting capacity of cuttings in *E. grandis* and *E. urograndis* (*E. urophylla* x *E. grandis*) is relatively higher than that of other *Eucalyptus* species (Denison and Kietzka 1993, Souvannavong 1992). In contrast, *E. globulus* is known to be difficult to propagate clonally because of the lower rooting ability of its cuttings (Borralho and Wilson 1994, Hartney 1980, Ito 2006, Ruaud et al. 1999).

Previous studies onh eucalyptus and other tree? species have pointed out the relationship between rooting ability and various cuttings characteristics, such as the size and maturity of leaves (Kawase 1972, Wilson 1994), growth activity (Fadl and Hartmann 1967, Howard 1968), proximity of nodes to the base of the cutting (Hansen 1986), exo- and/or endogenous levels of phytohormones (Fogaça and Fett-Neto 2005) and growth conditions of the cuttings and their donor trees (Mankessi et al. 2011, Marques et al. 1999, Schwambach et al. 2008). Cutting rooting ability varies also with stem size and original portion of the donor plant used (Howard 1991, Howard and Ridout 1991, Mankessi et al. 2011, Paton et al. 1970, Sach et al. 1988, Wilson 1993). There is a gradient in rooting ability that is dependent on the within-shoot position (Mankessi et al. 2011, Wilson 1993). In some eucalypt species as well as in most of other tree species, rooting ability is higher for cuttings removed from the juvenile parts than from the mature parts of the original plant from which they are coming (Paton et al. 1970, Wilson 1993). Furthermore, it has been observed that rooting efficiency varies with the season during which the cuttings are collected from the donor tree (Anand and Heberlein 1975, Bhusal 2001).

vVegetative propagation of *Eucalyptus* species by rooted cuttings has been already substantially investigated (ref,). , desirable conditions of propagation by rooted cuttings for difficult-rooting species were not fully clarified. To investigate the factors, in depth, that may influence rooting ability, the relationships between internode positions within shoots and seasonal variation and rooting efficiency of the cutting using a clone of *E. globulus* as a model for the difficult-rooting *Eucalyptus*. We believe that our present data provide some insight into process improvement for vegetative propagation of ornamental *Eucalyptus* species with lower rooting ability.

**Materials and methods**

***Growth conditions of donor trees***

All experiments were carried out in Tsukuba, Japan (36°09′N; 140°02′E) between 2010 and 2011. Annual mean temperatures were 14.8 degrees C and 14.3 degrees C, , with maximum (28.2 and 26.1 degrees C)e recorded in August and minimum (3.6 and 2.1 degrees C) in January for years 2010 and 2011, respectively.. 1 to 2-year-old *E. globulus* rooted cuttings derived from a cultivated mother tree (clone SI-10) were used as stock plants from which the cuttings were collected. These stock plants were grown individually in 24-liter containers. Trees were cultivated in an open-air field from spring to autumn and in a greenhouse during the winter. The soil used was a mixture of peat moss, vermiculite, akadama soil, and black soil (2:1:1:8, v/v).

***Preparation of cuttings for rooting***

Figure 1 summarizes the cutting preparation procedure. Cuttings with 1 node (about 3-4 cm) were prepared from main stems (Fig. 1A) of the donor trees?,, grown as described above (Fig. 1B). The leaves on each cutting were trimmed with scissors to approximately one-third of the total area to avoid wilting (Fig. 1C). A 2-mm-wide section of the cortex base of each cutting was scraped off with a knife to expose the cambium (Fig. 1D). After the cuttings were immersed for 10 seconds in a solution of benomyl fungicide (3 g/L, Sumitomo Chemical Company, Ltd., Tokyo) (Fig. 1E), the base of each cutting was treated with talc powder containing auxin (Fig. 1F), and cuttings were planted singly in an artificial medium made of coconut husk (Jiffy plugs®, Jiffy Pot Products Co. of Japan Ltd., Yokohama) (Fig. 1G). The propagation medium was placed in plastic containers (90 cm3) and filled with peat moss and vermiculite (1:1, v/v) (Fig. 1H). During experiments, cuttings were cultivated in a greenhouse without any artificial equipment for managements of relative humidity and temperature. Emergence of roots on cuttings was checked 8 weeks after the start of the experiment. The temperature in the greenhouse averaged 28°C in the summer and 13°C in the winter . Day length was approximately 12 h during the summer and 10 h during the winter season.

Fig.1

***within-shoot position experiments***

Experiments were performed during the summer (between July 24 and September 18, 2010) and spring (between March 16 to May 10, 2011) seasons. The cuttings prepared in the first experiment were designated as summer cuttings and the second as spring cuttings. Rooting ability was investigated using cuttings (about 3-4 cm) with a node prepared from different within-shoot positions. The average length of donor shoot was 66.8 cm in summer and 36.7 cm in spring. The internodes were defined as the first to thirteenth internodes from top to base of the shoot. Since the first to third internodes were generally short and soft, cuttings were prepared from the fourth to the thirteenth internodes.

Eight cuttings per each internode position were prepared from harvested shoots of clone SI-10. Three replications with 8 cuttings each were performed for a single experimental condition (24 cuttings in total for each condition). The basal parts of the cuttings were treated with talc powder containing 0.8% (w/w) indole-3-butyric acid (IBA, Wako Pure Chemical Industries, Ltd., Osaka). The powder was originally prepared by mix with talc and IBA (Wako Pure Chemical Industries).

***Seasonal variations experiments***A total of 10 experiments (set “A” to “J” in Table 1) were performed between July 27, 2010 and September 6, 2011. To examine the effect of soil temperature on the rooting ability of cuttings, an electric heating cable (1-250, Nihon Noden, Inc., Tokyo) with thermostat (ND-610, Nihon Noden, Inc., Tokyo) set at 50°C was used to heat the propagation medium between November 19, 2010 and January 7, 2011 (set F) and February 7 and April 3, 2011(set H). The control experiments for them were also performed without soil heating (sets E and G). Three replications of twenty-five cuttings each were performed in set A to J (75 cuttings in total were used for each experiment). The basal part of the cuttings was treated with talc powder containing 0.8% IBA as described above. The sensor of the data logger (TR-52S, T&D Co., Nagano) was buried in the soil at a depth of 5 cm. Maximum, minimum, and average soil temperatures were measured during each experimental period (See Table 1).

***Auxin treatment experiments***

The experiment was performed between March 28 and May 23, 2011. Auxin types used in this experiment were indole-3-butyric acid (IBA), naphthalene acetic acid (NAA, Junsei Chemical Co., Ltd., Tokyo), and indole acetic acid (IAA, Tokyo Chemical Industry Co., Ltd., Tokyo). The basal part of each cutting was treated with talc powder containing 0.2, 0.8, 1.6 and 3.2% of each auxin. The powders were originally prepared by mix with talc and each experimental-grade auxin. A control power without auxin was also included. Four replications with 25 cuttings each were conducted for thirteen different conditions of the experiment listed in Table 2. To estimate the root-promoting effects of these auxins, the number of roots, length of the longest root and the percentage of survival and rooting were examined.

***Combined application of auxin and N,N′-bis-(3,4-methylenedioxyphenyl)urea (3,4-MDPU)***

While 3,4-MDPU is a diphenylurea derivative with no hormonal activity, it can promote rooting when applied together with auxins (Ricci et al. 2001, Ricci et al. 2008). The experiment was performed between August 3 and September 28, 2011. The basal part of each cutting was treated with talc powder containing 0.8% IBA in addition to different levels (0.05, 0.25, 0.50, 0.75, 1.0 and 1.5%) of *N,N′*-*bis*-(3,4-methylenedioxyphenyl)urea(3,4-MDPU), synthesized according to the literature (RICCI et al. 2001). Three replications with twenty-five cuttings each (75 cuttings in total) at each concentration of 3,4-MDPU were performed (Table 3).

***Statistical analyses***

Except for temperature, all data are reported as means ± standard deviation of 3 or 4 replicates. The survival and rooting percentages, number of roots, and length of the longest root were analyzed by one-way analysis of variance (ANOVA) using KaleidaGraph 4.1 (Hulinks Inc., 2009, Tokyo). Bonferoni test was used to differentiate means of survival and rooting percentages. Tukey’s HSD test was used to compare means of number of roots, and length of the longest root. The significant level of statistical tests was set at *p* = 0.05.

**Results**

***within-shoot position influence***

Rooting ability of the cuttings varied according to their within shootposition particularly in summer and to lesser extent in spring (Figure2). For both seasons, cuttings derived from the seventh internode rooted better (83% in summer and (58% in spring than those from the other positions , the rooting scores increasing progressively from internode 4 to 7, then and decreasing gradually from internode 7 to lower internodes .. However, rooting percentages of the spring cuttings were relatively lower (29%–58%) than those from summer cuttings. The lower capabilities of the rooting in spring might be derived, in part at least, from physiological activity of donor trees in this season. Higher average temperature (26.8 degree C) in the summer experiment might contribute to increase biological activity of the donor tree compared to in the spring (the average temperature was 11.1 degree C).

Fig.2

***Seasonal variations influence***

Experiments were performed in 10 different periods throughout the year (A–J, Table 1). Although seventh internode was predicted to be the best donor for cuttings as indicated above, number of the donor tree for this experiment was restricted. Thus, we prepared cuttings from the fifth to ninth internodes of donor trees. Table 1 shows the survival and rooting percentages of cuttings, as well as the maximum, minimum, and average soil temperature, for each experimental period. Rooting percentages were overall relatively higher in summer than in other seasons (61% and 56% for rooting periods A and J respectively). In autumn (sets B and C), rooting percentages decreased gradually with decreasing soil temperature. From autumn to winter (sets D, E, and G), rooting percentages further decreased to less than 35%, although survival percentage was maintained at over 75%. When the soil was heated (sets F and H), rooting percentages of the cuttings almost doubled (sets E and G). Increasing the soil temperature during winter appears to promote rooting percentage in cuttings. Overall, *E. globulus* stem cuttings rooted better in summer (,) then in spring ( ) followed by autumn () and winter ( )..

Table1

***Auxin and 3,4-MDPU treatment influence***

Rooting efficiency of cutting could be improved by application of three auxins as indicated in Table 2. Although higher concentration of auxin applications is likely to exhibit negative effect on survival of cuttings, among the auxins tested, IBA was most effective to increase the rooting percentage of the cuttings. The rooting percentage under 0.8% IBA application was over 5 times higher than that without auxin. Root length was also increased at 0.8% IBA application, but not effective to number of developing roots. At the highest level of IBA application (3.2%), the survival percentage decreased drastically to 38%, which was the minimum value measured in this experiment.

Table2

NAA and IAA have also promoting effect of rooting ability as IBA. Rooting percentages were relatively higher with applications of 1.6% and 3.2% of NAA or IAA. However, their efficiencies were relatively lower than that of the best concentration of IBA. As found with IBA, survival efficiency decreased at higher concentrations of NAA and IAA. Among the auxins tested, a high concentration of IAA was the most effective in increasing of the number of roots induced.

***Supplemental effect of 3,4-MDPU on rooting***

Table3

Treatment of cuttings with auxin-containing talc powder with 3,4-MDPU has increased survival efficiency of cuttings during the experiment (Table 3). Survival percentage of the cutting under the application of 3,4-MDPU at 0.5 exhibited over 1.6 times higher than that without 3,4-MDPU. This chemical could also improve rooting percentage at 0.5% application, but the effect was not significant. Although the application of 3,4-MDPU might not be expect to increase rooting percentage itself, the application has certain effect to improve total productivity of rooted cuttings because of its positive effect to survival of cuttings during root development. Unfortunately, 3,4-MDPU did not show any additive effect on either root number or root elongation in our analysis.

**Discussion**

As in other plant species, the rooting ability of eucalyptus is affected by the cultivation conditions of the cuttings, in addition to the growth conditions of the donor trees from which the cuttings are taken (Assis et al. 2004). Using a lot of propagated donor plants derived from a clone of *E. globulus,* SI-10, we examined the effect of shoot position from which cuttings were prepared, seasonal variation, and response of cuttings to auxin application and to another additive on rooting ability. This clone exhibits superior traits such as good growth and straight trunk.

In the present study, cuttings were prepared using the fourth to thirteenth internodes and rooting ability of the cuttings was investigated. Although juvenile parts (i.e., first to third internodes) might have high rooting ability (Wilson 1993), using these parts was not possible due to a lack of mist equipment for water management. Our results show that the rooting ability of cuttings depends on their internode positions; furthermore, the seventh internode is the best part for cutting preparation in both summer and spring. The higher rooting activity of this part might be due to its high cambial activity and/or better state of nutritive storage. In contrast, the rooting ability of mature parts (twelfth and thirteenth internodes) during the summer was quite low compared to other parts (Figure 2). This was partially due to their poor ability of water transport. The color of cuttings from these parts easily turned purple and the leaves died rapidly during the experiment.

As shown in Table 1, the cutting experiments were performed throughout the year. Normally, the viability of donor tree is too low to perform cutting experiments in winter. Thus, we transferred donor tree to a greenhouse during this season. The survival and rooting efficiencies of cuttings varied between experimental periods (Table 1). The changes in the efficiencies were partially due to the air and soil temperatures. As indicated in previous studies (Bhusal 2001, Correa and Fett-Neto 2004, Stenvall et al. 2005), the survival and rooting efficiencies of cuttings may be affected by both air and soil temperatures via changes in endogenous conditions. Low rooting ability in winter appears to cause lower cambial activity (Anand and Heberlein 1975). Heating the soil with an electric cable during the winter could alleviate the reduced rooting activity. The rooting ability observed in our study increased up to the same level as reported in aspen (Stenvall et al. 2005) and Japanese black pine (Mori et al. 2004) during summer season. These results indicate that the potential rooting activity of cuttings in winter is the same as that in summer.

Auxin, a substance believed to promote rooting, was also examined in this study. Application of IBA at 0.8% was found to be a better rooting inducer than other types of auxins. While IAA and NAA did improve rooting efficiency, the effect was less than that of IBA. The usefulness of IBA treatment for the rooting of eucalyptus cuttings has also been reported in previous studies (Fogaça and Fett-Neto 2005, Wendling et al. 2000). As shown in Table 2, rooting efficiency was not correlated with survival efficiency or the number of roots developed. A similar tendency was observed in experiments conducted during different seasons (Table 1). These results indicate that inducing higher viability of cuttings does not ensure their rooting.

In addition to auxin, other plant regulators are known to effectively induce roots from cuttings. For example, growth retardants such as gibberellins-biosynthesis inhibitors have a positive effect on root induction in some plant species (Wiesman and Lavee 1994, Wiesman and Riov 1989). Diphenylurea derivatives are also known as root inducers in pine and apple (Ricci et al. 2001 and 2008). The chemicals are known to enhance adventitious root formation of cuttings in these plants. In this study, we examined the effect of one of the diphenylurea derivatives, 3,4-MDPU, on rooting of *E. globulus* cuttings. As shown in Table 3, application of 3,4-MDPU at a concentration of 0.5% increased rooting percentages of cuttings, but the effect is not significant (Table 3). By contrast, the application at 0.5% and 0.75% concentrations shows significant positive effect on survival of cuttings during the experiment. With our best knowledge, the positive effect on the survival by application of 3,4-MDPU has not been reported elsewhere so far. Although the mechanism of action of 3,4-MDPU has not fully revealed, our results suggest that combined application of 3,4-MDPU and IBA positively effects the vegetative production of *E. globulus*. Our data was obtained under a particular condition (IBA at 0.8% during the summer season) and thus the positive effect should be confirmed under application of different auxins and in different seasons. Furthermore, the confirmation with another genotype of *E. globulus* should be also needed in our future study since the present study was conducted only one E. globulus clone .

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***Figure legends***

Figure 1.

Overview of cutting preparation. (A) Axillary shoot harvested from a donor tree . (B) One node cutting with a pair of leaves. (C) Cutting with its leaves trimmed to approximately one-third of the total area. (D) Basal part of cutting scraped off with a knife on . (E) Cutting immersed in a solution of benomyl fungicide. (F) Cutting treated with auxin-added talc powder. (G) Cutting set for rooting in coconut husk-filled Jiffy plugs.. (H) Plastic containers filled with a mixture of peat moss and vermiculite (1:1, v/v).

Figure 2.

Rooting percentage of SI-10 clone cuttings according to their within shoot position. Experiments were performed during the summer (July 24 to September 18, 2010) and spring (March 16 to May 10, 2011). Twenty-four cuttings (8 cuttings × 3 same replicates) were used for each internode position. Each value was calculated based on initial number of cuttings tested. Bars represent standard deviations and different letters indicate scores that are significantly different at the 5% level (Bonferoni test was used in this analysis) among summer and spring data considering these two seasons independently.

Footnote of Table 1 . The experimental period was classified into 10 sets (A – J). 10 rooting periods referred to as A to J were compared. Survival and rooting percentages were calculated based on seventy-five cuttings (25 cuttings × same 3 replicates) used for each collection date. Means ± SD followed by different letters within a column indicate significant difference by Bonferoni test (α = 0.05). To examine the effect of soil temperature on the rooting ability of cuttings, an electric heating cable set at 50°C was used to heat the soil in the experiment set F and set H.

Response to reviewer’s major comments

- Please do not forget that the journal is “…Ornamental…” and with priority mention the ornamental use of *Eucalyptus globulus*

We have revised Abstract, Introduction and Discussion for fitting the general scope of the journal. Please see them.

- Key words: do not repeat the words existing in the title.

We have changed the keywords.

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We have changed the top page style following with the instructions for the authors.

- The correct citation of the authors is (Howard 1991, Howard and Ridout 1991, Mankessi et al. 2011, Paton et al. 1970, Sach et al. 1988, Wilson 1993) instead (Howard, 1991; Howard and Ridout, 1991; Mankessi et al., 2011; Paton et al., 1970; Sach et al., 1988; Wilson, 1993). Please do it everywhere in the text.

We have revised the way of writing for citation.

- Please send me your graphs as Excel and black and white files

We have added our Tables and Figures as separated files.

- Please use % instead mg/kg for the IBA and NAA concentrations.

We have revised the expressions.

- The statistical comparison of the results in the tables (the letters) must not be superscript.

We have revised the expressions.

- In the list of references: Please do not abbreviate the name of the cited journals.

We don’t use the abbreviation.

- Others

All samples of talc power with auxin were made by ourselves with talc and experimental-grade auxin.