Indian J. Hort. 70(3), September 2013: 423-427

An efficient plant regeneration system for *manuka* (*Leptospermum scoparium*) from seedlings

T.F. Fan, H.G. Liu, T.T. Wang, M. Du and L.S. Zhang*
College of Agronomy and Biotechnology, China Agricultural University, Beijing 100 094

ABSTRACT

***Leptospermum scoparium*, commonly known as *manuka*, is the most important indigenous shrub species in New Zealand, and has probably undergone the most varied development as an economic plant in the indigenous flora. The purpose of this research was to establish an efficient regeneration system for *manuka* from seedlings. Shoot tips and nodal segments of *manuka* seedlings were cultured on Murashige and Skoog (MS) medium, supplemented with several concentrations of 6-benzyladenine (BA) or in combination with gibberellic acid (GA_3). The best results for shoot proliferation were obtained with 2.0 mg l^{-1}BA and 0.1 mg $l^{-1}GA_3$, resulting in 89.3% shoot formation and a maximum shoot number (5.23 ± 0.06) per explant. Regeneration shoots were transferred to half-strength MS medium supplemented with several concentrations of α-napthalene acetic acid (NAA) or indole-3-acetic acid (IAA) for rooting. The best rooting was achieved with 0.5 mg l^{-1}NAA, resulting in a 96% root formation and a maximum root length (2.26 ± 0.41 cm). When transferred to outdoors, the well-rooted plantlets from NAA had a survival rate of 87.5% and continued to grow.**

Key words: *Leptospermum scoparium*, *in vitro* regeneration, *manuka*.

INTRODUCTION

Leptospermum scoparium, a shrub of the family Myrtaceae, commonly known as *manuka*, is the member of New Zealand indigenous woody flora and has probably undergone the most varied development as an economic plant (Yin *et al*., 18; Stephens *et al*., 16). *Manuka* has attractive white and pink to reddish coloured petals. Recently, it has largely been used for garden and flowering pot plant use. The floriferous nature of *manuka* makes it a good candidate for cut flower production (Seelye *et al*., 13). Traditionally, *manuka* was known for various medicinal uses as outlined by Brooker *et al*. (3). The leaves can be used for tea, which has antipyretic and diuretic effects. Essential oils from *manuka* showed moderate activity against *F. circinatum* (Lee *et al*., 8). *Manuka* honey is derived from nectar collected by honeybees (*Apis mellifera*) foraging on the *manuka* tree in New Zealand, which has been reported to show a significant antimicrobial activity against a wide range of bacterial species (Henriques *et al*., 5 & 6).

Propagation of *manuka* may be achieved by rather bulky semi-hardwood cuttings or seeds (Metcalf, 9). The interest in using *in vitro* culture techniques for the plantlet regeneration of *Leptospermum* spp. using explants from mature trees has already been reported (Shipton and Jackes, 14 ; Braun and Leung, 2; Seelye *et al*., 13). The purpose of this study was to establish an efficient regeneration system for *manuka* from seedlings. Comparing the former tissue culture reports about *Leptospermum* spp., here the regeneration system had its specialty and could be applied for the selection and clone of a desired cultivar.

*Corresponding author's present address: # 2, Yuanmingyuan Xi Lu, Haidian District, Beijing 100193, China; E-mail: lusheng@cau.edu.cn

MATERIALS AND METHODS

Manuka seeds were procured from New Zealand. Seeds were washed under running tap water for atleast 30 min. Thereafter, they were immersed by 200 mg $l^{-1}GA_3$ for 12 h. Before the seeds were cultured onto the half-strength Murashige and Skoog (11) medium for germination, they were surface sterilized with freshly prepared 10% (w/v) sodium hypochlorite (NaOCl) for 10 min. followed by rinsing for three times with sterile distilled water. Then the seeds were inoculated for germination. Shoot tips and nodal segments, 1.0-1.5 cm long, were excised from one-month-old seedlings and used as explants. The leaves were separated from the nodal segments carrying dormant axillary buds.

Shoot tips and nodal segments were cultured on MS basal medium supplemented with 3% (w/v) sucrose and 0.5% (w/v) agar. Rooting was achieved on half-strength MS medium supplemented with 3% (w/v) sucrose and 0.5% (w/v) agar. At least 25 explants per treatment were used at each stage. Plant growth regulators and their combinations were added to the medium as specified below. The pH was adjusted to 5.8, prior to autoclaving at 121°C for 20 min. When required, the appropriate auxins and

cytokinins were incorporated into the media before sterilisation. GA_3 was filter sterilized using 0.2 µm disposable filters (Millipore®) and added to the media following sterilisation. Molten medium (50 ml) were dispensed into 250 ml jars. All the cultures were incubated at 25 ± 2°C and 70% relative humidity under cool white fluorescent light with a photosynthetic photon flux density (PPFD) of 50 µmol $m^{-2}s^{-1}$ for a photoperiod of 16/8 h light and dark cycles.

At the proliferation stage the basal medium was supplemented various concentrations of BA 0, 0.5, 1.0, 1.5, 2.0, 2.5 mg l^{-1} or in combination with 0.1 mg l^{-1} GA_3. At the end of the fourth week, the number of explants forming shoots and shoot numbers were recorded. The effect of auxin on root induction was studied by placing excised shoots measuring 1.5-2.0 cm into 250 ml jars, each containing 50 ml half-strength MS medium with various concentrations of NAA 0.2, 0.5, 1.0, 1.5 mg l^{-1}. A second experiment was supplemented with various concentrations of IAA 0.2, 0.5, 1.0, 1.5 mg l^{-1}. The control involved transferring the shoots to a medium lacking growth regulators. Three weeks later, rooting percentage, the number of explants forming roots and root length were recorded.

Three weeks after incubation at the root forming stage, all jars with well-rooted shoots from all treatments were transferred to the mist system equipped with an electronic leaf device, for hardening. Plantlets from NAA or IAA were washed thoroughly in running tap water to remove any agar medium from the roots and transferred separately to plastic pots (20 cm) containing sterile garden soil and vermiculite (2:1) under diffused light (14/10 h photoperiod) conditions. Potted plantlets were covered with transparent plastic bags to ensure high humidity and watered every three days with half-strength MS salts solution for two weeks in order to acclimatize the plants to field conditions. At the end of the second week all plastic bags were removed and the plantlets uncovered. During this stage the shoots continued to grow as a sign of successful establishment. Three weeks after transplanting, the plants into the potting mixture, the plantlets were transferred outdoors where they continued to grow.

All the experiments were conducted with a minimum of 25 explants per treatment and were repeated three times. All data were analyzed using a one-way analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the least significant difference test at the 5% level of probability using the software SPSS 17.0.

RESULTS AND DISCUSSION

Only a few shoots developed in the absence of BA (Table 1). Explants demonstrated differential responses according to the concentrations and combinations of growth regulators in the culture media. For the various BA concentrations, the number of successful explants forming shoots and shoots number per explant increased with the increase in concentration of BA (Table 1). Maximum shoot regeneration frequency of 89.3% and maximum

Table 1. Effect of plant growth regulators on shoot proliferation from different explants of *manuka* seedlings.

Plant growth regulator (mg l^{-1})		Shoot regeneration (%)	No. of shoots per explant
GA_3	BAP		
0	0	5.3 ± 1.3 a	0.81 ± 0.05 a
0	0.5	2.7 ± 1.3 a	1.51 ± 0.11 b
0	1.0	13.3 ± 3.5 b	1.84 ± 0.06 c
0	1.5	41.3 ± 2.7 d	2.73 ± 0.03 d
0	2.0	74.7 ± 2.7 f	4.67 ± 0.02 e
0	2.5	89.3 ± 1.3 g	4.44 ± 0.05 e
0.1	0.5	6.7 ± 1.3 a	1.58 ± 0.07 b
0.1	1.0	25.3 ± 1.3 c	1.92 ± 0.11 c
0.1	1.5	64.0 ± 2.3 e	2.90 ± 0.07 d
0.1	2.0	89.3 ± 1.3 g	5.23 ± 0.06 f
0.1	2.5	90.1 ± 1.3 g	5.16 ± 0.05 f

*Each value represents the mean ± standard error of three replications (25 cultures for each replication). Values followed by different letters are significantly different (P = 0.05).

shoots number of 4.44 ± 0.05 per explant were both obtained when the concentration of BA was 2.5 mg l^{-1} (Table 1; Fig. 1A). The treatment with 0.1 mg l^{-1} GA_3 had the effect of promoting the multiplication of adventitious shoots. Comparing the 1.0 mg l^{-1}or 1.5 mg l^{-1}or 2.0 mg l^{-1}BA, addition of 0.1 mg l^{-1} GA_3 significantly increased the shoot multiplication respectively (Table 1). Another effect of stimulating shoot elongation was observed with the addition of 0.1 mg l^{-1} GA_3. The maximum number of shoots per explant was obtained on the medium supplemented with 2.0 mg l^{-1}BA and 0.1 mg $l^{-1}GA_3$ or 2.5 mg l^{-1} BA and 0.1 mg $l^{-1}GA_3$ (Table 1). However, we observed that the growth rate of shoots was faster on the medium containing 2.0 mg l^{-1}BA and 0.1 mg $l^{-1}GA_3$ than 2.5 mg l^{-1} BA and 0.1 mg $l^{-1}GA_3$. The best results for shoot proliferation were obtained with 2.0 mg l^{-1}BA and 0.1 mg $l^{-1}GA_3$ (Fig. 1B).

Different concentrations of BA have been used for proliferation of *Leptospermum* shoots (Braun and Leung, 2; Seelye *et al*., 13) and Myrtaceae shoots (Yadav *et al*., 17; Speer, 15; Khan *et al*., 7) as noted in the present study. In a previous study carried out by Braun and Leung (2), the best BA concentration for *Leptospermum scoparium* proliferation from mature shoots was 1.0 or 2.0 mg l^{-1}, but they used one-tenth strength MS salts and data on actual shoot numbers were not presented. Addition of GA_3 is known to promote internodal length in many plants (Bidwell, 1). However, in most plants, the use of gibberellins in shoot culture medium was detrimental, producing elongated shoots with narrow leaves. Sometimes, at the proliferation stage, GA_3 can enhance growth and/or increase the rate of shoot proliferation (Moshkov *et al*., 10), hence the addition of GA_3 with BA caused high-frequency bud breaks and shoot multiplication in apical shoot bud and nodal segment explants of *Morus cathyana* (Pattnaik and Chand, 12). Our results on shoot regeneration of *manuka* were similar. In the previous study on *Leptospermum scoparium* proliferation (Braun and Leung, 2), GA_3 was used alone and the effect was negative. In contrast, our study showed that addition of GA_3 with BA to the proliferation medium was essential for optimal proliferation.

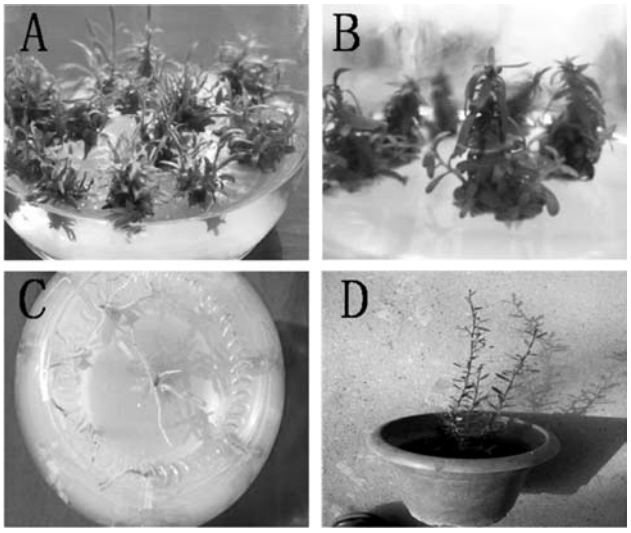

Fig. 1. Regeneration from *Manuka* seedling explants: Proliferation of shoots on MS + BA (2.5 mg l^{-1}) (A); Regeneration of shoots on MS + BA (2.0 mg l^{-1}) + GA_3 (0.1 mg l^{-1}) (B); Rooted plantlet on half-strength MS + NAA (0.5 mg l^{-1}) (C); 35 cm high plant after a 5-month of acclimatization (D).

About 88.3% of shoots developed roots on half-strength MS medium without growth regulators. The rooting of regeneration shoots demonstrated differential responses according to the concentrations of growth regulators in the culture media (Table 2). Of the various NAA or IAA concentrations, about 90 to 96% of individual shoots produced roots when the concentration of NAA or IAA was 0.5 or 1.0 mg l^{-1}. There was no significant difference in root number per shoot among the media supplemented with 0.5 or 1.0 mg l^{-1} NAA or IAA (Table 2). However, the roots had a faster growth rate with longer length (2.26 ± 0.41 or 2.15 ± 0.32 cm) when the concentration of NAA or IAA was 0.5 mg l^{-1} (Table 2). Root production was suppressed with the higher concentration of NAA (1.5 mg l^{-1}) or lower concentration of NAA (0.2 mg l^{-1}) (Table 2). Moreover, it was observed that the roots induced by NAA were thick and white, while roots induced by IAA were thin and brown (Fig. 2A, B).

Incubation of adventitious shoots on an NAA or IAA or IBA-supplemented medium induced the formation of roots in many different *Leptospermum* species (Shipton and Jackes, 14; Braun and Leung, 2; Seelye *et al*., 13). However, NAA was found to be more effective in root induction than IBA in *manuka* (Braun and Leung, 2). About 88.3% of shoots developed roots on a growth regulator-free medium, similar results were also found in the previous studies about *Leptospermum* species carried out by Braun and Leung (2), and Seelye *et al*. (13). Through it was seen that number of shoots per shoot and root length were both not good on growth regulator-free medium (Table 2). This may be attributed to the low endogenous hormone levels. The most effective root induction was obtained on the medium supplemented with 0.5 mg l^{-1}NAA or IAA. There were no previous reports about the thick and white roots induced by

Table 2. Effect of plant growth regulators on root induction from shoots of *manuka*.

Plant growth regulator (mg l^{-1})		Rooting (%)	No. of roots per shoot	Root length (cm)
NAA	IAA			
0	0	88.3 ± 4.6 d	1.2 ± 0.08 a	1.12 ± 0.32 c
0.2	0	30.7 ± 1.3 b	1.6 ± 0.1 a	0.76 ± 0.22 b
0.5	0	96.0 ± 2.3 d	3.5 ± 0.2 b	2.26 ± 0.41 e
1.0	0	90.7 ± 1.3 d	3.7 ± 0.3 b	1.25 ± 0.38 c
1.5	0	18.7 ± 2.7 a	1.6 ± 0.2 a	0.40 ± 0.10 a
0	0.2	44.0 ± 2.3 c	1.5 ± 0.1 a	0.75 ± 0.25 b
0	0.5	93.3 ± 1.3 d	3.6 ± 0.2 b	2.15 ± 0.32 e
0	1.0	90.7 ± 2.7 d	3.8 ± 0.2 b	1.50 ± 0.43 d
0	1.5	22.7 ± 1.3 a	1.4 ± 0.1 a	0.30 ± 0.08 a

[1]Each value represents the mean ± standard error of three replications (25 cultures for each replication). Values followed by different letters are significantly different (P = 0.05).

NAA, which maybe have a better absorbing ability than the thin and brown roots induced by IAA for the plantlets from NAA had a higher survival rate than the plantlets from IAA.

Plantlets with roots were easy to transfer into the potting mixture without injury. The plantlets with fully expanded leaves and well-developed roots induced on NAA had a survival rate of 87.5% (Fig. 2C) in the first three weeks and they continued to grow well, five month later, they grew (Fig. 1D). However, the explants with expanded leaves and well-developed roots induced by IAA only had a survival of 32% (Fig. 2D). Hence, the best root forming response was achieved on half-strength MS medium supplemented with 0.5 mg l^{-1}NAA.

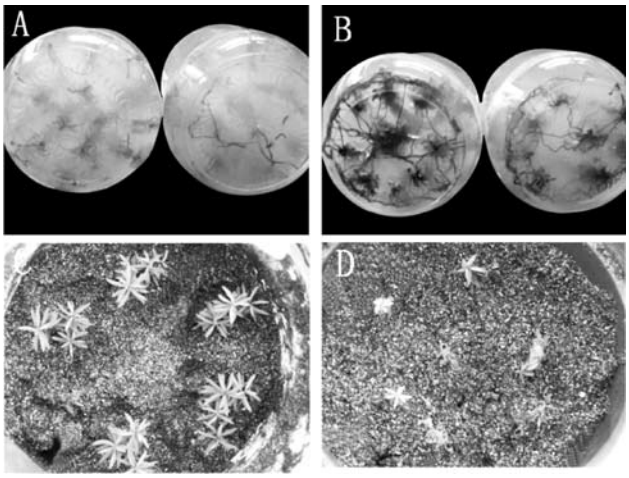

Fig. 2. The different response in root induction between NAA and IAA: Thick and white roots induced by NAA (A); Thin and brown roots induced by IAA (B); Plantlets with roots induced by NAA after 18 days (C); Plantlets with roots induced by IAA after 18 days (D).

In summary, this paper reports an effective method and culture medium for the rapid multiplication of *manuka* from seedlings along with a relatively simple procedure of acclimatizing rooted explants under mist with a high survival rate. The system appears to be highly effective and could be used as a means of the selection and clone of a desired cultivar.

ACKNOWLEDGEMENTS

Authors are thankful to China Agricultural University for financial assistance. Seeds received from Massey University and AgrResearch of Palmerston North is acknowlwdged. Thanks are also due to Mr. Steven Nichols and Mr. Mike Seawright, for their help to review the manuscript.

REFERENCES

1. Bidwell, R.G.S. 1974. *Plant Physiology*, MacMillan Publishers, New York, 321 p.

2. Braun, R.H. and Leung, D.W.M. 1991. Establishment and clonal propagation of *in vitro* plantlets of *Leptospermum scoparium*. *New Zealand Nat. Sci.* **18**: 39-43.

3. Brooker, S.G., Cambie, R.C. and Cooper, R.C. 1987. *New Zealand Medicinal Plant*, Heinemann Publishers, Auckland, 170 p.

4. De Fossard, R.A. and Fossard, H. 1988. Micropropagation of some members of the Myrtaceae. *Acta Hort.* **227**: 346-51.

5. Henriques, A.F., Jenkins, R.E., Burton, N.F. and

Cooper, R.A. 2010. The intracellular effects of manuka honey on *Staphylococcus aureus*. *European J. Clinical Microbiol. Infectious Dis.* **29**: 45-50.

6. Henriques, A.F., Jenkins, R.E., Burton, N.F. and Cooper, R.A. 2011. The effect of *manuka* honey on the structure of *Pseudomonas aeruginosa. European J. Clinical Microbiol. Infectious Dis.* **30**: 167-71.

7. Khan, P.S.S.V., Hausman, J.F. and Rao, K.R. 1999. *In vitro* morphogenesis and plantlet regeneration from seeds of *Syzygium alternifolium. Biol. Plant.* **42**: 177-84.

8. Lee, Y.S., Kim, J., Lee, S.G., Oh, E., Shin, S.C. and Park, I.K. 2009. Effects of plant essential oils and components from Oriental sweet gum (*Liquidambar orientalis*) on growth and morphogenesis of three phytopathogenic fungi. *Pesticide Biochem. Physiol.* **93**: 138-43.

9. Metcalf, L.J. 1987. *The Cultivation of New Zealand Trees and Shrubs*, Reed Publishing, Methevn, 152 p.

10. Moshkov, I.E., Novikova, G.V., Hall, M.A. and George, E.F. 2008. Plant growth regulators, III: gibberellins, ethylene, abscisic acid, their analogues and inhibitors; miscellaneous compounds. **In**: *Plant Propagation by Tissue Culture*, Vol. 1. E.F. George, M.A. Hall, G.J. De Klerk (Eds.). Springer, the Netherlands, pp. 227-82.

11. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-97.

12. Pattnaik, S.K. and Chand, P.K. 1997. Rapid clonal propagation of three mulberries, *Morus cathayana* Hemsl., *M. ihou* Koiz. and *M. serrata* Roxb., through *in vitro* culture of apical shoot buds and nodal explants from mature trees. *Plant Cell Rep.* **16**: 503-8.

13. Seelye, J.F., Hofmann, B.L., Burge, G.K. and Morgan, E.R. 2001. *In vitro* propagation of *Leptospermum hybrida. New Zealand J. Crop Hort. Sci.* **29**: 233-37.

14. Shipton, W.A. and Jackes, B.R. 1986. Clonal propagation of *Leptospermum* spp. by tissue culture. *Plant Cell Rep.* **5**: 5-8.

15. Speer, S.S. 1993. Micropropagation of some Myrtaceae species which show potential as new ornamental plants. *Australian J. Exp. Agric.* **33**: 385-91.

16. Stephens, J.M.C., Molan, P.C. and Clarkson, B.D. 2005. A review of *Leptospermum scoparium* (Myrtaceae) in New Zealand. *New Zealand J. Bot.* **43**: 431-49.

17. Yadav, U., Lal, M. and Jaiswal, V.S. 1990. *In vitro* micropropagation of the tropical fruit tree *Syzygium cuminii* L. *Plant Cell Tissue Organ Cult*. **21**: 87-92.

18. Yin, R.H., Mark, A.F. and Wilson, J.B. 1984. Aspects of the ecology of the indigenous shrub *Leptospermum scoparium* (Myrtaceae) in New Zealand. *New Zealand J. Bot.* **22**: 483-7.

Received: October, 2012; Revised: February, 2013; Accepted: June, 2013