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Tissue Culture of Red Bayberry, A New Industry for Australia

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Summary

The successful establishment and propagation of red bayberry (*Myrica rubra*) in vitro cultures require precise optimization of initiation, multiplication, and rooting stages. This study aimed at developing a high-throughput clonal propagation system for red bayberry. Here, the influence of stem growth stage, seasonal variation for clean culture initiation success and tissue culture media composition for bud induction, multiplication and rooting were evaluated.

Un-hardened red stems consistently outperformed semi-hardened stems, achieving a maximum clean culture establishment rate of 90% compared to a 10% maximum for semi-hardened stems across four rootstocks trialled in the study. Seasonal analysis revealed summer and autumn as the optimal material collection periods, with overall success rates of 47.25% and 45%, respectively. Media optimization during the initiation phase enhanced axillary bud vigour, addressing initial poor growth observed on

BB01 media. Multiplication challenges, including chlorosis and shoot die-back, were mitigated using I09.1 media, which supported robust shoot quality and a multiplication rate of 2,244-fold, over seven subculture cycles for the tested genotype. Rooting experiments demonstrated an efficient auxin treatment protocol, achieving 100% rooting success within six weeks without adverse effects on shoot quality. Rooted plants acclimatized successfully under misting conditions, reached 100% survival and vigorous growth post-acclimatization.

These findings establish a comprehensive commercial tissue culture protocol for red bayberry, from initiation to nursery transfer, demonstrating potential for large-scale propagation. This research provides critical insights into optimizing in vitro systems for woody perennials, enhancing their application in horticultural biotechnology.

INTRODUCTION

Myrica rubra, commonly known as Chinese bayberry or yangmei, belongs to the Myricaceae family. The genus *Myrica*, to which *M. rubra* belongs, comprises approximately 50 species globally, many of which are found in temperate and subtropical regions. *M. rubra* is the most economically significant species within this genus (Zhang et al., 2015). This species holds significant horticultural, economic and cultural importance in East Asia, particularly its native region of China (Mo et al., 2024). *M. rubra* is a dioecious, evergreen tree characterized by its vibrant bright red to dark purple sweet-tart fruit that has a unique textural delight, which is highly valued for its unique flavor and nutritional benefits. The taxonomy of this species has been well-studied, revealing its close genetic relationships within the Myricaceae family and its adaptation to various climatic conditions. It has a long history of cultivation and usage in traditional medicine, making it a subject of growing interest for both agricultural and scientific communities (Zhang et al., 2022).

Myrica rubra is a dioecious species, meaning that individual trees are either male or female. This characteristic necessitates the presence of both male and female plants for successful pollination and fruit production. The tree typically reaches a height of 10 to 20 meters, with a broad, dense canopy. The leaves are leathery, lanceolate, and dark green, measuring 5 to 12 cm in length. The flowers are small, inconspicuous, and appear in early spring. The fruit is a drupe, 1.5 to 2.5 cm in diameter, with a rough, waxy surface and a vivid red to dark purple color when ripe (He et al., 2016; Jia et al., 2019; Liu et al., 2014). The tree thrives in well-drained, acidic soils and prefers a subtropical climate with moderate rainfall and mild temperatures. Traditional cultivation methods have been employed for centuries, but recent advancements in agricultural practices are being adopted to enhance yield and fruit quality (Wang et al., 2017).

Propagation of red bayberry (RB) is commonly done through grafting, which ensures the consistency of desirable traits such as fruit size, taste, and color. Seed propagation is less common due to the genetic variability and the extended time required for trees to reach fruit-bearing age (Chen et al., 2008; Fang-Yong and Ji-Hong, 2014). RB is a difficult to root woody species with no commercial success in rooted

cuttings as a propagation method. In China propagation is achieved through grafting. It takes 1.5 years to raise rootstock seedlings and also suffers with inefficiencies due to low/variable seed germination rates (50-60% maximum). Graft is also problematic; success depends on optimum budwood condition making the propagation process highly inefficient (Perkins, 2014).

The primary challenge for the progression of the RB industry in Australia is the "lack of efficient propagation technology" (Joyce and Sanewski, 2010; Perkins and Joyce, 2018). RB is an outcrossing woody species, meaning that genetically divergent seeds from parent plants are unsuitable for commercial cultivation. Vegetative propagation, which replicates exact genetics, is crucial to maintaining consistent fruit characteristics such as colour, flavour, and post-harvest qualities that are vital for market success. (Perkins, 2014).

Micropropagation presents a promising alternative for the mass production of RB plants with desirable characteristics. By exploiting the inherent totipotency of plant cells, micropropagation enables the regeneration of numerous identical plantlets from a small explant, such as a shoot tip or leaf segment. Moreover, the use of tissue culture techniques allows for the production of disease-free plant material, thus reducing the risk of pathogen transmission and ensuring the establishment of healthy orchards and landscapes (Hiti-Bandaralage, 2019; Hiti-Bandaralage et al., 2017).

The successful micropropagation of RB relies on the optimization of several key factors, including the selection of suitable explant sources, the development of appropriate culture media formulations, and the manipulation of growth regulators to induce shoot proliferation and root formation. Additionally, the establishment of proper environmental conditions, such as temperature, light intensity, and humidity, is crucial for the in vitro growth and development of the plantlets. To date commercial tissue culture propagation of this recalcitrant species is not found anywhere in the world due to the less efficient protocols for it to be viable with respect to consistency of results as well as economic feasibility.

This manuscript presents the latest advancements in developing a commercially viable tissue culture platform for the mature rootstock MR06. The developed protocols and some specific details are withheld due to high commercial value and project funders' agreements. However, the results are presented in detail where possible to benefit the scientific community by highlighting the availability of this advanced tissue culture platform and to attract prospective future collaborations. The developed tissue culture platform holds significant potential not only for the propagation of *Myrica rubra* but also for its conservation and genetic improvement, underscoring its economic and ecological importance.

MATERIALS AND METHODS

Explant Sterilisation

Elite RB rootstock mother plants were maintained in grow bags with irrigation at a private property in Brisbane, Queensland, Australia. Three days prior to stem section collection, the mother plants were treated with 1 g/L Mancozeb, systemic fungicide. Initial efforts to establish an effective sterilization process began in mid-summer (January), using 10 cm softwood stem sections with all leaves removed. A series of exper-

iments were conducted to optimize the sterilization process, involving various pre-treatments (e.g., 1 g/L fungicide-Mancozeb soak for 1-24 hours, hot water treatments, and soap water soak), followed by standard 70% ethanol washes and bleach treatments at different concentrations and durations.

However, the material proved to be highly sensitive to ethanol and bleach, often resulting in successful sterilization at the cost of losing bud viability. After numerous trials, the following procedure was identified as the most effective, yielding at least 20% clean cultures with viable axillary buds.

Optimized Sterilization Procedure

a) Remove all leaves by snipping and collect 5-7 stem sections into a 500 ml tall container filled with 200 ml of a 1 g/L solution of systemic fungicide Mancozeb.

b) Incubate the stem sections in the fungicide solution for 24 hours.

c) Decant the fungicide and add 2 ml of antibacterial hand soap to the container. Add approximately 300 ml of 40 °C water and gently shake for 10 minutes. Decant the hot soapy water solution and repeat the hot soapy water washing step one more time.

d) Decant the hot soapy water solution and wash the stem sections under running tap water for 30 minutes.

e) After the tap water wash, transfer the container with washed material into a laminar hood. Under sterile conditions in a laminar hood, add 400 ml of 70% ethanol to the container and gently shake for 3 minutes.

f) Decant the ethanol and wash three times with sterile distilled water.

g) Add 2 drops of Tween 20 solution onto the stem sections and add 300 ml of a 4% commercial bleach solution (Concentrate containing 42 g/L sodium hypochlorite, and 9 g/l NaOH; active chorine 4.0% m/V). Shake gently for 3 minutes.

h) Decant the bleach solution and wash 5 times with sterile distilled water to remove all traces of bleach.

After sterilization, stem sections were cut into 1-1.5 cm nodal sections, ensuring each section contains at least one axillary bud. The nodal sections were inoculated into BB01 media (Commercial IP not disclosed) contained in test tubes (one nodal section per tube). Inoculated cultures were then incubated for 7 days and meticulously inspected them for any signs of fungal or bacterial growth. Bacterial or fungal contaminations were discarded and clean cultures were continued to maintain under the optimum incubation conditions for up to 4 weeks.

Initiated clean cultures were incubated under fluorescent lights with light intensity of PAR 575 uW/cm^2 with 16 h light and 8 h dark regime. The temperature of the growth room was maintained at 25 ^{0}C ± 1 ^{0}C.

Mother Plant Quality Assessment

Initially four different rootstocks were subjected to the assessment: MR06, MR07, MR09 and MR24. Two different types of material; semi hardened green stems and red soft stems were collected from each rootstock to identify the type of material suitable for tissue culture initiations. To assess the quality of plant material in different growth cycles, initiations were carried out in all four seasons to understand any seasonal effect on establishment of clean cultures and viability of axillary buds in culture. The optimized sterilization procedure

explained above was used for all experiments.

Optimisation of Initiation Media

A series of experiments were conducted with MR06 rootstock to optimize initiation media that can support fast axillary bud growth. Due to the high commercial value of the protocols developed, the media composition is not presented in detail for this manuscript. The factors included the type of cytokinin used, concentration of cytokinin, combination of cytokinin with one-two types of auxins, sugar type and concentration, other additives such as coconut water, banana pulp and potato extract tested in independent experiments. Effect of different light spectrums (red/far red/blue) on bud initiation and growth were also tested (data not presented).

Optimisation of Shoot Multiplication

Clean cultures established were used to excise 0.1 – 0.5 cm shoot tips for multiplication. A series of independent experiments were conducted to test individual factors; basal media, type of cytokinin, concentration of cytokinin and combination of cytokinin and auxin to achieve commercially viable multiplication rates. Ten tubs from each multiplication cycle were randomly collected to calculate the multiplication rate (number of explants produced/number of explants used) for each subculture cycle to identify the best media formulation and plant growth regulator (PGR) combination.

Optimisation of Root Induction

MR06 shoots cultured and elongated on I09.1 media with at least 3 open leaves and about 1 – 1.5 cm length were subjected to rooting treatment. Rooting trials were carried out as in vitro agar based rooting trials with the use of different concentrations of naphthaleneacetic acid (NAA), indole-3-butric acid (IBA) or indole-3-acetic acid (IAA) as rooting hormones. Once the effective type and the concentration of auxin were identified in individual experiments the effect of charcoal at 1 g/L concentration was also trialed to improve the root number and growth vigour of the plantlets.

Acclimatisation of Rooted Plantlets

MR06 rooted plants were removed from agar medium and directly planted into 50 mm net pots filled with seed raising potting mix added with perlite at 3:1 ratio. Plants were maintained in a misting chamber at 100% humidity for 7 days and at 65-70% humidity thereafter for another 3 weeks.

RESULTS AND DISCUSSION

Mother Plant Quality Assessment

The comparison between green semi-hardened stems and red un-hardened stems revealed that the higher success rate in establishing clean cultures is associated with newer young stems rather than older stems. Un-hardened red stems achieved over a 50% success rate in clean culture establishment, while semi-hardened stems from all four rootstocks recorded a maximum of only 10% success (**Table 1, Fig. 1**).

Figure 1. Optimal growth stage of un-hardened red bayberry stems for in vitro culture success.

Table 1. Effect of growth stage on culture initiation success.

Rootstock	Percentage clean culture, semi-hardened stem	Percentage clean culture, un-hardened stem
MR06	10%	52%
MR07	9%	78%
MR09	3%	90%
MR24	0%	53%

The higher success rate observed with un-hardened red stems aligns with findings from other woody perennials, where newer growth often exhibits superior culture establishment due to reduced microbial contamination and higher physiological activity (Leelavathy and Sankar, 2015*)*. Similarly, studies on avocado and grapevine have demonstrated the pivotal role of growth stage in tissue culture success, underscoring the advantage of juvenile/young tissues for optimal in vitro outcomes (Cyndi, 2023; Hiti-Bandaralage et al., 2022*)*. These results emphasize the critical need for precise selection of donor plant material to maximize clean culture establishment rates and propagation efficiency.

The season in which the material was collected had a significant impact on the percentage of clean culture establishment for red bayberry (**Fig. 2**). The highest percentages of clean cultures were achieved in the summer and autumn seasons, with overall success rates of 47.25% and 45%, respectively, as a cumulative average across all varieties and multiple initiation trials.

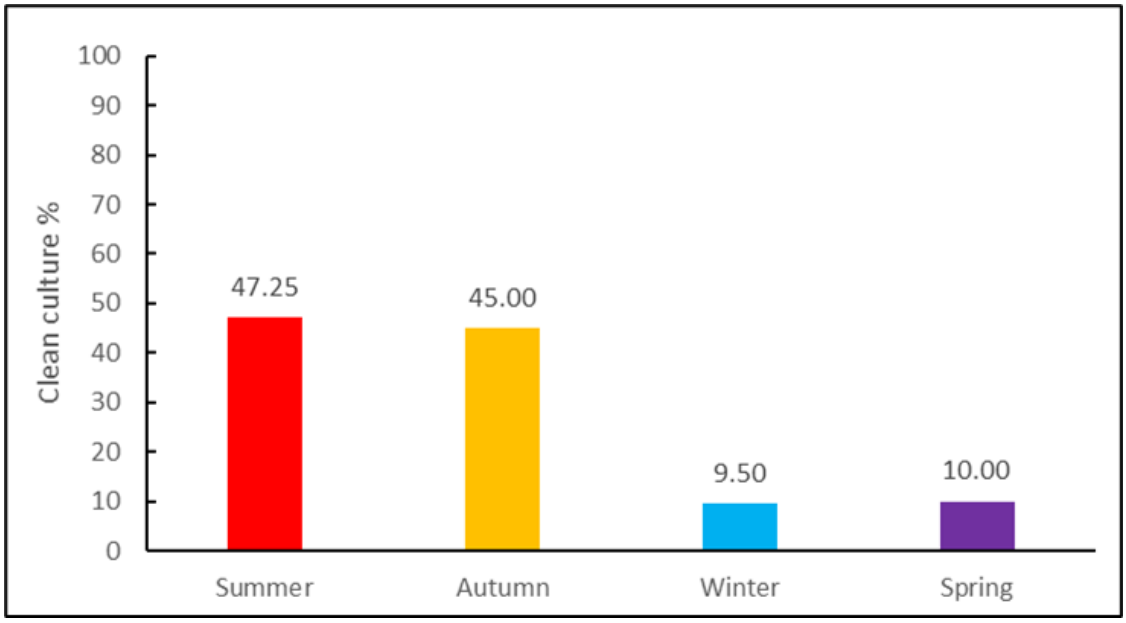

Figure 2: Percentage of clean culture establishment in different growth seasons for red bayberry.

The significant seasonal impact on culture establishment aligns with findings in woody perennials, where summer and autumn promoted active growth and reduced microbial contamination. Seasonal variations affect endogenous hormone levels and microbial load, enhancing tissue responsiveness and clean culture rates during these periods, as observed in mango and fig tissue culture studies (Ray and Savage, 2021).

Red bayberry initiation was successful using BB01 media, but the growth of the axillary buds was notably slow and exhibited poor vigor. The series of optimisations could improve the growth vigour of the axillary buds (**Figure 3**). Similar obasevations were made by Asghari et al. (2013) highlighting the importance of fine optimisation of sterilisation process and initiation media for success with red bayberry.

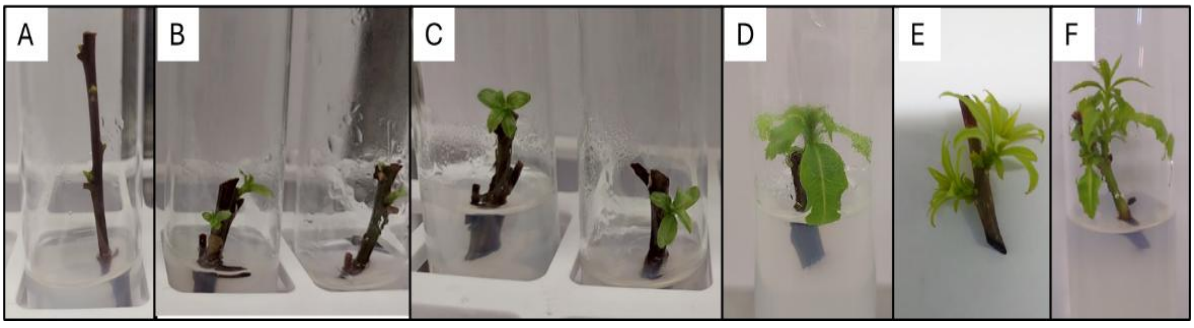

Figure 3. Bud breaking and shoot quality improvement by fine optimization of initiation media composition. (**A** to **F**) are shoot development at 2 weeks in different initiation media from inoculation.

Several problems were encountered in the multiplication stage of red bayberry. Chlorosis, shoot die-back and stunted growth were common to many of the formulations trialed in multiplication experiments. The best shoot quality and multiplication was achieved with media formulation I09.1 which resulted in best quality shoots with highest multiplication rates over the 8 subculture cycles (**Fig. 4**). The multiplication rate achieved over the 7 subculture cycles starting from a single shoot tip was almost close to 2500 times (**Table 2**). Asghari et al. (2013) in their study recorded 5.6 – 6.8 maximum rate of shoot emergence, however, did not report the total multiplication factors for long term culture required for commercial tissue culture process. Woody plants in general are difficult to be maintained in continuous subculture with high multiplication rates and good health (Hiti-Bandaralage, 2019).

Figure 4. Successful shoot multiplication of MR06 on I09.1 media.

Table 2. Multiplication rate for MR06 with I09.1 media from initiation to 7th subculture cycle.

Initiation (I_0)	I_0 - T1	T1-T2	T2-T3	T3-T4	T4-T5	T5-T6	T6-T7	T7-T8	Total multiplication per shoot tip initiated
1	1	2	4.5	2	4.5	2.6	3.2	3.33	2,244

In general, woody plants display leaf defoliation, tip die back or necrosis when subjected to rooting treatments with auxins (Hiti-Bandaralage, 2019; Xue et al., 2023). Treatments applied on MR06 shoots did not have any adverse effect on shoot quality such as leaf defoliation, tip die back or necrosis. At 3 weeks from inoculation into rooting media, root initiation was visible (**Fig. 5**). The best treatment recorded 80% rooting at 3 weeks and 100% rooting at 6 weeks.

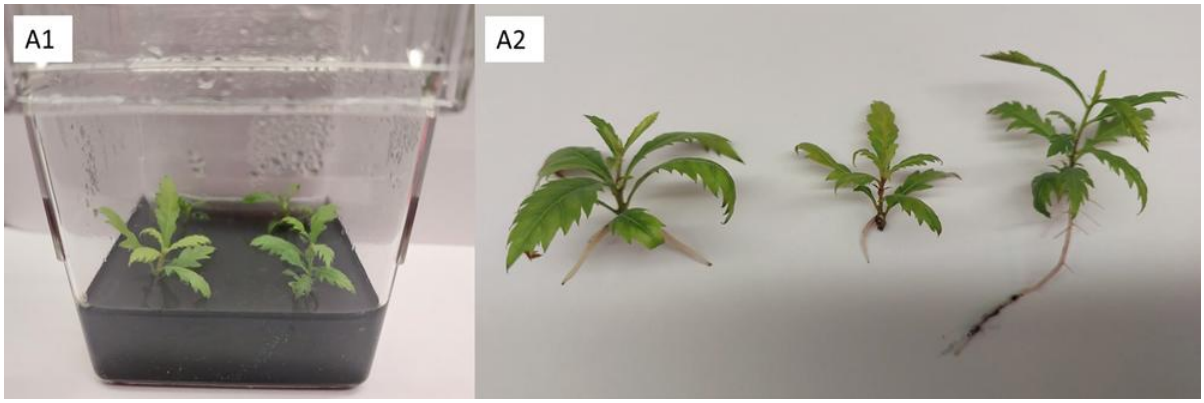

Figure 5. Rooting of red bayberry genotype MR06. **A1**) Microcuttings inoculation to rooting media and **A2**) Rooted plantlets.

Red bayberry plants developed a good root system within 6 weeks of rooting treatment. These plants acclimated with 100% survival under misting conditions. After 4 weeks, roots emerged from the pot and shoot growth was visible with new leaves (**Fig. 6**). The only significant work published on *Myrica rubra* propagation do date is by Asghari et al. (2013), who focused on two commercial cultivars, "Biji" and "Dongkui". For red bayberry tissue culture, successful rooting and acclimatization have only been reported by them and achieved a 95% rooting rate, demonstrating the importance of high-quality shoots to ensure successful multiplication and commercially viable rooting rates.

Figure 6. Fully acclimatized MR06 plantlet ready to move to nursery for repotting.

CONCLUSION

This study emphasizes the importance of optimizing plant material selection, seasonal timing, and media composition for successful red bayberry micropropagation. Un-hardened red stems and summer/autumn collections demonstrated superior clean culture establishment rates, supported by physiological activity and reduced microbial contamination. Improved initiation and multiplication media significantly enhanced shoot vigor, multiplication rates, and rooting success, achieving nearly 2500-fold multiplication. Rooting treatments produced high-quality plantlets with 100% acclimatization success. This research introduces the first successful commercial tissue culture protocol for the challenging red bayberry species. These findings provide crucial insights into overcoming culture establishment challenges, advancing the development of efficient propagation methods, and enabling large-scale production for horticultural purposes. The protocol will facilitate improved growth and mass propagation of red bayberry for commercial applications within and beyond Australia.

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LITERATURE CITED

Asghari, Sayed, J.A., Ligeng, C., Xinhua, H., and Yonghua, Q. (2013). Micropropagation of *Myrica rubra* Sieb. and Zucc. using shoot tips and nodal explant. Afr. J. Agric. Res. *8*: 1731–1737.
DOI:10.5897/AJAR10.1160

Chen, S.Y., Kuo, S-R., and Chien, C.T. (2008). Roles of gibberellins and abscisic acid in dormancy and germination of red bayberry (*Myrica rubra*) seeds. Tree Physiology *28*:1431–1439.
DOI: 10.1093/treephys/28.9.1431

Cyndi, C. (2023). The application of tissue culture for grapevine disease elimination. The Grapevine Magazine.
https://thegrapevinemagazine.net/2023/06/the-application-of-tissue-culture-for-grapevine-disease-elimination/
(accessed 12.1.24).

Joyce, D. and Sanewski, G. (2010). The Commercial Potential of Red Bayberry in Australia. RIRDC Publication No. 10/200 RIRDC Project No. PRJ-000527.
https://agrifutures.com.au/wp-content/uploads/publications/10-200.pdf

Fang-Yong, C., and Ji-Hong, L. (2014). Germplasm genetic diversity of *Myrica rubra* in Zhejiang Province studied using inter-primer binding site and start codon-targeted polymorphism markers. Scientia Horticulturae. *170*: 169–175.
DOI: 10.1016/j.scienta.2014.03.010

He, K., Li, X., Xiao, Y., Yong, Y., Zhang, Z., Li, S., Zhou, T., Yang, D., Gao, P., and Xin, X. (2016). Hypolipidemic effects of *Myrica rubra* extracts and main compounds in C57BL/6j mice. Food Funct. *7*: 3505–3515. DOI: 10.1039/c6fo00623j

Hiti-Bandaralage, J., Hayward, A., and Mitter, N. (2022). Structural disparity of avocado rootstocks in vitro for rooting and acclimation success. International Journal of Plant Biology *13*: 426–442.
DOI: 10.3390/ijpb13040035

Hiti-Bandaralage, J.C.A. (2019). Micropropagation as an alternative for avocado clonal propagation (PhD Thesis). The University of Queensland.
DOI:10.14264/uql.2019.531

Hiti-Bandaralage, J.C.A., Hayward, A., and Mitter, N. (2017). Micropropagation of avocado (*Persea Americana* Mill.). American Journal of Plant Sciences *08*: 2898.
DOI:10.4236/ajps.2017.811197

Jia, H.M., Jia, H.J., Cai, Q.L., Wang, Y., Zhao, H.B., Yang, W.F., Wang, G.Y., Li, Y.H., Zhan, D.L., Shen, Y.T., Niu, Q.F., Chang, L., Qiu, J., Zhao, L., Xie, H.B., Fu, W.Y., Jin, J., Li, X.W., Jiao, Y., Zhou, C.C., Tu, T., Chai, C.Y., Gao, J.L., Fan, L.J., van de Weg, E., Wang, J.-Y., and Gao, Z.S. (2019). The red bayberry genome and genetic basis of sex determination. Plant Biotechnology Journal *17*:397–409.
DOI: 10.1111/pbi.12985

Leelavathy, S. and Sankar, P.D. (2015). Curbing the menace of contamination in plant tissue culture. Journal of pure and applied microbiology *10*:2145-2152.

Liu, H., Qi, X., Cao, S., and Li, P. (2014). Protective effect of flavonoid extract from Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) fruit on alcoholic liver oxidative injury in mice. J Nat Med *68*:521–529.
DOI: 10.1007/s11418-014-0829-9

Mo, J., Rashwan, A.K., Osman, A.I., Eletmany, M.R., and Chen, W. (2024). Potential of Chinese bayberry (*Myrica rubra* Sieb. et Zucc.). Food Bioprocess Technol *17*:3506–3524.
DOI: 10.1007/s11947-023-03313-9

Perkins, M. (2014). Pilot production and sales of red bayberry in Australia. RIRDC, Barton, A.C.T.Perkins, M.L., and Joyce, D.C. (2018). Red bayberry – the journey to its commercialisation in Australia. Acta Hortic. *1205*: 281–288.
DOI:10.17660/ActaHortic.2018.1205.1

Ray, D.M., and Savage, J.A. (2021). Seasonal changes in temperate woody plant phloem anatomy and physiology: implications for long-distance transport. AoB PLANTS *13*, plab028.
DOI: 10.1093/aobpla/plab028

Wang, J., Tian, C., Zhang, C., Shi, B., Cao, X., Zhang, T.Q., Zhao, Z., Wang, J.W., and Jiao, Y. (2017). Cytokinin signaling activates WUSCHEL expression during axillary meristem initiation. Plant Cell *29*:1373–1387. DOI: 10.1105/tpc.16.00579

Xue, Y., Hiti-Bandaralage, J.C.A., and Mitter, N. (2023). Micropropagation of *Duboisia* species: A review on current status. Agronomy *13*:797.
DOI: 10.3390/agronomy13030797

Zhang, S., Yu, Z., Sun, L., Ren, H., Zheng, X., Liang, S., and Qi, X. (2022). An overview of the nutritional value, health properties, and future challenges of Chinese bayberry. PeerJ 10, e13070.
DOI: 10.7717/peerj.13070

Zhang, T.Q., Lian, H., Tang, H., Dolezal, K., Zhou, C.M., Yu, S., Chen, J.H., Chen, Q., Liu, H., Ljung, K., and Wang, J.W. (2015). An intrinsic MicroRNA timer regulates progressive decline in shoot regenerative capacity in plants. The Plant Cell tpc.*114*.135186.
DOI:10.1105/tpc.114.135186