

A plant regeneration protocol from callus cultures of medicinal and ornamental shrub *Rhodomyrtus tomentosa*

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Research Article

Keywords: *Rhodomyrtus tomentosa*, plant regeneration, PGRs, tissue culture, adventitious shoot bud

Posted Date: July 14th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1825313/v1>

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Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on February 25th, 2023. See the published version at <https://doi.org/10.1007/s11240-023-02464-z>.

Abstract

Rhodomyrtus tomentosa (Ait.) Hask is a multifunctional shrub of the Myrtaceae, which uniquely combines ornamental, medicinal and health purposes. In this study, for the first time, a regeneration system was established in *R. tomentosa*. At least three types of calli varied in color, shape and texture were induced from explants of leaf, stem, petal or stamen, among which, the light yellow-green, rigidity and granule calli had great potential for differentiation. Adventitious shoots were steady obtained when the calli were grown on 1/2 MS medium with 6-BA 1 mg/L and NAA 0.1 mg/L at 26 °C and 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity. Unexpectedly, two types of adventitious roots initiated from the stems above the cutting sites were observed within 15 d when the regenerated plantlets were transferred to PGR-free 1/2 MS medium. When rooted plantlets were transferred to a sandy soil, over 90% survived within 2 months. The established *in vitro* regeneration system provided a solution for the germplasm preservation and large-scale clonal propagation of elite lines of *R. tomentosa*.

Introduction

Myrtaceae, a large family of dicotyledonous woody plants, approximately consists of 5650 species from 130~150 genera worldwide, the distribution overlaps the world's biodiversity hotspots partially, such as southern and southeast Asia (Grattapaglia et al. 2012). Several genera of Myrtaceae are well known for their economic value, and are cultivated worldwide for their fleshy fruit, antiseptic oils, and as important sources of timber or fiber for multiple industrial purposes (Biffin et al. 2010).

Rhodomyrtus tomentosa (Ait.) Hask is an evergreen shrub belong to the family Myrtaceae, which forms a potential resource for exploring novel, structure diversified and biological active metabolites (Biffin et al. 2010; Zhao et al. 2020). Recently, many studies have focused on the functional properties of *R. tomentosa* extracts. These extracts have been traditionally used to combat various diseases, and possess the pharmacological activities to protect the plant from biotic or abiotic stresses (Mordmuang and Voravuthikunchai 2015). Rhodomyrtone, an extract from *R. tomentosa*, is used for treating the infections of methicillin-resistant *Staphylococcus aureus* (MRSA) (Srisuwan and Voravuthikunchai 2017; Zhao et al. 2018). Moreover, *R. tomentosa* is useful to make food products, such as wine, jam and tea (Vo and Ngo 2019). Volatile terpenoids are substantial in *R. tomentosa*, of which play important roles in our life and health, such as the respiratory medicine Myrtol Standardized and the marketable perfume Mirto di Panarea (Asgarpanah and Ariamanesh 2015). The continuously increasing demand and lack of protection awareness causes a sharp decline in *R. tomentosa*, which make it urgent to develop efficient propagation strategy for the sustainable use of this precious resource.

Conventionally, the propagation of *R. tomentosa* mainly relies on seed. The seeds of *R. tomentosa* are quite tiny and have a very low germination rate in natural conditions (Hué et al. 2013). Propagating through seeds faces several problems, including poor seed set, short seed viability, low germination and highly heterogeneous seedling populations (Kaur et al. 2022). Culm cuttings and rootstocks allow us to avoid the problem of seed propagation, but the availability of propagules and seasonal dependence are

still the major constraints (Liang et al. 2013). Therefore, new plant propagating strategy is especially required for *R. tomentosa*.

In vitro regeneration by tissue culture is a potential approach to obtain mass production of excellent plantlets. Typically, it is necessary to obtain high-quality embryogenic callus through suitable explants, and then proliferate and differentiate into new shoots or roots and other tissues (Pinto et al. 2002; Ikeuchi and Iwase 2013). However, tissue culture and rapid propagation technologies are mostly based on the "bud to bud" method in *R. tomentosa* currently (Latiff 1992), which is with low shoot proliferation coefficient. It is beneficial to achieve a synchronization on plantlets propagation, and obtain a high number of regenerated plantlets rapidly and efficiently. Furthermore, callus induction and plant regeneration are the basis for genetic transformation to investigate their giant characteristics for most plants.

The establishment of plant regeneration is effected by varied factors. The type of explants, the age and maturity of mother tree are limiting factors for the success of propagation programs. Recently, Mela et al. (2020) reported the successful callus induction from leaves of *R. tomentosa* seedlings by using different concentrations and combinations of 2, 4-D, TDZ and 6-BA growth regulators, but lacked a further investigation of adventitious buds differentiation. Most *in vitro* regeneration researches in Myrtaceae has been reported in *Eucalyptus*, which include several trees with great economic values. For example, Nugent et al. (2001) published research on somatic embryogenesis in *E. globulus*, and Guan et al. (2004) studied the ability to differentiate adventitious shoot bud differed among the leaves of *E. camaldulensis* and explants with shorter leaf age were desired. These studies provide new insights into the *in vitro* regeneration of *R. tomentosa*.

Here we used tender leaves and stems from seedlings as explants to establish a stable system for callus induction, differentiation, rooting and transplanting of *R. tomentosa*. We investigated the effect of different explant, PGRs (NAA, 6-BA, TDZ, IBA), the type of basal medium, temperature and light intensity on the regeneration capacity and obtained regenerated plantlets with normal growth. Unconventionally, we found a high light density is necessary for the callus differentiation and PGR-free 1/2 MS medium was the best choice for rooting in *R. tomentosa*. Additionally, the rapidly formed adventitious root from the exposed stem near the incision provided a new clue to obtain regenerated plantlets quickly in *R. tomentosa*. The successful of plants regeneration from embryonic callus of *R. tomentosa* provided a theoretical basis for its transgenic or genome-editing breeding and molecular biology study.

Materials And Methods

Plant material

Seeds and the explant materials of *R. tomentosa* were collected from South China Botanical Garden Chinese Academy of Sciences in Guangzhou, China (113° 21' 46.77" E, 23° 10' 52.68" N).

The flower bud, tender leaves and stems from branch shoots of mature tree were disinfected with 70% (v/v) ethanol for 1 minutes, then disinfected 3 minutes with 0.1% (v/v) mercury bichloride and 8 minutes with 3% (v/v) sodium hypochlorite. After that the explants were washed with sterile water, rinsed and dried. Seeds were disinfected with 75% (v/v) ethanol for 1 minutes, then disinfected with 0.1% (v/v) mercury bichloride and 3% (v/v) sodium hypochlorite with Tween (5 drops in 100 ml) for 10 minutes, respectively, washed with sterile water, rinsed and dried (Kaur et al. 2016). Seeds were germinated and grown on the MS (Murashige and Skoog 1962) medium. Sterile cultured young seedlings about 6 cm in height were used as the source of explants directly.

Medium and growth condition

All medium were supplemented with 30 g/L sucrose and 5 g/L plant agar. The pH of media was adjusted to 5.8 with 1M KOH, autoclaved at 121 °C for 20 min. All the cultures were maintained in a growth chamber under cold white fluorescent light.

Optimization of callus induction

The injured parts due to sterilization were excised from explants, then the tissue of different explants, such as the tender leaves (from seedlings, branch shoots of mature tree, or *in vitro* culture), tender stems (from seedlings, branch shoots of mature tree, or *in vitro* culture), petal, and stamen, were inoculated in MS media containing PGRs (6-BA, NAA and TDZ), and cultured adaxial-side down on medium for callus induction. calli were subcultured to induce adventitious buds on MS medium with PGRs (6-BA and NAA).

Differentiation and proliferation of adventitious buds

To study the effects of different combinations of PGRs concentrations and different basal medium on the induction of adventitious buds, we selected light yellow-green, rigidity and granule callus, used MS, 1/2 MS, and B5 as basal medium, with different conditions of 6-BA and NAA concentrations, respectively, to investigate the differentiation of adventitious buds. Each treatment consisted of 50 clumps of callus with three replicates, five clumps of callus were placed on one plate and grew at 26 °C and 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$ illumination under a 16h-light/8h-dark cycle. The percentage of adventitious shoots were counted after two months of culture. To investigate the effect of temperature and light intensity on regeneration, we cultured callus with similar growth state at different temperatures (20 °C, 26 °C or 32°C) and light intensity (60 $\mu\text{mol m}^{-2}\text{s}^{-1}$ or 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$) to induce adventitious bud. The orthogonal experimental test was repeated three times. Periodic subcultures placed on glass flask were carried out for two months, then the number and growth state of calli and shoots were recorded.

Rooting medium, transplanting and acclimatization

To study the effect of PGRs (IBA, NAA) on rooting of the differential bud of *R. tomentosa*, the differentiated plantlets were cut and cultured on rooting medium with different PGRs ratios. Four plantlets were placed on one glass flask (6 cm diameter and 9 cm height) for rooting, and one treatment

used ten glass flasks with three replicates. Plantlets were grown at 26 °C and 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$ illumination under a 16h-light/8h-dark cycle. When the roots were grown to about 2 cm in length, the plantlet were transplanted into sandy soil, and maintained a moist soil environment. The rooting rate and growth status were recorded after 60 days.

Statistical analysis

All experiments were repeated three times within one week. The values used for statistical analysis were the means obtained for each treatment. The treatment effects were analyzed by ANOVA and means were compared by Duncan's multiple range test ($P = 0.05$).

Results

Effect of different explants in callus induction

During the period of callus induction, the callus induced by different types of explants (leaf, stem, petal and stamen) showed significant differences. We found that the tender leaves from seedlings formed light yellow-green callus quickly, with about 15 days cultured in dark, which started to differentiate to adventitious buds within 30 days (Fig. 1a). Afterwards, light green granular callus gradually grew around the periphery with tight texture surface (Fig. 1b). After subcultured 60 days, the ratios of callus induction (98.48%) and shoot initiation (77.07%) were higher than that of other explants (Table 1). The tender stems from seedlings swelled and formed light yellow-green callus after 30 days (Fig. 1c). Then the callus became green or light pink, rigidity and granule during the light culture for 45 days (Fig. 1d). At the end of this period, the callus induction and shoot initiation rate were reached to 96.93% and 66.94%, respectively (Table 1). But the tender leaves and stems from *in vitro* cultures formed white or light yellow callus slowly (Fig. 1e, f), and the shoot initiation rate were reached to 58.54% and 35.80%, respectively (Table 1). The tender leaves and stems from branch shoots of the adult plant failed to differentiate to adventitious buds and formed white or light yellow, incompact and friable callus, which browning occurred easily (Fig. 1g, h). The stamens formed light pink, transparent and loose calli after 45 days (Fig. 1i), while the petals formed pink or light yellow, transparent and tight callusing tissue on the edge of injury after 45 days (Fig. 1j). And they both failed to differentiate into adventitious buds although the callus induction rate up to 85.43% and 83.73%, respectively (Table 1). Collectively, tender leaves and tender stems from the seed seedlings and *in vitro* culture as explants were appropriate for the regeneration of *R. tomentosa*, especially the tender leaves.

Effect of various combinations of 6-BA, NAA and TDZ in callus induction

To investigate the effect of PGRs on regeneration, we tested twelve MS medium with different combination of PGRs (6-BA, NAA and TDZ). Treatment of 6-BA 0.5 mg/L + NAA 0.05 mg/L and 6-BA 1 mg/L + NAA 0.5 mg/L were both promoted callus induction, but excessive concentrations of PGRs (6-BA 2 mg/L + NAA 2 mg/L) inhibited the formation of effective buds. In addition, high TDZ concentration (0.02 mg/L) caused less callus formation. Our results indicated that optimum callus occurred at the basal

medium with 0.5 mg/L 6-BA + 0.05 mg/L NAA + 0.005 mg/L TDZ, while the callus induction reached to 97.33% with 88.36% shoot initiation rate (Table 2).

Adventitious shoots differentiation potential of different type of calli

Calli originated from different explants varied greatly in color, texture and shape. To examine the differentiation potential of different type of calli, we cultured 50 clumps of each callus on the same medium and calculated the differentiation rate. Based on three independent experiments, we found that more than 90% of the group A calli (light yellow-green, rigidity and granule) generated adventitious shoots after 60 d induction, which is significantly higher than that of the group B calli (dark brown, rigidity and nubbly) (Table 3). The callus of group C (white, incompact and friable) failed to differentiate into embryoids and buds (Table 3). The results indicated that the light yellow-green, rigidity and granule calli were competent to form adventitious shoots and embryoids.

Effect of basal medium and PGRs on adventitious shoots differentiation

Among the different combination of basal media, 6-BA and NAA, we found that 1/2 MS supplemented with 1 mg/L 6-BA and 0.1 mg/L NAA was the best recipe to produce cluster or single shoot organogenesis with the frequency of regeneration response rate of 78.00% (Fig. 2a, b, Table 4). MS and B₅ media both induced cluster adventitious shoots with a low level efficiency down to 24.67% and 28.67%, respectively (Fig. 2c, d, Table 4). Together, we identified that the basal medium and PGRs affected the induction of adventitious shoots, especially in the proliferation of cluster or single shoots.

Effect of temperature and light intensity on the proliferation of shoot bud in *R. tomentosa*

The proliferation and differentiation of the adventitious shoots were suitable at 26 °C and 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$ or 32 °C and 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$ with comparable regeneration response rate around 85%. Meanwhile, cluster and single shoot bud grew quickly with many embryoids and few glassy plantlets under such conditions (Table 5). Low temperature and light intensity (20 °C, 60 $\mu\text{mol m}^{-2}\text{s}^{-1}$) strongly induced light green glassy plantlets and inhibited buds differentiation with 4.33% frequency of regeneration response rate (Table 5). Cluster bud grew slowly at low light intensity while the single bud growth was dramatically inhibited. The results indicated that high light intensity and temperature promoted the proliferation of shoot bud in *R. tomentosa*.

Rooting and acclimatization

Clusters of shoots were divided into individual shoots and transferred to rooting medium for rooting. We surprisingly found that roots arose in all shoots on PGR-free 1/2 MS medium (Table 6, Fig. 3a). Moreover, the adventitious root was also occurred rapidly on the stem near the incision exposed to the medium within 15 days (Fig. 3a). Rooting on the medium with NAA 0.05 mg/L was inhibited and down to 56.67% (Table 6, Fig. 3b). The addition of IBA 0.5 mg/L effectively reduced the rooting rate to 71.11% with 6 ~ 8 roots per shoot (Table 6, Fig. 3c). Rooting was occurred about 1 cm above the cut site after 15 days and

the rooting rate was about 45% with 2 ~ 3 roots per shoot under the medium with IBA (0.5 mg/L) and NAA (0.05 mg/L) (Table 6, Fig. 3d). Callus was easily formed from the cutting site but rooting was at a low level with a percentage of 11.11% when treated with 0.5 mg/L NAA (Table 6). The regenerative plantlets with roots were transplanted to sandy soil and 90% plantlets were survived (Fig. 3e, f). Our results indicated that the PGR-free 1/2 MS medium was suitable for rooting in *R. tomentosa*, moreover the rapidly formed adventitious root from the exposed stem near the incision provided a novel and quick rooting solution to obtain regenerated plantlets in *R. tomentosa*.

Discussion

Plant regeneration system is an important asexual propagation method and forms the basis for genetic transformations for most plant species. We have established the *R. tomentosa* regeneration system from callus for the first time, which would undoubtedly fasten the molecular breeding and functional study for this new resource plant (Vo and Ngo 2019; Zhao et al. 2020). We found that the tender leaves and stems from different part of maternal plants, petals and stamens were all suitable for callus induction but only the callus originated from tender leaves and stems of young seedlings were potent for shoots differentiation. The various potential of dedifferentiation-differentiation of explants have also been reported in *E. globulus*, a tree plants from the same family Myrtaceae, in which the shoot initiation was succeeded from hypocotyls but failed for leaf or stem explants (Azmi et al. 1997). Composition and proportion of PGRs plays critical roles during callus induction and proliferation. TDZ, a novel cytokinin like PGRs have been widely used in woody plant tissue culture (Huetteman and Preece 1993; Azmi et al. 1997; Anzidei et al. 2004; Yucesan et al. 2007). However, TDZ played a distinctive role in callusing in *R. tomentosa*, which depended on the concentration. A high concentration of TDZ (2 mg/L) combined with auxin was used to *R. tomentosa* callus induction and gained good results (Mela et al. 2020), but no further bud differentiation was observed. We found the shoot initiation rate was more than 88% when a low level of TDZ (0.005 mg/L TDZ, 0.5 mg/L 6-BA and 0.05 mg/L NAA) was used in callus induction. Too high concentration of TDZ is interruptive to *R. tomentosa* buds differentiation, which have also been reported in other plants (Dewir et al. 2018).

Typically, the addition of PGRs is critical for rooting during plant regeneration. However, the additional PGRs in rooting medium inhibited rooting in *R. tomentosa* differentiated shoots, which was consistent with the results in *Cerasus serrulata* (Luo et al. 2021). We speculated that PGRs were involved in all periods before rooting of plantlets which led to hormone sensitivity and reduced the hormone dependence of plantlets at a later stage in *R. tomentosa*. It's also possible that hormone autotrophic adaptation occurred during the regeneration process in tissue culture and acquired the ability of hormone independence. In consistence with this, the regenerated plantlets had more branches and grew vigorously, as shown in Fig. 3c.

The root primordium was grown from the cut site and then differentiated into roots in *E. globulus* (Fett-Neto et al. 2001), which was general in the rooting process during *in vitro* regeneration. Surprisingly, Two kinds of root (darker sturdier adventitious root and translucent fragile aerial root) grew from the stem that

uncontacted with the medium were observed in the case of *R. tomentosa*. There was few roots originated from the cutting site that merged into the medium. The regenerated plantlets grow normally after transplanting into sandy soils. Cutting off the regenerated plantlets usually caused browning and callusing from the incision, which hindered the absorption of water and nutrient. We speculated that the plantlets generated roots on the stem to reduce the damage and ensure water and nutrient absorption in *R. tomentosa*. The rooting characters greatly shortened the rooting time and increase the survival rate of regeneration plantlets. Understanding the molecular mechanism of this distinctive rooting system would conduce to resolve the recalcitrance to rooting during the regeneration and promote the application of *in vitro* propagations.

Conclusions

In this study, we have investigated the effect of explant, PGRs, basal medium, temperature and light intensity on the regeneration of *R. tomentosa*. Taken together, it indicated that tender leaves and tender stems were among the suitable explants for regeneration and the tender leaves of seedlings were the first choice. TDZ had a significant influence on the differentiation of *R. tomentosa*. Unconventionally, high light intensity was required for the calli induction and PGR free 1/2 MS medium benefited for rooting of *R. tomentosa*. We obtained regenerated plantlets with normal growth, which contributed a solution for the germplasm preservation and large-scale clonal propagation of elite lines of *R. tomentosa*.

Abbreviations

NAA: Naphtha acetic acid; 6-BA: 6-Benzyladenim; TDZ: Thidiazuron; PGRs: Plant growth regulators; IBA: 3-Indolebutyric acid; ANOVA: Analysis of Variance; MS medium: Murashige and Skoog (1962) medium; GA₃: Gibberellic acid

Declarations

Authors contributions: Conceptualization of research (S. D and S. Q); Designing of the experiments (S. D, L. Y and K. W); Contribution of experimental materials (H. C and S. Q); Execution of feld/lab experiments and data collection (L. Y and K. W); Analysis of data and interpretation (S. D, L. Y and K. W); Preparation of manuscript (L. Y and S. D).

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Funding

This study was supported by the Guangzhou Innovation Leading Team Project (No. 202009020004), "One Center and Three Bases" Project for Flora and Fauna Conservation of Guangdong Province and Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) (GML2019ZD0408).

References

1. Anzidei M, Bennici A, Schiff S, Tani C, Mori B (2004) Organogenesis and somatic embryogenesis in *Foeniculum vulgare*: histological observations of developing embryogenic callus. *Plant Cell Tissue Organ Cult* 61:69–79
2. Asgarpanah J, Ariamanesh A (2015) Phytochemistry and pharmacological properties of *Myrtus communis* L. *Indian J Trad Knowl* 14:82–87
3. Azmi A, Noin M, Landré P, Prouteau M, Boudet AM, Chriqui D (1997) High frequency plant regeneration from *Eucalyptus globulus* Labill. hypocotyls: Ontogenesis and ploidy level of the regenerants. *Plant Cell Tissue Organ Cult* 51:9–16
4. Biffin E, Lucas E, Craven LA, Costa IRd, Harrington M, Crisp M (2010) Evolution of exceptional species richness among lineages of fleshy-fruited Myrtaceae. *Ann Bot* 106:79–93
5. Dewir YH, Nurmansyah, Naidoo Y, Silva JA (2018) Thidiazuron-induced abnormalities in plant tissue cultures. *Plant Cell Rep* 37:1451–1470
6. Fett-Neto AG, Fett JP, Vieira G, Giancarlo P, Termignoni RR, Ferreira AG (2001) Distinct effects of auxin and light on adventitious root development in *Eucalyptus saligna* and *Eucalyptus globulus*. *Tree Physiol* 21:457–464
7. Grattapaglia D, Vaillancourt RE, Shepherd M, Thumma BR, Foley W, Külheim C, Potts BM, Myburg AA (2012) Progress in Myrtaceae genetics and genomics: *Eucalyptus* as the pivotal genus. *Tree Genet Genomes* 8:463–508
8. Guan Y, Huang M, Wang M (2004) In vitro culture and plantlet regeneration from leaves of *Eucalyptus urophylla*. *Plant Physiol Commun* 5:591–592
9. Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tissue Organ Cult* 33:105–119
10. Huế T, Abdullah TL, Sinniah UR, Abdullah NA (2013) Seed traits and germination behaviour of kemunting (*Rhodomyrtus tomentosa*) populations as affected by different temperatures. *Seed Sci Technol* 41:199–213
11. Ikeuchi M, Iwase SA (2013) Plant callus: mechanisms of induction and repression. *Plant Cell* 25:3159–3173
12. Kaur N, Dhawan M, Sharma I, Pati PK (2016) Interdependency of reactive oxygen species generating and scavenging system in salt sensitive and salt tolerant cultivars of rice. *BMC Plant Biol* 16:1–13

13. Kaur K, Dolker D, Behera S, Pati PK (2022) Critical factors influencing *in vitro* propagation and modulation of important secondary metabolites in *Withania somnifera* (L.) dunal. Plant Cell Tissue Organ Cult 149:41–60
14. Latiff AM (1992) *Rhodomyrtus tomentosa* (Aiton) Hassk. In E.W.M. Verheij and R.E.
15. Coronel Plant Resources of South East Asia. No 2: Edible fruits and nuts pp: 276–277
16. Liang HL, Liu HN, Yang QH, Huang RZ, Wei X (2013) Seed germination of *Rhodomyrtus tomentosa*. Seed Sci Technol 41:188–198
17. Luo Q, Zhang Y, Ou J (2021) Callus induction and plant regeneration of *Cerasus serrulata* var. lannesiana cv. 'Grandiflora'. Chin Bull Bot 56:451–461
18. Mela R, Aswaldi A, Swasti E (2020) Karamunting (*Rhodomyrtus tomentosa*) callus induction in vitro. Int J Environ Agric Biotechnol 5:459–465
19. Mordmuang A, Voravuthikunchai SP (2015) *Rhodomyrtus tomentosa* (Aiton) Hassk. leaf extract: An alternative approach for the treatment of staphylococcal bovine mastitis. Res Vet Sci 102:242–246
20. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
21. Nugent G, Chandler S, Whiteman P, Stevenson T (2001) Somatic embryogenesis in *Eucalyptus globulus*. Plant Cell Tissue Organ Cult 67:85–88
22. Pinto G, Santos C, Neves L, Araújo C (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21:208–213
23. Srisuwan S, Voravuthikunchai SP (2017) *Rhodomyrtus tomentosa* leaf extract inhibits methicillin-resistant staphylococcus aureus adhesion, invasion, and intracellular survival in human HaCaT keratinocytes. Microb Drug Resis 23:1002–1012
24. Vo TS, Ngo DH (2019) The health beneficial properties of *Rhodomyrtus tomentosa* as potential functional food. Biomol 9:76–92
25. Yucesan B, Turker AU, Gurel E (2007) TDZ-induced high frequency plant regeneration through multiple shoot formation in witloof chicory (*Cichorium intybus* L.). Plant Cell Tissue Organ Cult 91:243–250
26. Zhao LY, Liu HX, Wang L, Xu ZF, Tan HB, Qiu SX (2018) Rhodomyrtosone B, a membrane-targeting anti-MRSA natural acylphloroglucinol from *Rhodomyrtus tomentosa*. J Ethnopharmacol 228:50–57
27. Zhao Z, Wu L, Xie J, Feng Y, Tian J, He X, Li B, Wang L, Wang X, Zhang Y, Wu S, Zheng X (2020) *Rhodomyrtus tomentosa* (Aiton.): A review of phytochemistry, pharmacology and industrial applications research progress. Food Chem 309: 125715–125749

Tables

Table 1 Callus induction and differentiation from different explants in *R. tomentosa*

Kinds of explant	Callus induction (%)	Shoot initiation (%)
Tender leaves from seedlings	98.48 ± 0.37 ^a	77.07 ± 1.91 ^a
Tender stems from seedlings	96.93 ± 0.70 ^a	66.94 ± 2.06 ^b
Tender leaves from <i>in vitro</i> cultures	88.01 ± 3.59 ^b	58.54 ± 0.70 ^c
Tender stems from <i>in vitro</i> cultures	89.47 ± 3.81 ^b	35.80 ± 3.04 ^d
Tender leaves from branch shoots of the adult plant	43.95 ± 2.15 ^c	0
Tender stems from branch shoots of the adult plant	36.00 ± 2.61 ^d	0
Stamen	83.73 ± 0.82 ^b	0
Petal	85.43 ± 1.32 ^b	0

Results are presented as mean ± SE, within each column, mean value with the same letter(s) are not statistically significant based on Duncan's multiple range test at $P = 0.05$.

Table 2 Effect of various combinations of PGRs (6-BA, NAA, TDZ) on adventitious shoots regeneration in *R. tomentosa*

PGR (mg/L)			Callus induction (%)	Shoot initiation (%)
6-BA	NAA	TDZ		
0.1	0.01	0	58.67 ± 4.37 ^d	47.85 ± 8.66 ^d
0.5	0.05	0	78.67 ± 1.76 ^c	76.39 ± 2.83 ^b
1	0.5	0	77.33 ± 4.37 ^c	58.47 ± 2.89 ^c
2	2	0	94.00 ± 2.00 ^a	51.81 ± 1.96 ^d
0.1	0.01	0.005	47.33 ± 3.71 ^e	77.26 ± 1.78 ^b
0.5	0.05	0.005	97.33 ± 0.67 ^a	88.36 ± 2.43 ^a
1	0.5	0.005	87.33 ± 1.33 ^b	74.02 ± 1.02 ^b
2	2	0.005	96.00 ± 1.15 ^a	32.60 ± 1.46 ^e
0.1	0.01	0.02	70.00 ± 4.16 ^c	38.83 ± 1.91 ^e
0.5	0.05	0.02	92.67 ± 3.53 ^a	26.85 ± 2.99 ^f
1	0.5	0.02	72.00 ± 4.16 ^c	14.61 ± 3.65 ^g
2	2	0.02	62.67 ± 5.21 ^d	9.86 ± 2.21 ^g

Results were presented as mean ± SE, within each column, mean value with the same letter(s) were not statistically significant based on Duncan's multiple range test at $P = 0.05$.

Table 3 Differentiation potential of various calli from *R. tomentosa*.

Callus	No. of total adventitious shoot/50 callus	No. of regeneration bud/50 callus	Frequency of regeneration response (%)
A: Light yellow-green, rigidity and granule	49	47	93.33 ± 1.76 ^a
	48	45	
	49	48	
B: Dark brown, rigidity and nubby	13	2	2.67 ± 0.67 ^b
	12	1	
	9	1	
C: White, incompact and friable	0	0	0 ^c
	0	0	
	0	0	

Results were presented as mean ± SE, within each column, mean value with the same letter (s) were not statistically significant based on Duncan's multiple range test at $P = 0.05$.

Table 4 Effect of basal medium and PGRs (6-BA and NAA) concentrations in adventitious shoots differentiation in *R. tomentosa*

Medium	PGR (mg/L)		Frequency of regeneration response (%)
	6-BA	NAA	
MS	0.5	0.1	6.67 ± 1.76 ^e
MS	1	0.1	24.67 ± 1.76 ^c
MS	1	0.5	12.67 ± 1.76 ^d
1/2 MS	0.5	0.1	36.67 ± 1.33 ^b
1/2 MS	1	0.1	78.00 ± 2.31 ^a
1/2 MS	1	0.5	14.67 ± 1.76 ^d
B ₅	0.5	0.1	14.00 ± 2.31 ^d
B ₅	1	0.1	28.67 ± 2.40 ^c
B ₅	1	0.5	14.67 ± 1.33 ^d

Results are presented as mean $\pm$ SE, within each column, mean value with the same letter(s) are not statistically significant based on Duncan's multiple range test at $P = 0.05$.

Table 5 Effect of different temperature and illuminance on shoot bud proliferation in *R. tomentosa*

Temperature (°C)	Illuminance ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Frequency of regeneration response (%)	State of growth
20	60	4.33 ± 0.88^e	Easy to induce white or light pink glassy plantlets
	120	24.67 ± 0.88^d	Uneasy to induce glassy plantlets, clustered and single shoot bud grew slowly
26	60	34.67 ± 1.20^c	Clustered shoot bud grew slowly and browning easily
	120	86.67 ± 1.45^a	Cluster and single shoot bud grew quickly, with many embryoids
32	60	53.00 ± 1.15^b	Many light red fleshy glassy plantlets, callus browning easily
	120	84.33 ± 0.88^a	Few glassy plantlets, normal growth of single shoot bud

Results are presented as mean $\pm$ SE, within each column, mean value with the same letter(s) are not statistically significant based on Duncan's multiple range test at $P = 0.05$.

Table 6 Effect of IBA, NAA on rooting of the adventitious shoots in *R. tomentosa* within 60d

PGR (mg/L)		Rooting rate (%)	State of growth
IBA	NAA		
0	0	100 ^a	Normal root system on the stem near the incision exposed to the medium, 10 ~ 12 roots
0.5	0	71.11 ± 1.11^b	6 ~ 8 roots occurred at the stem on the media surface
0	0.05	56.67 ± 1.93^c	3 ~ 5 roots occurred at the stem on the media surface
0	0.5	11.11 ± 2.94^e	More callus formed on cut, rooting inhibited
0.5	0.05	45.56 ± 1.11^d	Callus formed on cut, 2 ~ 3 roots occurred at the stem on the media surface

Results are presented as mean $\pm$ SE, within each column, mean value with the same letter(s) are not statistically significant based on Duncan's multiple range test at $P = 0.05$.

Figures

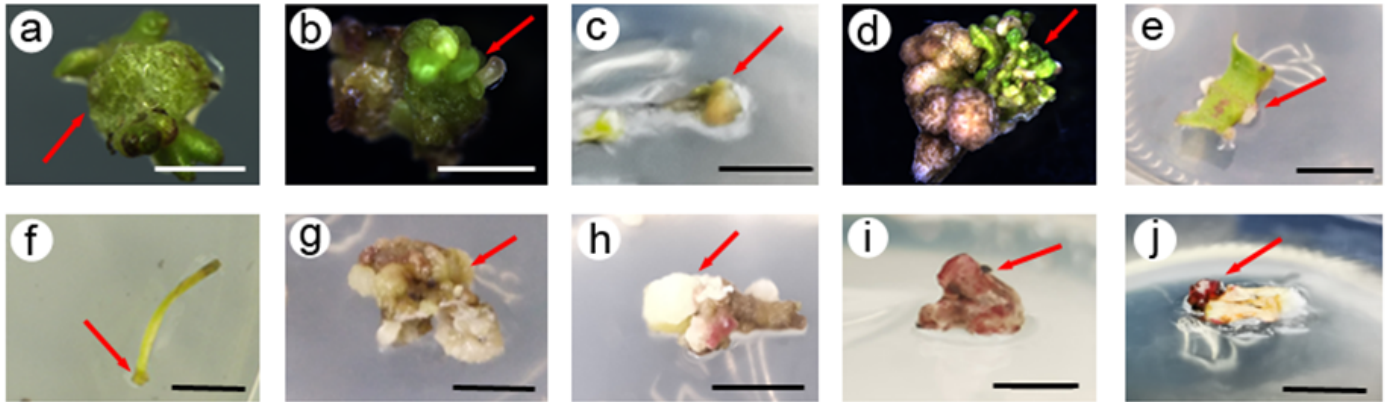


Figure 1

Callus induction with different explants in *R. tomentosa*. (a) Callus from leaves of seedlings after 30 d; (b) Callus from leaves of seedlings after 45 d; (c) Callus from tender stems of seedlings after 30 d; (d) Callus from tender stems of seedlings after 45 d; (e) Callus from tender leaves from *in vitro* cultures after 30 d; (f) Callus from tender stems from *in vitro* cultures after 30 d; (g) Callus from tender leaves from branch shoots of the adult plant after 45 d; (h) Callus from stems from branch shoots of the adult plant after 45 d; (i) Callus from stamen after 45 d; (j) Callus from petal after 45 d. All callus were induced on MS medium with 0.5 mg/L 6-BA, 0.05 mg/L NAA and 0.005 mg/L TDZ. Bar = 0.5 cm.

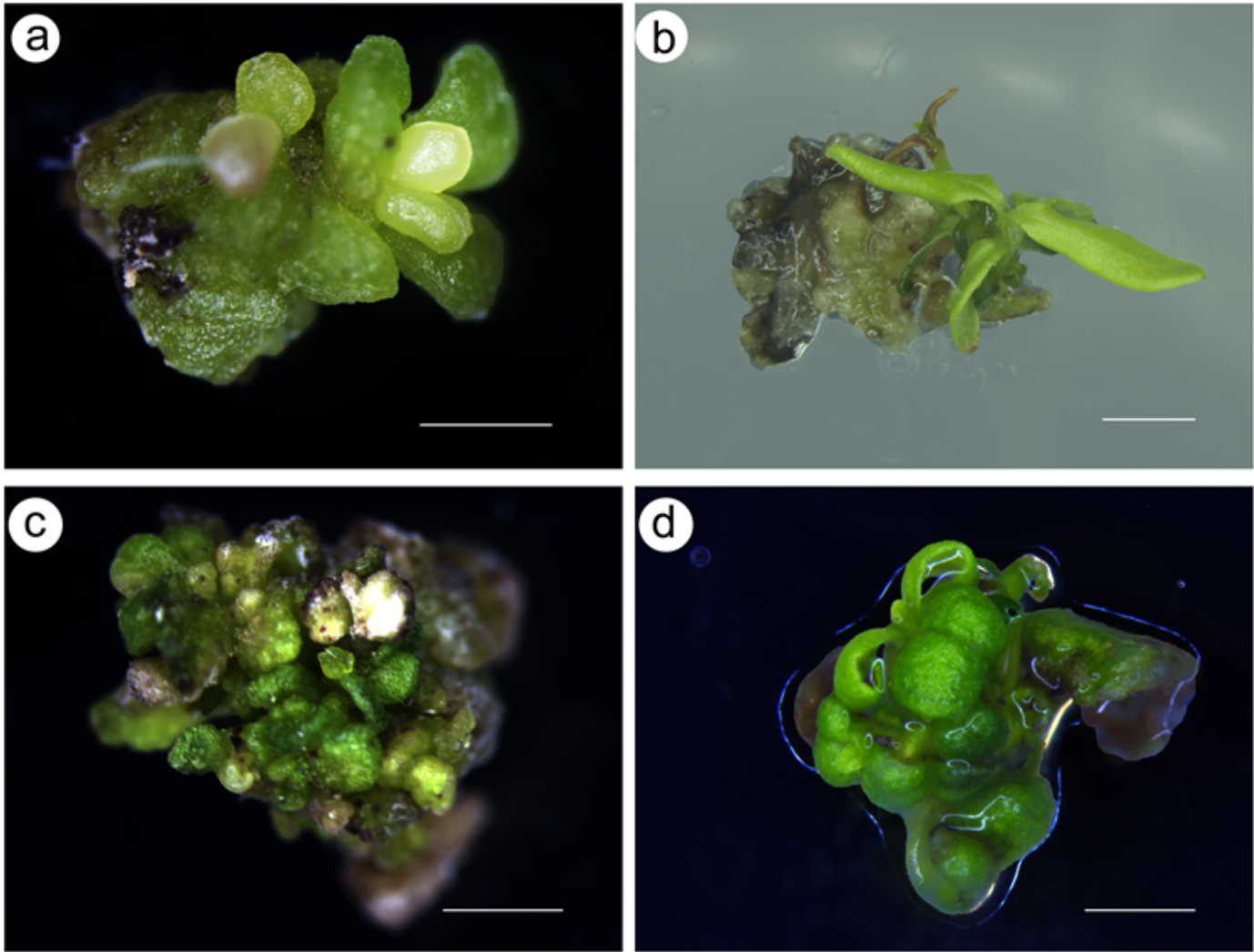


Figure 2

Shoot organogenesis from tender leaf and stem explants of *R. tomentosa*. (a) Shoot organogenesis at 1/2 MS medium after 15d; (b) Single adventitious shoot at 1/2 MS medium after 30d; (c) Cluster adventitious shoots at MS medium after 30d; (d) Cluster adventitious shoots at B₅ medium after 30d. Shoots proliferation and differentiation were induced with PGRs (1 mg/L 6-BA, 0.1 mg/L NAA) and cultured at 26 °C and 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Bar = 0.5 cm.

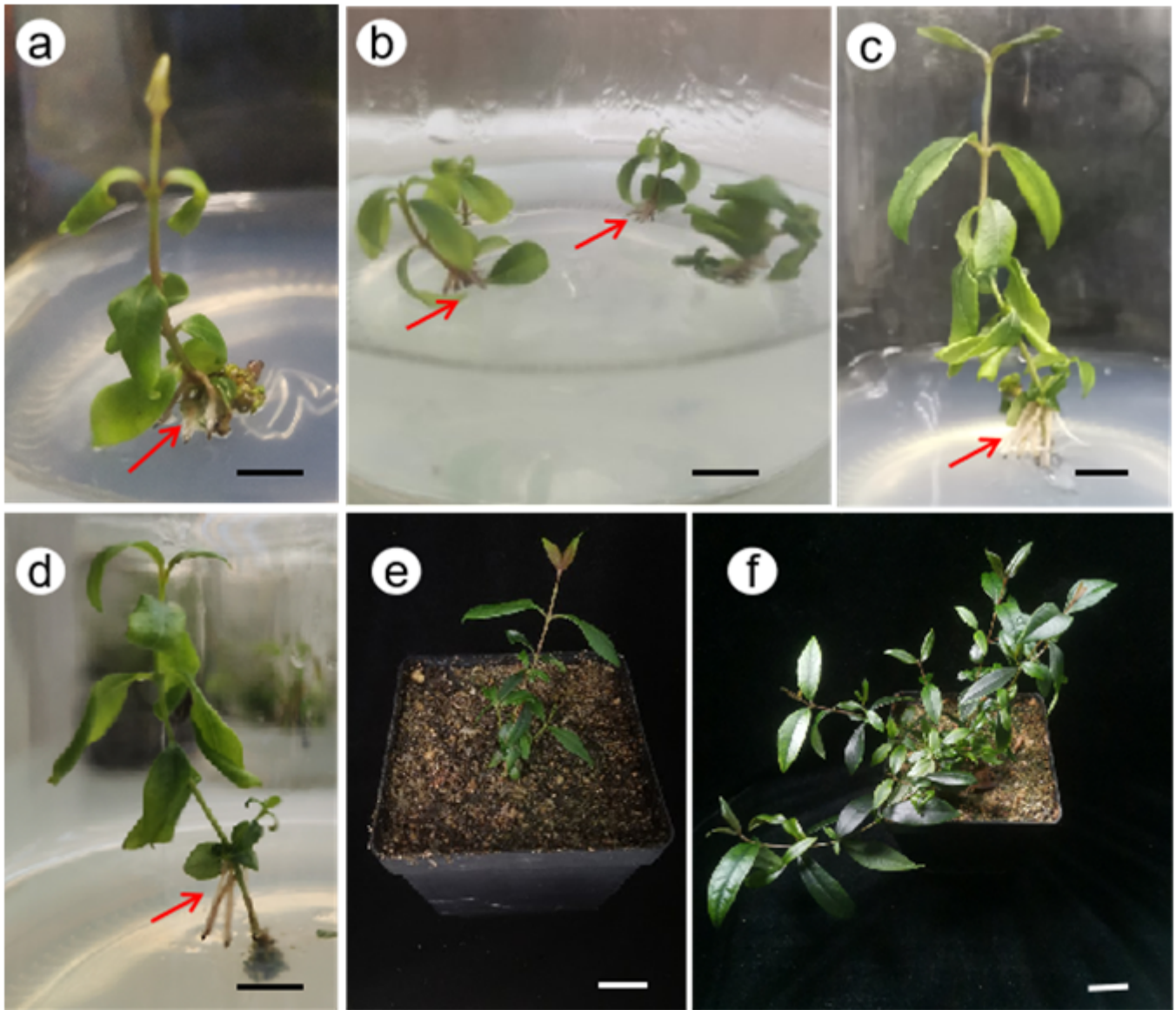


Figure 3

Rooting and plant regeneration in *R. tomentosa*. (a) Rooting of regenerated plantlets on PGR-free 1/2 MS medium after 60d; (b) Rooting of regenerated plantlets on the medium with NAA 0.05 mg/L after 60d; (c) Rooting of regenerated plantlets on the medium with IBA 0.5 mg/L after 60d; (d) Rooting of regenerated plantlets on the medium with IBA 0.5 mg/L and NAA 0.05 mg/L after 60d; (e, f) Plantlets were transferred into sandy soil with a moist sandy soil environment after 30d and 60d. Bar = 1 cm.