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REVIEW

Micropropagation of tree peony (*Paeonia* sect. *Moutan*): A review

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Abstract

Tree peony is a well-known ornamental plant that is also valued for its medical uses and edible oil production. A long breeding period and low propagation rate are the main hurdles hindering the development of the tree peony industry, for which micropropagation can offer a solution. This article reviews the advances in tree peony micropropagation during the past three decades, providing a detailed analysis of the conditions required for the four stages of micropropagation (initiation, multiplication, in vitro rooting, and acclimatization). Additionally, potential of tree peony micropropagation for commercial application was evaluated. This review provides valuable information for the successful micropropagation of tree peony, permitting more targeted and in-depth research into tree peony micropropagation.

Key message

The review summarized in detail the results achieved in tree peony micropropagation over the last three decades. Furthermore, the potential of the existing micropropagation protocol was evaluated and the problems and the future perspectives were summarized.

Keywords Tree peony · *Paeonia* · Micropropagation · Bud

Abbreviations

2-iP	2-Isopentenyladenine
AMF	Arbuscular mycorrhizal fungi
BA	6-Benzylaminopurine
CCFL	Cold cathode fluorescent lamps
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
iPA	Isopentenyladenosine
KT	Kinetin
LEDs	Light-emitting diode
MS	Murashige and Skoog medium
*m*T	*meta*-Topolin
NAA	Naphthaleneacetic acid
PGRs	Plant growth regulators
WPM	Woody plant medium

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Why is micropropagation important in tree peony breeding and propagation?

Tree peony (*Paeonia* sect. *Moutan*) is a perennial woody plant native to China (Wister 1995), now grown extensively throughout temperate regions of the world as outdoor ornamental, pot flower, and cut flower (Cheng 2007). Recently, tree peony has been proposed as a new source for edible oil due to the high oil content of its seeds (> 25%) with abundant unsaturated fatty acids (> 90%), which are especially beneficial for human health (Li et al. 2015; Gao et al. 2018).

The disadvantages of conventional propagation methods (seeding, division, and grafting) have highly constrained the breeding and propagation of tree peony. Currently, seedling selection and hybridization are the main strategies for tree peony breeding; however, its long breeding cycle is the major obstacle for these procedures (Cheng 2007). The tree peony seeds take 2 to 3 years to germinate due to dormancy (Barton and Chandler 1958). Moreover, tree peony is an inherently slow grower with a long juvenile stage (Zhu et al. 2018), and it takes 4 to 6 years for the seedlings to reach the flowering stage and then another 3 to 4 years to obtain stable and typical blooms (Barton and Chandler 1958), whereby breeding a new cultivar can take at least 10 years.

In addition, vegetative propagation of tree peony remains problematic: division has a low multiplication rate because it can only be practiced every 3 to 4 years (Yu 1998), and grafting is complex and time-consuming (Aoki and Inoue 1992). Therefore, alternative and efficient large-scale propagation methods are urgently needed in tree peony.

Micropropagation is a useful tool for the rapid and large-scale propagation of plants which has been widely used to overcome the limitations of conventional propagation methods. Although the first definition of micropropagation reads as 'any aseptic procedure involving the manipulation of plant organs, tissues or cells that produces a population of plantlets', now it is widely defined as 'clonal propagation of plants from small plant parts (0.2–10.0 mm) under in vitro conditions' (Read and Preece 2009). Micropropagation of tree peony has been extensively exploited since the pioneering work of Li et al. (1984) who induced axillary shoot from buds. Both Teixeira da Silva et al. (2012) and Qin et al. (2012) reviewed advances in the tissue culture of tree peony, and proposed micropropagation as the most promising way for its large scale propagation. However, they failed to report the detailed in vitro conditions essential for tree peony micropropagation. Additionally, rapid strides have been achieved in tree peony micropropagation in recent years, which changed the problems and perspectives in this domain. Therefore, it is essential to give a new review on the micropropagation of tree peony.

The present study aims to summarize in detail the results achieved in tree peony micropropagation over the last three decades, including establishment of aseptic culture, axillary shoot multiplication, rooting and acclimatization. Furthermore, the potential of the existing micropropagation protocol was evaluated for commercial application, and the problems and the future perspectives were summarized. This review provides useful information for the micropropagation of tree peony and should be of great value for the researchers concerned with the breeding and propagation of tree peony.

Establishment of aseptic culture

Explant selection

Li et al. (1984) published the first protocol for in vitro production of tree peony starting with the blade, petiole and bud; they proposed buds as the optimal explant and obtained viable plantlets. Since then, buds have been commonly used for micropropagating the tree peony (Table 1). Among axillary buds, terminal buds and underground buds used for tree peony micropropagation, underground buds showed the best differentiation with 65–80% success (Kong and Zhang 1998; Meng 2011). A significant linear correlation ($p < 0.01$) was found between the developmental stage of the bud and the in vitro performance; for instance, just-emerged buds, with progressively expanded leaves, were associated with a substantially higher success rate (64%) than buds with unexpanded leaves (43%) (Beruto and Curir 2007). However, the proper time for bud collection is then limited from November to March (in the Northern hemisphere), when the buds have been released from dormancy and become fully differentiated (He et al. 2009).

Surface sterilization

By optimizing the sterilizing agent type and sterilizing time, a successful surface sterilization technique has been established which produced 80–100% survival for tree peony buds (Zhang 2008; Qiu 2010). First, the surface bud scales are excised, buds are washed under running tap water (30–60 min), and then soaked in commercial liquid detergent (1% v/v; 10 min). Second, they are transferred to a clean bench, sterilized by dipping in ethanol (70% v/v; 25–30 s) and then a solution of NaClO (0.2% v/v; 11–15 min), and then rinsed three times with sterile distilled water. Finally, the buds are excised of bud scales and expanded young leaves and then inoculated in an initial culture medium.

With the above mentioned techniques, aseptic culture has been successfully established in a wide range of tree peony cultivars. However, almost all of the existing studies have focused on bud culture (Table 1), which as said can only be collected for a limited period (He et al. 2009). To date, there is still a lack of explants with year round availability, which should be of great value for annual production.

Initiation and multiplication

The formation of healthy shoots and high multiplication rates are the prerequisites of an economically viable micropropagation protocol, and the in vitro shoot formation of tree peony is usually influenced by the basal medium, plant growth regulators (PGRs), culture conditions, and some other factors.

Basal medium

Although the woody plant medium (WPM) (Lloyd and McCown 1980) has been considered as optimal for the in vitro shoot proliferation of tree peony (Table 1), the concentration of Ca^{2+} and the ratio of NO_3^-/NH_4^+ in WPM need to be modified for better in vitro performance (Bouza et al. 1994a; Wang and Van Staden 2001; Li and Cheng 2008). Doubling the concentration of Ca^{2+} (by adding $CaCl_2$) increased the multiplication rate and decreased the shoot apical necroses in tree peony (Bouza et al. 1994a; Wang and Van Staden 2001). Increasing the ratio of NO_3^-/NH_4^+ not

Table 1 Micropropagation of tree peony with buds as explants

Species or cultivar	The best performing medium (concentration: mg/L)	Culture conditions	Results	References
P. suffruticosa 'Qinglong Wo Mochi', '18th'	I, II. 1/2MS + KT 0.2–1 + BA 0.5–1 + GA_3 0.1–0.5 III. RI: IBA 50–100 treated for 2–3 h; RD: 1/2MS	I, II, III. 25 ± 1 °C, 12-h PP, 19–25 μmol m^2 s^{-1}	MR 4–5; rooting 90%; survival rate unquantified	Li et al. (1984)
P. suffruticosa	I, II. Lepoivre + BA 1 + GA_3 0.1 III. Lepoivre + IBA 0.1	I, II. 15 °C, 16-h PP, 35 μmol m^2 s^{-1} III. 15 °C, in the dark	MR 1.3-2.9; rooted shoots	Kunneman and Albers (1989), Albers and Kunneman (1992)
P. suffruticosa var. *papaveracea*	I, II. MS + BA 1 + 2ip 1 III. MS + IBA 1	I, II, III. 19 °C, 16-h PP, 160 μmol m^2 s^{-1}	MR 2.7; rooting 60%	Harris and Mantell (1991)
P. suffruticosa 'Madame de Vatry'	I, II. MS + BA 1 III. RI: MS + IBA 15; RD: MS + AC 3000	I, II. 15 °C, 16-h PP, 35 μmol m^2 s^{-1} III. Not mentioned	Rooting rate unquantified	Bouza et al. (1992)
P. suffruticosa 'Madame de Vatry'	I, II. MS ($CaCl_2$ 880) +BA 1	Not mentioned	MR unquantified	Bouza et al. (1993)
P. suffruticosa	II. MS + BA 1 + NAA 1	I, II. 26 °C, 16-h PP, 10–19 μmol m^2 s^{-1}	MR unquantified	Chu and Li (1992)
P. suffruticosa 'Madame de Vatry'	I, II. MS ($CaCl_2$ 880) +BA 1 + GA_3 0.5	I, II. 24 ± 1 °C, 16-h PP, 50 ± 5 μmol m^2 s^{-1}	MR 4.1	Bouza et al. (1994a)
P. suffruticosa 'Madame de Vatry'	I, II. MS ($CaCl_2$ 880) + BA 1 + GA_3 0.5–1 III. RI: 1/2MS + IBA 15; RD: MS + AC 3000	I, II, III. 24 ± 1 °C, 16-h PP, 50 ± 5 μmol m^2 s^{-1}	Rooting 87%	Bouza et al. (1994b)
P. suffruticosa 'Madame de Vatry'	I, II. MS ($CaCl_2$ 880) +BA 1 + GA_3 0.5-1 III. RI: 1/2MS + IBA 15; RD: MS + AC 3000	I, II. 24 ± 1 °C, 16-h PP, 50 ± 5 μmol m^2 s^{-1} III. RI: 24 ± 1 °C, in darkness; RD: 24 ± 1 °C, 16-h PP, 50 ± 5 μmol m^2 s^{-1}	Rooting 87%	Bouza et al. (1994c)
P. suffruticosa 'Madame de Vatry'	I, II. MS ($CaCl_2$ 880) +BA 1 + GA_3 0.5-1 III. RI: 1/2MS + IBA 15; RD: MS + AC 3000	I, II. 24 ± 1 °C, 16-h PP, 50 ± 5 μmol m^2 s^{-1} III. RI: 18 °C, in darkness; RD: 24 ± 1 °C, 16-h PP, 50 ± 5 μmol m^2 s^{-1}	Rooted shoots	Bouza et al. (1994d)
P. suffruticosa 'Yao Huang', 'Yanzhi Hong', 'Yeguang Bai', 'Luoyang Hong'	I, II. Modified MS + BA 1 + GA_3 0.5 III. 1/2MS + IBA 1	I, II, III. 25 ± 2 °C, 12-h PP, 19–25 μmol m^2 s^{-1} III. 15 °C, in darkness	MR 2.7–4.7; rooting 78–86%	Kong and Zhang (1998)
P. suffruticosa 'Comtesse de Tuder'	I, II. MS + BA 1 + NAA 0.2	I, II. 22 °C, 16-h PP, 19–25 μmol m^2 s^{-1}	MR 4.8	Černá et al. (2001)
P. suffruticosa 'Hei Huakui', 'Dou Lv', 'Jinxing Xuelang', 'Da Huhong'	I, II. MS + BA 0.5–1 + NAA 0.1–0.2	I, II. 25 ± 1 °C, 16-h PP, 25 μmol m^2 s^{-1}	MR unquantified	Zhang et al. (2001)
P. rockii	I, II. MS + BA 1 + NAA 0.5 + 2,4-D 0.5	I, II. Temperature not mentioned, 4-h PP, 25 μmol m^2 s^{-1}	MR unquantified	Chen et al. (2003)

Table 1 (continued)

Species or cultivar	The best performing medium (concentration: mg/L)	Culture conditions	Results	References
P. suffruticosa 'Bai Yu', 'Fengdan Bai', 'Shiyuan Bai', 'White Pearl', 'Yao Huang', 'Huang Hu', 'Orange Yellow', 'Golden Palace', 'Hu Hong', 'Da Jinfeng', 'Fish Scale Pink', 'Zhuangyuan Hong', 'Zi Erqiao', 'First Red', 'Red Diamond', 'Zhusha Lei', 'Orange', 'Red', 'Old Pink', *P. rockii* 'Xue Lian'	I, II. Modified WPM + BA 1 + citric acid 75 + Vc 50 III. Cold treatment: Modified WPM; RI: Modified WPM + IBA 1 + citric acid 75 + Vc 50; RD: WPM + AC 3000	I, II. 19 ± 1 °C, 12-h PP, 50 μmol m^2 s^{-1} III. Cold treatment: 2 °C, in darkness (7 days); RI: 19 ± 1 °C, in darkness; RD: 19 ± 1 °C, 16-h PP, 50 μmol m^2 s^{-1}	MR 1.0–3.9; rooting 50%; survival 80 ± 10%	Beruto and Curir (2007), Beruto et al. (2004)
P. suffruticosa 'Wulong Pengsheng', 'Da Huhong', 'Jin Ge'	I, II. 1/2MS + BA 1 + NAA 0.2 + IAA 0.3 + AC 3000 III. WPM + IBA 1 + IAA 0.5	I, II, III. 23 ± 1 °C, 12-h PP, 25 μmol m^2 s^{-1} IV. 15–20 °C, 12-h PP, 25 μmol m^2 s^{-1}	MR 5.4; rooting 55%; survival 30%	Wang (2008)
P. suffruticosa	I, II. MS + BA 0.5–1 III. MS + IBA 2	I, II, III. 23 ± 3 °C, 14-h PP, 25–38 μmol m^2 s^{-1}	MR 1.6-2.6	Zhang and Luo (2006)
P. suffruticosa	II. Modified MS + BA 1 + GA_3 0.2-0.5	I, II. 25 ± 2 °C, 14-h PP, 50 μmol m^2 s^{-1}	MR 4.0–5.0;	Li and Cheng (2008)
P. suffruticosa 'Hu Hong', 'Wulong Pengsheng', 'Luoyang Hong'	II. MS + BA 0.3 + NAA 0.4	I, II, III. 25 ± 1 °C, 12-h PP, 25–38 μmol m^2 s^{-1} (LED, 'Hu Hong' 50% red light + 50% blue light, 'Wulong Pengsheng' 75% red light + 25% blue light)	rooting rate unquantified	Yue (2008)
P. suffruticosa 'Wulong Pengsheng', *P.* × *lemoinei* 'High Noon'	I, II. Modified WPM + BA 0.5 + GA_3 0.2 + $AgNO_3$ 2 III. Cold treatment: 1/2MS ($CaCl_2$ 296); RI: 1/2MS ($CaCl_2$296) + IBA 1 + PUT 1-5 mg/L; RD: 1/2MS ($CaCl_2$ 296) + AC 4000	I, II. 25 ± 3 °C, 14-h PP, 19–25 μmol m^2 s^{-1} III. Cold treatment: 4 °C, in darkness (8 days); RI: 25 ± 3 °C, in darkness; RD: 25 ± 3 °C, 14-h PP, 19–25 μmol m^2 s^{-1} IV. Cold treatment: 4 °C, in darkness (30 days); transplanting substrate: vermiculite/peat/perlite (1/1/1, v/v/v)	MR 4.4; rooting 87–100%; survival 59%	Zhang (2008)
P. suffruticosa 'Wulong Pengsheng', 'Rou Furong', 'Lu Hehong', 'Luoyang Hong', 'Zhao Feng', 'Hu Hong', *P. ostii* 'Fengdan Bai'	I. Modified MS + BA 1.5 + NAA 0.5 II. Modified MS + BA 2 + IAA 0.3	I, II. 23 ± 1 °C, 12-h PP, 25 μmol m^2 s^{-1}	MR 5.1	He et al. (2009)
P. suffruticosa 'Wulong Pengsheng'	I. MS + BA 0.3 + NAA 0.3 II. MS + BA 2 + NAA 0.5 + PVP + lactoalbumin hydrolysate 500	I, II. 24 ± 1 °C, 12-h PP, 50 μmol m^2 s^{-1}, (LED, 80% red light + 20% blue light)	MR unquantified	Yan (2009)

Table 1 (continued)

Species or cultivar	The best performing medium (concentration: mg/L)	Culture conditions	Results	References
P. suffruticosa 'Wu Long Peng Sheng'	II. MS + BA 0.3 + NAA 0.3	I, II. 25 °C, 10-h PP, 45 μmol m^2 s^{-1}, 70% red light + 30% blue light (CCFL)	MR unquantified	Ding et al. (2010)
P. × *lemoinei* 'High Noon'	I, II. Modified WPM + BA 0.5 + GA_3 0.2 + $AgNO_3$ 2 III. Cold treatment: 1/2MS ($CaCl_2$ 296) (4 °C, 7 days, in the dark); RI: 1/2MS ($CaCl_2$ 296) + IBA 1 + PUT 1; RD: 1/2MS ($CaCl_2$ 296) + AC 4000 IV. Transplanting substrate: vermiculite/peat/perlite (1/1/1, v/v/v)	I, II. 25 ± 4 °C, 14-h PP, 19–25 μmol m^2 s^{-1} III. Cold treatment: 4 °C, 8 days, in darkness; RI: 16 ± 1 °C, in darkness; RD: 25 ± 4 °C, 14-h PP, 19–25 μmol m^2 s^{-1} IV. A cold treatment was applied before transplanting (4 °C, in darkness, 30 days)	MR 4.8–7.0; rooting 83%; survival 33–85%	Qiu (2010)
P. suffruticosa 'Wulong Pengsheng'	I, II. WPM + BA 3 + IAA 0.2 III. Modified 1/2MS + IBA 4 + NAA 1	I, II. 25 ± 1 °C, 14-h PP, 31 μmol m^2 s^{-1} III. Day 20 ± 1 °C, night 16 ± 1 °C; 3 days in darkness and then at 25–31 μmol m^2 s^{-1}	MR 7.8; rooting 81%; survival 90%	Liu and Jia (2010)
P. suffruticosa 'Taiping Hong'	III. WPM + IBA 4 + PVP 1000 + Vc 50 + gellan gum 2000	III. 24 ± 1 °C, 12-h PP, 40 μmol m^2 s^{-1}	Rooting 80%	He et al. (2011)
Paeonia suffruticosa 'Linghua Zhanlu'	I, II. WPM + 6-BA 1.5 + NAA 0.5 + GA_3 0.1 + $AgNO_3$ 10 III. Modified 1/2WPM + IBA 10 + $AgNO_3$ 10 + AC 1000	I, II, III. 22 ± 1 °C, 12-h PP, 25 μmol m^2 s^{-1}	MR 3.7	Meng (2011)
P. suffruticosa 'Wulong Pengsheng'	I, II. MS + BA 2 + NAA 0.5 + PVP 1000 III. WPM + IBA 4	I, II, III. 25 ± 1 °C, 16-h PP, 45 μmol m^2 s^{-1}	MR unquantified; rooting 93%	Wang et al. (2012)
P. suffruticosa 'Wulong Pengsheng'	III. RI: 1/2MS + IBA 3 + NAA 0.6; RD: 1/2MS	III. Cold treatment: 4 °C, in darkness, 10 days; RI, RD: 24 ± 1 °C, 14-h PP, 25 μmol m^2 s^{-1}	Rooting 80%	Jia et al. (2013)
P. ostii 'Fengdan Bai'	I, II. MS + BA 0.5 + NAA 0.5 III. WPM + IBA 4 III. 1/2MS + IBA 4 + NAA 0.6 + PVP 1000 + Vc 50 + gellan gum 2000	I, II, III. 24 ± 1 °C, 12-h PP, 40 μmol m^2 s^{-1}	Rooting rate unquantified	Wang et al. (2014)
P. ostii 'Fengdan Bai'	III. RI: 1/2MS + IBA 4 + PVP 1000 + gellan gum 2000	III. 24 ± 1 °C, 12-h PP, 25 μmol m^2 s^{-1}	Rooting 15–45%	Fu et al. (2016)

Table 1 (continued)

Species or cultivar	The best performing medium (concentration: mg/L)	Culture conditions	Results	References
P. suffruticosa 'Wulong Pengsheng', 'Jinpao Hong', *P.* × *lemoinei* 'High Noon'	I. WPM ($CaCl_2$ 290) +BA 1 II. WPM ($CaCl_2$ 290) +BA 1 + GA_3 0.5 III. RI: 1/2MS + IBA 1 + PUT 1–5; RD: 1/2MS + AC 4000 IV. The rooted shoots were transplanted in vermiculite/peat/perlite (1/1/1, v/v/v) and inoculated with *Glomus mosseae*	I, II. 25 ± 1 °C, 14-h PP, 25 μmol m^2 s^{-1} III. RI: 7 days at 4 °C and then at 25 ± 1 °C, in darkness; RD: 25 ± 1 °C, 14-h PP, 25 μmol m^2 s^{-1} IV. A cold treatment was applied before transplanting (4 °C, in darkness, 30 days)	MR 4.4–4.8; rooting 80–100%; survival 83%	Wen et al. (2016b)
P. × *lemoinei* 'High Noon'	I. WPM ($CaCl_2$ 290) + BA 0.5 + GA_3 0.2 II. WPM [$Ca(NO_3)_2 \cdot 4H_2O$ 1668] + BA 0.5 + GA_3 0.2 III. Rejuvenation culture: 1/2MS ($CaCl_2$ 296) + AC 500; RI: 1/2MS ($CaCl_2$ 296) +IBA 1 + PUT 1; RD: 1/2MS ($CaCl_2$ 296) + AC 4000 IV. Transplanting substrate: vermiculite/peat/perlite (1/1/1, v/v/v)	I, II. 25 ± 1 °C, 14-h PP, 32 μmol m^2 s^{-1} III. RI: 8 days at 4 °C and then at 25 ± 1 °C, in darkness; RD: 25 ± 1 °C, 14-h PP, 25 μmol m^2 s^{-1} IV. Cold treatment: 4 °C, 30 days, in darkness	MR 3.0; rooting 77%; survival 92%	Wen et al. (2016a)
P. × *lemoinei* 'High Noon'	I. WPM [$Ca(NO_3)_2 \cdot 4H_2O$ 1112] + *m*T 1 + GA_3 0.5 II. WPM [$Ca(NO_3)_2 \cdot 4H_2O$ 1112] + *m*T1 + GA_3 0.5 III. Cold treatment: 4 °C in darkness (8 days); RI: 1/2MS ($CaCl_2$ 296) +IBA 2 + PUT 1; RD: 1/2MS ($CaCl_2$ 296) + AC 4000 IV. Transplanting substrate: vermiculite/peat/perlite (1/1/1, v/v/v)	I, II. 25 ± 1 °C, 14-h PP, 25 μmol m^2 s^{-1} III. RI: 7 days at 4 °C and then at 25 ± 1 °C, in darkness; RD: 25 ± 1 °C, 14-h PP, 25 μmol m^2 s^{-1} IV. A cold treatment was applied before transplanting (4 °C, in darkness, 30 days)	MR 2.7; rooting 62%; survival 91%	Wen et al. (2016c)
P. suffruticosa 'Feng Dan'	I, II. WPM [$Ca(NO_3)_2 \cdot 4H_2O$ 1668] +BA 0.5 + GA_3 0.2 III. RI: 1/2MS + IBA1 + PUT 1; RD: 1/2MS + AC 4000	I, II. 24 ± 1 °C, 14-h PP, 32.4 μmol m^2 s^{-1} III. RI: 4 °C (8 days) and then 24 ± 1 °C, in darkness RD: 25 ± 1 °C, 14-h PP, 32.4 μmol m^2 s^{-1}	MR 3.9; rooting 57%; survival 67%	Wang et al. (2016)

Table 1 (continued)

Species or cultivar	The best performing medium (concentration: mg/L)	Culture conditions	Results	References
P. suffruticosa 'Feng Dan'	I. Modified WPM + 6-BA 1 + ascorbic acid 50 + citric acid 75 + MES 0.5 II. Modified WPM + 6-BA 0.5 + IAA 0.05 + V_c 15 + V_{B2} 20 III. Modified 1/2 WPM + IBA 20 IV. Transplanting substrate: perlite/peat (1/2, v/v/v)	I, II. 22 ± 1 °C, 16-h PP, 54 μmol m^2 s^{-1} III. Not mentioned IV. 20 ± 5 °C	MR 2.73; rooting 40% Survival 97.8%	Wang et al. (2018)

2ip 2-isopentenyladenine, *AC* activated charcoal, *BA* 6-benzylaminopurine, *CCFL* cold cathode fluorescent lamps, *GA_3* gibberellic acid, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *LED* light emitting diodes, *MR* multiplication rate, *NAA* naphthaleneacetic acid, *KT* kinetin, *PP* photoperiod, *PUT* putrescine, *PVP* polyvinylpyrrolidone, *RI* root induction, *RD* root development, *Vc* vitamin C

I. initiation, II. multiplication, III. in vitro rooting, IV. acclimatization

The light source in each study is fluorescent lamps, except where otherwise indicated. The original light intensity reported in each study has been converted to μmol m^2 s^{-1}; for fluorescent lamps, 1 μmol m^2 s^{-1} = 80 lx; for high voltage sodium lamp, 1 μmol m^2 s^{-1} = 71.4 lx (Thimijan and Heins 1983)

only improved the multiplication rate, but also reduced vitrification and browning in tree peony shoots (Li and Cheng 2008). Considering the addition of $Ca(NO_3)_2$ in WPM can improve both the concentration of Ca^{2+} and the ratio of NO_3^-/NH_4^+, Wen et al. (2016a) increased the multiplication rate of *P.* × *lemoinei* 'High Noon' from 1.7 to 3.0 by adding three times the original quantity of $Ca(NO_3)_2$ in WPM (Wen et al. 2016a). Currently, the modified WPM, containing 2–3 times $Ca(NO_3)_2$, is commonly used for the multiplication of tree peony (Li 2007; Zhang 2008; Qiu 2010; Wen et al. 2016a, b, c; Wang et al. 2016). All of these studies show that high concentrations of Ca^{2+} and the ratio of NO_3^-/NH_4^+ could be effective in enhancing the morphogenic responses of tree peony, and the specific underlying mechanism is worth further research. In the future study, design of experiments methodology could be used for further mineral nutrition optimization of tree peony by testing many factors at various levels simultaneously, and advanced statistical techniques as machine learning could be utilized for the optimization process which has been reported in hazelnut (Akin et al. 2016) and apricot (Kovalchuk et al. 2018).

Plant growth regulators (PGRs)

Compared with zeatin (Z), 2-isopentenyladenine (2-iP), isopentenyladenosine (iPA), and kinetin (KT), BA was the most effective cytokinins to stimulate multiplication of tree peony shoots (Bouza et al. 1994a). To improve the multiplication rate, researchers have combined BA with other PGRs, including KT (Li et al. 1984), 2-iP (Harris and Mantell 1991), naphthaleneacetic acid (NAA) (Černá et al. 2001), 3-Indole acetic acid (IAA) (Wang et al. 2018) and gibberellic acid (GA_3) (Bouza et al. 1994a; Wen et al. 2016b; Wang et al. 2016), among which GA_3 was the most effective. Although GA_3 alone could not induce shoots in tree peony, when combined with BA, it dramatically increased the multiplication rate (3.9–4.8) compared to BA alone (0.6–2.9) (Bouza et al. 1994a; Wen et al. 2016b; Wang et al. 2016). In addition, a recent report showed that *meta*-topolin (*m*T), a natural hydroxylated BA, could produce similar in vitro shoot multiplication as BA (Wen et al. 2016c); however, further screening of the efficacy of *m*T with a wide range of tree peony genotypes is required to confirm its broader application.

Culture conditions

The in vitro culture conditions, particularly the temperature and light (photoperiod and intensity), are important factors affecting the proliferation and growth of shoots (Table 1). A suitable temperature of 25 ± 1 °C was determined, with higher or lower temperatures causing a reduction in the multiplication rate and increasing vitrification (Zhang

et al. 2001). Both Yan (2009) and Wen (2016) proposed 50 µmol m^{-2} s^{-1} as the optimal light intensity for multiplication of tree peony, resulting in the highest multiplication rate, fresh weight, leaf number, and chlorophyll content. In addition, recent studies have proposed light-emitting diodes (LEDs) and cold cathode fluorescent lamps (CCFL) as alternatives to traditional fluorescent lamps, but the optimal ratio of red to blue light varies among cultivars (Yue 2008; Ding et al. 2010; Wen 2016). Both *P. suffruticosa* 'Wulong Pengsheng' and *P.×lemoinei* 'High Noon' showed a better multiplication and growth at 7:3 ('Wulong Pengsheng' in CCFL; 'High Noon' in LEDs) (Ding et al. 2010; Wen, 2016), while 3:1 (LEDs) for *P. suffruticosa* 'Luoyang Hong' (Yue 2008) and 1:1 (LEDs) for *P. suffruticosa* 'Hu Hong' were optimal (Yue 2008).

Other factors

Constantine (1986) proposed that a multiplication rate of 2.5–3.5-fold for a four week cycle was realistic for the commercial production of many ornamentals. However, in tree peony, a 3 week culture with a frequent supply of fresh medium is considered as the optimal subculture regime, which produced an increased number of shoots by encouraging further shoot proliferation (Harris and Mantell 1991). The inclusion of 2.0 mg/L silver nitrate in the medium was found to reduce browning and promote multiplication (Li et al. 2008a, b). Additionally, lateral bud cutting and carving promoted the in vitro shoot induction rate by 2.6–4.8 times, with the maximum reaching 96% (Liu and Jia 2010).

In vitro rooting

For a long time, rooting has been considered as a bottleneck problem hindering the micropropagation of tree peony. According to previous studies, in vitro rooting of tree peony depends on the medium, rooting methods, culture conditions, and some other factors.

Medium and rooting method

Half-strength MS (Murashige and Skoog 1962) is the most commonly used basal medium in tree peony (Table 1). Addition of auxins to the medium is essential for adventitious rooting of tree peony, because there is no root primordium in its in vitro shoots (He et al. 2011; Jia et al. 2013). Compared with IAA and NAA, indole-3-butyric acid (IBA) is the only auxin that triggers adventitious rooting in tree peony and can be used in different ways (Li et al. 1984). Bouza et al. (1994b, c) compared three methods of rooting, i.e., quick dip rooting, one-step rooting, and two-step rooting; two-step rooting was the most effective means. Later, Beruto et al. (2004) improved the two-step rooting protocol by applying a cold treatment (2 °C in darkness for 7 days) prior to root induction, which greatly promoted the rooting of 20 tree peony cultivars. Based on this improved two-step rooting protocol, recent studies further improved the in vitro rooting of tree peony by adding polyamines (1–5 mg/L) (Wen et al. 2016b) and caffeic acid (1 mg/L) (Shang et al. 2017) to the root induction medium. Therefore, the optimal in vitro rooting method for tree peony is the improved two-step rooting protocol with IBA, putrescine, and caffeic acid used in the root induction phase; however, the concentration and root induction time need to be optimized for each cultivar.

Culture conditions

Environmental conditions (low temperature and darkness during root induction) are also critical factors for the in vitro rooting of tree peony. For the two-step rooting of tree peony, the optimal temperature differed in the two phases: (i) 17 ± 1 °C was suitable for root induction (Albers and Kunneman 1992; Bouza et al. 1994b) with cold treatment (2 °C, 7 days) usually applied at the initial induction period (Beruto and Curir 2007), and (ii) 25 ± 1 °C was favored for root development (Bouza et al. 1994b). In addition, the entire rooting stage needs to be maintained in darkness due to the low photostability of IBA (Bouza et al. 1994b).

Other factors

In tree peony, BA is the most widely used cytokinin for promoting shoot multiplication and can be used alone or in combination with other PGRs (Table 1). Recently, BA was found to cause negative carryover effects on subsequent rooting in many species including tree peony (Aremu et al. 2012; Wen et al. 2016c). Furthermore, Wen et al. (2016c) found that substituting BA with *m*T, a natural hydroxylated BA, during the multiplication stage could produce a substantially higher rooting percentage in tree peony, and proposed that the inhibitory carryover effect of BA could be an important reason for the poor in vitro rooting of tree peony. In addition, shoots selected from a five-week shoot multiplication subculture regime exhibited higher rooting performance (Harris and Mantell 1991). These studies show that the physiological state of shoots is an important factor affecting in vitro rooting of tree peony, and further work on rooting enhancement can also be undertaken during the multiplication stage.

Acclimatization

The ultimate success of micropropagation depends on the ability to acclimatize plantlets at a large-scale and with high survival rates under ex vitro conditions. In tree peony,

previous research has mainly focused on releasing the apical dormancy of the shoot and helping the plantlets gradually adapt to ex vitro conditions.

Dormancy release of plantlets

Shoot apical dormancy is a typical problem in the acclimatization of tree peony. It is marked by decreased mitotic activity and endogenous abscisic acid (ABA) accumulation in shoot tips, and the dormant plantlets did not grow and eventually perished during ex vitro acclimatization (Bouza et al. 1992). To release the dormancy, a cold treatment was applied prior to transplantation, but the cold-treated plantlets went into dormancy again and eventually died after 60 days of ex vitro acclimatization (Bouza et al. 1994c; Wen et al. 2016b; Wang et al. 2016). A recent study demonstrated that arbuscular mycorrhizal fungi (AMF) inoculation may be a useful tool for overcoming the poor survival and arrested growth of tree peony plantlets, as the plantlets inoculated with *Glomus mossea* (an important AMF living symbiotically with tree peony under natural conditions) had a significantly higher survival rate and better growth than the non-inoculated plantlets during ex vitro establishment (Wen et al. 2016b).

Transplanting techniques

Shoot dormancy hinders survival during acclimatization in tree peony, yet few studies have reported on transplanting techniques. Beruto and Curir (2007) developed a two-step procedure that yielded true-to-type blooming micropropagated plants. They found that (i) it is necessary to allow the propagules to gradually adapt to the non-tissue culture conditions with a sterilized mixture instead of agar medium; and (ii) the plantlets should be transferred into normal substrate, and the propagules should be watered adequately and carefully. Pearlite, vermiculite, and turfy soil at 1:1:1 is typically used as the matrix for the transplantation of in vitro-derived tree peony plantlets (Zhang 2008; Qiu 2010).

Potential evaluation of micropropagation for commercial application

Based on the above mentioned studies, a complete micropropagation protocol has been developed for tree peony (Fig. 1); however, whether it can be used for commercial application remains unknown. In this study, potential of tree peony micropropagation for commercial application was evaluated.

Genetic stability assessment of the micropropagated plantlets

The assessment of genetic stability is essential for a micropropagation protocol, because genetic variations can be easily induced by in vitro processes (e.g., stress, auxin-cytokinin ratio, and nutritional conditions). Due to their desirable properties (reproducibility, level of polymorphism, information content, and inheritance; Agarwal et al. 2008), SSR markers have been widely used to evaluate the genetic stability of the micropropagated plantlets, such as in *Olea* spp. (Lopes et al. 2009; Brito et al. 2010), *Jatropha curcas* (Rathore et al. 2014), and *Asparagus* spp. (Regalado et al. 2015). In 2016, the genetic stability of micropropagated tree peony plantlets was assessed using 20 SSR markers, and genetic profiling data suggested the absence of genetic variations in the micropropagated plants (Wen et al. 2016c). Therefore, the micropropagation protocol is reliable for producing true-to-type tree peony plants.

Productivity evaluation of the micropropagation protocol

To date, a wide range of cultivars have been successfully multiplied in vitro with high rates ranging from two to eight, and several cultivars have been reported to root in vitro and survive after being transplanted to *ex vitro* conditions. According to the method by Kaur and Sandhu (2015) in the industrialized production of sugarcane micropropagation, the productivity of the reported tree peony micropropagation protocols was evaluated (Table 2). It was found that the productivity varies from 1536 to 75,570,220 among different researches. In *P. suffruticosa* 'Wu Long Peng Sheng', 'Jin Pao Hong', *P.* × *lemoinei* 'High Noon', more than 10,000 plantlets can be produced from one bud after one year's in vitro culture (Wang 2008; Zhang 2008; Qiu 2010; Wen et al. 2016b; Wang et al. 2016), which demonstrates the great potential of micropropagation for commercial application in tree peony.

Problems and future perspectives

After the development in the past three decades, some of the existing micropropagation protocols have demonstrated great potential in the commercial application of tree peony; however, they are still unviable for commercial use due to the rooting and acclimatization problems. Moreover, there remains numerous tree peony genotypes, which are unable to proliferate under in vitro conditions due to the following issues.

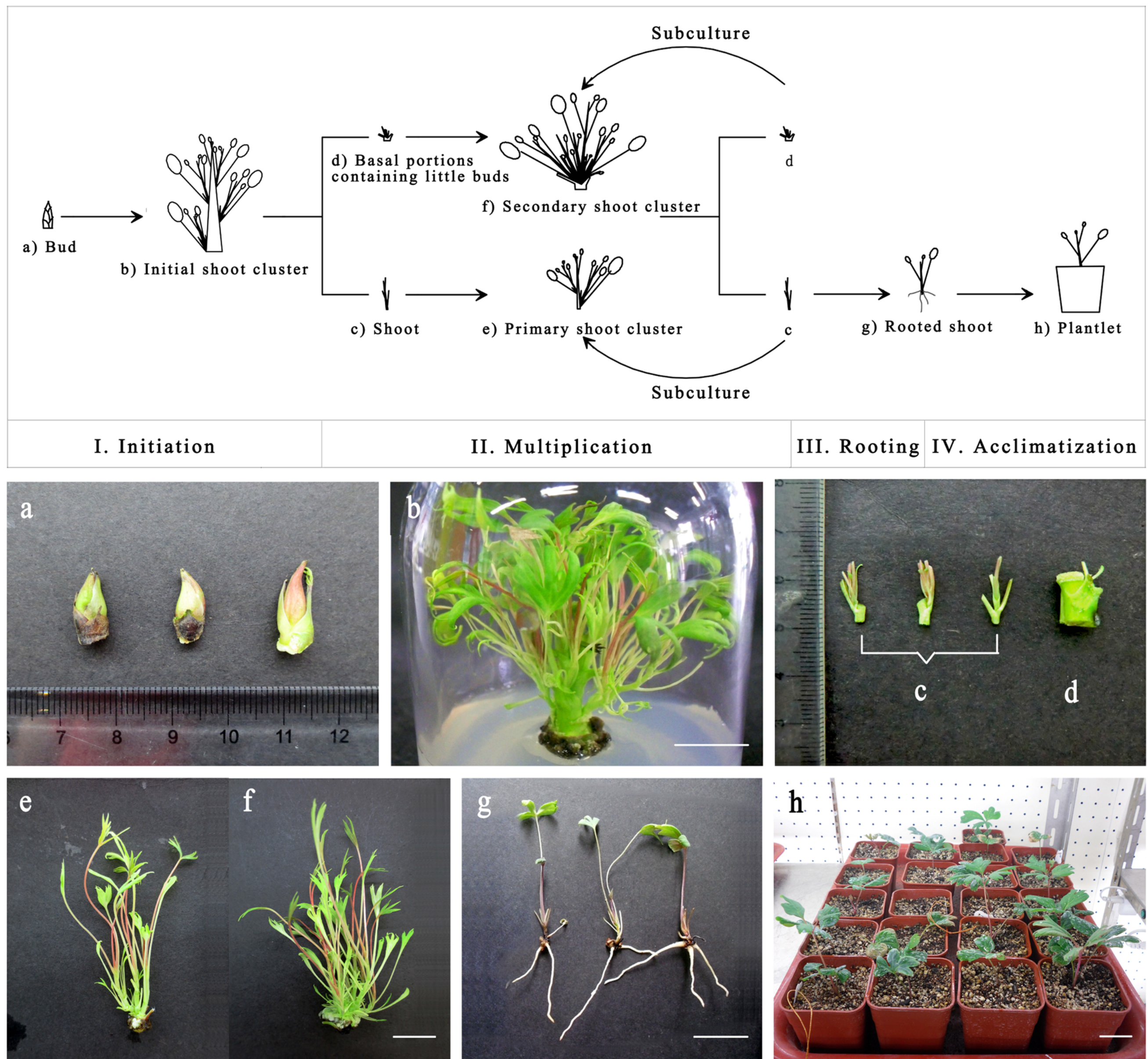

Fig. 1 Micropropagation of tree peony though bud culture. I. Initiation: buds (**a**) are used as explants and develop into shoot clusters (**b**) after initial culture. II. Multiplication: the shoot cluster is subdivided into single shoots (1–1.5 cm in length) (**c**) and the basal portions containing little buds (**d**) for the subculture, which develop into primary shoot clusters (**e**) and secondary shoot clusters (**f**), respectively. III. Rooting: shoots (1–1.5 cm in length) (**c**) are excised from the shoot cluster and cultured for adventitious rooting. IV. Acclimatization: the rooted shoots (**g**) are transplanted to ex vitro conditions, and the plantlets (**h**) are acclimatized under ex vitro conditions. The bars in the figure equal to 2 cm

In vitro rooting problems

Although some of the existing protocols have obtained rather high rooting percentage (80–100%) (Table 1), the commonly used two-step rooting method is complex and time-consuming thus increasing the production costs. Moreover, poor rooting was previously reported (≤ 60%) for more than half of the cultivars tested (Table 1), and many cultivars are still unable to root under the existing technical conditions.

As one of the most difficult-to-root plant, it is essential to explore the in vitro rooting mechanism of tree peony. By observing the histological changes that occur during in vitro rooting, it was found that no root primordia exist in the shoots before rooting (He et al. 2011; Jia et al. 2013), and thus rooting needs to be induced by exogenous auxin (IBA) (Table 1). By analyzing the endogenous hormone and enzyme changes during IBA-induced rooting, researchers proposed deficiencies in auxin content, transport, or

Table 2 Productivity evaluation of the micropropagation protocol

Species or cultivar	Multiplication		Rooting		Survival during acclimatization (S: %)	Plantlets obtained from one bud for year (P)	References
	MR	Subculture duration (days)	R (%)	Rooting duration (days)			
P. suffruticosa 'Bai Yu', 'Fengdan Bai', 'Shiyuan Bai', 'White Pearl', 'Yao Huang', 'Huang Hu', 'Orange Yellow', 'Golden Palace', 'Hu Hong', 'Da Jinfeng', 'Fish Scale Pink', 'Zhuangyuan Hong', 'Zi Erqiao', 'First Red', 'Red Diamond', 'Zhusha Lei', 'Orange', 'Red', 'Old Pink', *P. rockii* 'Xue Lian'	1.0–3.9	50	50	50	70–90	22–6820	Beruto and Curir (2007), Beruto et al. (2004)
P. suffruticosa 'Wulong Pengsheng', 'Da Huhong', 'Jin Ge'	5.4	30	55	50	30	3,374,374	Wang (2008)
P. suffruticosa 'Wulong Pengsheng', *P.* × *lemoinei* 'High Noon'	4.4	40	87–100	50	59	80,398	Zhang (2008)
P. × *lemoinei* 'High Noon'	4.8–7.0	35	81	58	33-85	359,364–27,615,386	Qiu (2010)
P. suffruticosa 'Wulong Pengsheng', 'Jinpao Hong', *P.* × *lemoinei* 'High Noon'	4.4–4.8	35	80–100	50	83	90,482–226,871	Wen et al. (2016b)
P. × *lemoinei* 'High Noon'	3.0	35	77	70	92	4508	Wen et al. (2016a)
P. × *lemoinei* 'High Noon'	2.7	35	62	50	91	4173	Wen et al. (2016c)
P. suffruticosa 'Feng Dan'	3.9	40	57	50	67	19,826	Wang et al. (2016)
P. suffruticosa 'Feng Dan'	2.7	35	40	30	98	8743	Wang et al. (2018)

MR multiplication rate, *R* rooting percentage, *S* Survival during acclimatization, *C* contamination rate

$P = MR^{n} \cdot R \cdot S \cdot (1 - C)$ (Kaur and Sandhu 2015)

Contamination rate (C) was taken as 3% for calculation

perception mechanisms as the major explanations for the rooting recalcitrance of tree peony (Bouza et al. 1992; He et al. 2011). In addition, non-auxin related factors can also block rooting. For instance, Fu et al. (2016) attributed the poor rooting of tree peony to deficiencies in phenolic acid contents, as some of the phenolic acid (paeoniflorin, benzoic acid, and 4-hydroxy benzoic acid) contents were higher in easy-to-root tree peony cultivars. However, those studies are still insufficient to reveal the mechanism for the rooting recalcitrance of tree peony; the molecular mechanisms involved remain unknown and should be the emphasis for future research.

Acclimatization problems

Although several tree peony cultivars have been reported to acclimate ex vitro, poor survival rates ranging from 30 to 80% occured during acclimatization (Table 1). According to previous studies, the main reasons for the poor survival of plantlets can be concluded as follows: (i) apical bud dormancy is induced during the root induction (Bouza et al. 1992, 1994c; Wen et al. 2016b; Wang et al. 2016); and (ii) in comparison with in vitro conditions, the *ex vitro* conditions have substantially lower relative humidity, higher light levels, and a septic environment, which are stressful to micropropagated plants (George et al. 2008). Recently, although AMF inoculation have been proposed as a useful tool for overcoming these problems (Wen et al. 2016b), the study was conducted using pure and expensive AMF, and commercial AMF fertilizer is still unavailable in the market. Therefore, the development of commercial AMF fertilizer is required for the improved acclimatization of tree peony plantlets. In addition, as the response to AMF was found to be dependent on both the genotype and the AMF species used for inoculation (Zeng et al. 2011), future studies also need to develop suitable host-endophyte combinations in a wide range of tree peony cultivars.

Browning

Browning is a common problem in the tissue culture of woody plants such as tree peony and is usually attributed to phenolic compounds being oxidized to highly toxic quinones by polyphenol oxidase (PPO) (An and Zhao 2005). Browning is a stumbling block for the micropropagation of tree peony, which has been reported to inhibit growth, cause lower rates of multiplication or rooting, and even lead to cell/tissue/plant death (George et al. 1984).

To prevent or ameliorate browning in tree peony, some advances have been made, including the sampling of explants at low temperature in the winter or early spring (Chen 2005; Kong and Zhang 1998), using dark culture (e.g., during the root induction stage of tree peony) (Chen 2005), and using low-salt medium (WPM) and suitable PGRs (He et al. 2005; Lang et al. 2007). In addition, some more targeted approaches of amending the culture medium with anti-browning agents have also been employed. The majority of anti-browning agents can be divided into two categories: (i) antioxidants, such as ascorbic acid, melatonin, or citric acid, which reduce oxidative stress and prevent the oxidation of phenolic compounds; (ii) adsorbents, such as activated charcoal or polyvinylpyrrolidone, which bind phenolic compounds, rendering them less toxic (Zhang and Luo 2006; Li et al. 2008a, b). While the aforementioned approaches have reduced browning in several micropropagation systems, the problem persists in many cultivars. More efficient methods are still needed to address this fundamental challenge in tree peony micropropagation.

Vitrification

Vitrification, also known as hyperhydricity, is a common morphological, anatomical, and physiological disorder during plant in vitro culture (Kevers et al. 2004). The typical 'glassy' morphology is characterized by a translucent, less green, turgid, wrinkled, curled, and brittle appearance, which is the result of chlorophyll deficiency, poor lignification, and excessive hydration of the tissues (Sreedhar et al. 2009; Huang et al. 2010; Hassannejad et al. 2012).

In tree peony micropropagation, the degree of vitrification varies from 0 to 76.2% among cultivars (Li and Kong 2010). Moreover, micropropagation of *P. rockii* (an important tree peony species for oil production) was completely hindered by vitrification, as 8 cultivars showed 100% vitrification under in vitro conditions (unpublished results of our laboratory). The vitrified shoots, including those that are only very slightly vitrified, are difficult to proliferate and almost impossible to root and survive after transplantation (Chu and Li 1992). Tree peony pollen after vitrification cryopreservation has a variety of change trends in viability, with most pollens showing decreased viability (Ren et al. 2019). Although some methods have been tested to reduce vitrification in tree peony, including modifying the concentrations of gelling agents, changing the light intensity, and reducing the BA concentration (An 2005; Li and Kong 2010), using one or several methods together described above usually cannot suitably prevent vitrification. Currently, research concerning the vitrification of tree peony is insufficient and further studies are required to control vitrification for commercial clonal propagation.

Conclusion

Over the past three decades, the great advances in tree peony micropropagation have brightened the prospects of the efficient propagation and breeding of this plant (Table 1; Fig. 1). The advances include that (i) aseptic culture has been developed for most of the cultivars tested; (ii) a wide range of cultivars have been successfully multiplicated in vitro with high multiplication rates ranging from 2 to 8; and (iii) several cultivars have been reported to root in vitro and survive after transplantation to *ex vitro* conditions. However, the protocol is still unviable for commercial application, and there are still major issues concerning in vitro rooting, acclimatization, browning and vitrification. This review should facilitate a more targeted and in-depth level of research for the successful micropropagation of tree peony, thus promoting its propagation and breeding.

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