Efficient plant regeneration via meristematic nodule culture in *Paeonia ostii* ‘Feng Dan’

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Abstract

Tree peony (*Paeonia* sect. *Moutan*) is an economically important multipurpose woody plant in terms of its medical, ornamental and oil values, but its breeding and industrial development are severely limited due to inefficient traditional propagation methods and existing in vitro regeneration systems. Meristematic nodules (MNs) are an attractive alternative to solve this problem. This study first presented a protocol for in vitro regeneration of *P. ostii* ‘Feng Dan’ via MN culture with four consecutive steps, including embryogenic callus (EC) formation, MN induction and leaf cluster differentiation, shoot elongation, rooting and acclimatization. The highest EC induction rate (81.25%) was achieved when cotyledons were cultured on modified Murashige and Skoog (mMS) medium with 4.04 µM N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) + 5.37 µM α-naphthylacetic acid (NAA) for 30 days. The optimal MN induction rate (100%) and leaf cluster differentiation rate (45.83%) were obtained when ECs were cultured on modified woody plant medium (mWPM) supplemented with 2.02 µM CPPU + 2.27 µM thidiazuron (TDZ) for a subculture time of 10 days. The combination of 1.29 µM 6-benzyladenine (BA) + 0.58 µM gibberellin (GA3) yielded the best shoot elongation (13.40 shoots per nodule), rooting rate (43.33%) and consequently survival rate (45.83%). The study will be beneficial to the mass propagation, breeding and genetic improvement of tree peony.

Key message

A protocol for in vitro regeneration of *Paeonia ostii* ‘Feng Dan’ via meristematic nodules culture was firstly presented.

Keywords Tree peony · *Paeonia ostii* ‘Feng Dan’ · Organogenesis · Meristematic nodule

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Abbreviations

AC Activated carbon
BA 6-Benzyladenine
CIM Callus induction medium
CK Cytokinin
CPPU N-(2-chloro-4-pyridyl)-N-phenylurea
DAA Days after anthesis
EC Embryogenic callus
GA3 Gibberellin
IBA Indole-3-butyric acid
MSIM MN/shoot induction medium
MN Meristematic nodule
mMS Modified Murashige and Skoog
mWPM Modified woody plant medium
NAA α-Naphthylacetic acid
Put Putrescine
PGR Plant growth regulator
TDZ Thidiazuron
2,4-D 2,4-Dichlorophenxyacetic acid
Introduction

Tree peony (*Paeonia sect. Moutan*) is an economically important woody plant in China because of its medical, ornamental and oil value (Yu et al. 2016). *P. ostii* is main option for oil tree peony because of the high yield and adaptability. However, the low efficiency and long cycle of conventional propagation methods, such as grafting and division, severely constrain its breeding and are insufficient to address the increasing commercial demands. Thus, an efficient and stable *in vitro* regeneration system is urgently needed. In general, no regeneration system published to date can meet the needs for the propagation and genetic manipulation of tree peonies due to various obstacles, such as vitrification, low multiplication, poor rooting and difficult acclimatization in micropropagation (Beruto et al. 2004; Wen et al. 2020), rare differentiation in callus culture (Zhu et al. 2018), and high deformity rates and low germination in somatic embryogenesis (Du et al. 2020a, b); hence, innovative breakthroughs are needed to overcome this problem.

Meristematic nodules (MNs) are special structures comprising of organization centers (OCs), a cortical-like area of parenchymatous cells and an epidermal-like area under histological observation, which have high regeneration potential, genetic stability and long-term cellular dynamics, making them an attractive alternative for plant regeneration via organogenesis, *in vitro* phytochemical production and plant transformation (McCown et al. 1988; Batista 2008). Successful in vitro regeneration through MN culture has been reported in several woody and herbaceous plants, such as *Eucalyptus globulus* (Dobrowolska et al. 2017), *Liquidambar orientalis* (Bayraktar et al. 2015), *Populus euphratica* (Ferreira et al. 2009), *Humulus lupulus* (Fortes and Pais 2000), *Sclerocarya birrea* (Moyo et al. 2009) and *Billbergia zebrina* (Dal Vesco et al. 2011). The regeneration patterns were significantly affected by genotype, plant growth regulators (PGRs) and subculture time (Xie and Hong 2001; Trindade and Pais 2003). Therefore, the optimal culture conditions should be screened with genotype changes.

There are scarce reports of MNs in *Paeonia*. Zhong (2011) established a nodule induction and multiplication system of *P. rockii* through calli induced from petiole sections. Subsequently, Qin et al. (2012) further optimized the system in *P. lemoinei* ‘Golden Era’ and *P. itoh* ‘Barzella’ but only found a 16% differentiation rate in *P. itoh* ‘Barzella’, and no rooted shoots and surviving plantlets were obtained. Overall, efficient *in vitro* shoot organogenesis via MN culture is still needed for tree peony. Thus, the aim of the present study was to establish a protocol for *in vitro* regeneration of *P. ostii* ‘Feng Dan’ based on an MN culture system. This study could be beneficial to the large-scale propagation, genetic improvement, gene identification and functional analysis of tree peony.

Materials and methods

Plant material and sterilization

Mature seeds of *P. ostii* ‘Feng Dan’ were collected in August 2018 at 90 days after anthesis (DAA) from living adult plants grown in Beijing Guose Peony Garden in Beijing, China (40°45′N, 115°97′E) (Fig. 1A) and washed under running tap water for 15 min before soaking in commercial liquid detergents (1% v/v; 5 min). Then, seeds were sterilized by dipping in ethanol (70% v/v; 30 s), followed by dipping in a solution of NaOCl (0.2% v/v; 5 min) and rinsed three times with sterile distilled water.

Medium and culture conditions

The basal media was modified Murashige and Skoog medium (mMS, half-strength macroelements and full-strength Ca²⁺) (Murashige and Skoog 1962),1/2 MS (all macroelements at half-strength) and modified woody plant medium (mWPM, double strength of Ca²⁺) (Lloyd and McCown 1980). All media were supplemented with 3% sucrose and 0.7% agar, and the pH was adjusted to 5.8–6.0 before autoclaving (at 118 kPa and 121 °C for 20 min). All reagents were supplied by Biodee (Beijing, China). The cultures, if not otherwise stated, were maintained at 24 ± 1 °C under a 16 h photoperiod of 50 µmol·m⁻²·s⁻¹ illumination intensity provided by LED light (70% red light +30% blue light) (TLD 36 W Philips, Beijing, China).

Callus induction

Zygotic embryos were aseptically isolated from seeds and inoculated on germination medium [mMS + 2.57 µM 6-benzyladenine (BA) + 2.89 µM gibberellin (GA₃)] for 15 days. The expanded cotyledons (Fig. 1B) were cut...
into 1×1 cm pieces and inoculated on callus induction medium (CIM) [mMS + 5.37 μM α-naphthalacetic acid (NAA) + 2.02 μM N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU)] with the abaxial side touching the medium, under the dark conditions. The embryogenic callus (EC, yellow compact calli with densely arranged clumps) (Fig. 1C) induction rate (%) and browning rate (%) were calculated after cotyledons were inoculated on CIM with different induction times (20, 30, 40 and 50 days). Each experiment was conducted in three replications with 16 explants in each replication.

To screen optimal PGRs for callus induction, two experiments were carried out in chronological order. The EC induction rate (%), browning rate (%) and callus biomass (g) were calculated after 30 days. Each experiment was conducted in three replications with 16 explants in each replication. Step 1: Two auxins alone, 5.37 μM NAA, 4.52 μM 2,4-dichlorophenxyacetic acid (2,4-D), and in combination with different cytokinins (CKs), including 2.27 μM thidiazuron (TDZ), 2.02 μM CPPU and 2.57 μM BA, were used to screen suitable PGRs. Step 2: Based on the results obtained in step 1, the combination of NAA (1.34, 2.69, 5.37, 10.74 μM) with CPPU (1.00, 2.02, 4.04 μM) was used to further select the best concentration of PGRs.

**Meristematic nodule induction and leaf cluster differentiation**

The ECs induced on optimal CIM after 30 days of culture were inoculated into MN/shoot induction medium (MSIM) (mWPM + 2.02 μM CPPU) to optimize the subculture time for MN induction and leaf cluster differentiation. The MN induction rate (%), leaf cluster differentiation rate (%) and browning rate (%) were determined after 12 subcultures with different subculture times (10, 15, 20, 25 d), respectively. Each experiment was conducted in three replications with 16 explants in each replication.

To examine the effects of PGRs and callus induction time on MN induction and leaf cluster differentiation, two experiments were designed. The MN induction rate and leaf cluster differentiation rate were determined after 12 subcultures with different subculture times (10, 15, 20, 25 d), respectively. Each experiment was conducted in three replications with 16 explants in each replication. Experiment 1: ECs induced on optimal CIM with different days of culture (20, 30, 40, 50 d) were inoculated into MSIM containing different PGR combinations (2.02 μM CPPU, 2.02 μM CPPU + 2.27 μM TDZ, 2.02 μM CPPU + 2.57 μM BA, 2.02 μM CPPU + 2.69 μM NAA, 2.02 μM CPPU + 0.58 μM GA3), with a subculture time of 20 days. Experiment 2: ECs induced on optimal CIM with 30 days of culture were used. Based on experiment 1, an orthogonal test with three factors and three concentrations of CPPU (1.00, 2.02, 4.04 μM), TDZ (0, 2.27, 4.54 μM) and BA (0, 2.57, 5.15 μM) was established, with a subculture time of 10 days.

**Shoot elongation, rooting and acclimatization**

Nodules with leaf clusters cultured on the optimal MSIM after 12 subcultures with a subculture time of 10 days, were cultured on mWPM containing 1.29 μM BA and (0.29, 0.58, 0.87, 1.15 μM) GA3 for 5 months with a subculture time of 30 days. Shoots induced from leaf clusters and elongated to 1–3 cm were successively excised and cultured for rooting. The mean number of shoots per nodule (n) was calculated, and each experiment included three replications of 5 nodules per treatment. According to Wang et al. (2016), shoots were cultured on root induction medium [1/2 MS + 4.92 μM indole-3-butric acid (IBA) + 11.34 μM putrescine (Put)] for 38 days in the dark and then transferred to root expression medium [PGR-free 1/2 MS + 0.4% activated carbon (AC)] for 20 days in the light. During the root induction phase, the shoots were subjected to cold treatment (4 °C, 8 days) in the dark and then cultured at 24 ± 1 °C for another 30 days. The rooting rate (%) was calculated, and each experiment included three replications of 30 shoots per treatment. The rooted plantlets were removed carefully from the medium, washed thoroughly with tap water, and finally transferred to pots containing a mix of an autoclaved vermiculite, peat, and perlite (1:1:1, v/v/v) substrate. The plantlets were grown in a culture chamber at 20 ± 1 °C under a 16 h photoperiod of 50 μmol·m−2·s−1 photosynthetic photon flux density provided by fluorescent lamps, and the survival rate (%) was recorded two months after acclimatization with three replications of 30 rooted plantlet per treatment.

**Statistical analysis**

Prior to data analysis, arcsine transformation was carried out for any percentage data, and were inversely transformed for presentation after analysis. Statistical analysis was performed with SPSS 22.0 (SPSS Inc., Chicago, USA), and Microsoft Excel 2013 software (Microsoft Corp., Richmond, ca., USA) was used for data statistics and charts. The data were submitted to analysis of variance (ANOVA) followed by Duncan’s multiple range test at p ≤ 0.05 and expressed as the mean ± standard error.

**Results**

**Effect of induction time on callus formation**

A small proportion of calli (33.33%) expanded into clumps of ECs after 20 days, and the browning rate was 6.25% (Fig. 2). The EC induction rate and browning rate gradually
increased with induction time but with no significant difference between 30 and 50 days of treatment. The selection of the optimal induction time should comprehensively consider the EC induction rate, browning rate and differentiation potential (following in this paper).

**Effect of plant growth regulators on callus induction**

The EC induction rate (12.50%, 8.33%) and biomass (1.20 g, 1.05 g) when NAA and 2,4-D alone were applied were significantly lower than those grown on the combination with CKs, but the browning rate (93.75%, 97.92%) was significantly higher. The highest EC induction rate (81.25%) and biomass (1.73 g) were obtained under 2.02 µM CPPU + 5.37 µM NAA with a low rate of browning (12.50%) (Fig. 3).

The optimal concentrations of obtained suitable PGR combinations were further studied in step 2. The EC induction rate and biomass were generally improved with CPPU and NAA concentration, but decreased at higher level (10.74 µM) in NAA. The highest EC induction rate was 87.50%, with the highest biomass (1.58 g) and lowest browning rate (10.42%) when 4.04 µM CPPU + 5.37 µM NAA was used (Fig. 4).

**Meristematic nodule formation and leaf cluster differentiation**

Morphological study revealed that development of MN and leaf cluster differentiation were asynchronous and chronologically included four main stages: ①Pre-nodular structures: During 1–4 cycles of subculture on MSIM, ECs turned green and visible small protuberances were gradually observed on the surface (Fig. 5A). ②MNs: After subculturing for 5–6 times, protuberances greatly increased in diameter (Fig. 5B). ③Nodular clusters: During 7–10 subcultures, MNs proliferated with small grooves that appeared on the surface of nodules, and then progressively deepened, without observing nodule separation. Several MNs displaying different levels of development were loosely attached to each other and developed into nodular clusters in appearance (Fig. 5C). ④Leaf cluster differentiation: After 11–12 subcultures, differentiated leaf clusters started to develop and elongate (Fig. 5D).

**Effect of subculture time on meristematic nodule induction and leaf cluster differentiation**

The MN induction rate and leaf cluster differentiation rate decreased, while the browning rate significantly increased with prolonged subculture time (Fig. 6). The optimal
The rate of MN induction and leaf cluster differentiation significantly decreased with callus induction time among all PGR treatments (Fig. 7). Comprehensively considering the effects of induction time on the EC induction rate and browning rate, 30 days was selected as the best callus induction time in this paper.

Among all the media tested, combinations of CPPU and GA$_3$ failed to induce MNs and subsequently leaf. The promoting effect of CPPU + NAA treatment on the MN induction rate and differentiation rate was conspicuously lower than that with CPPU alone or in combination with CKs, while the combinations of CPPU + TDZ and CPPU + BA resulted in significantly higher rates than that of CPPU alone with no significant difference between them, which indicated combinations of CPPU + BA + TDZ might be conducive to MN induction and differentiation.

The concentrations of CPPU, BA and TDZ were screened by orthogonal test to obtain the optimal PGRs combination (Table 1). CPPU and TDZ had significant effect both on the MN induction and leaf cluster differentiation rates ($p < 0.01$), but BA showed no significant effect on the MN induction rate ($p > 0.05$) (Table 2). The best performance of MN induction rate was obtained at 4.04 µM CPPU, 0 µM BA and 4.54 µM TDZ respectively, but no significant difference between 2.02 and 4.04 µM CPPU, 0 and 5.15 µM BA as well as 2.27 µM and 4.54 µM TDZ (Table 3). For the leaf cluster differentiation rate, the optimal concentration of CPPU, BA and TDZ was 2.02 µM, 0 µM and 2.27 µM respectively, but no significant difference between 2.27 and 4.54 µM TDZ (Table 3). Based on above results, the suitable medium for MN induction was mWPM + 4.04 µM CPPU + 4.54 µM TDZ, and the optimal medium for enhancing leaf cluster differentiation was mWPM + 2.02 µM CPPU + 2.27 µM TDZ, with the highest MN induction rate (100%) and leaf cluster differentiation rate (45.83%).

**Effect of GA$_3$ concentration on shoots elongation, rooting and seedling acclimatization**

Nodules with clusters of leaves were not conductive to promote shoot elongation when cultured in the same MSIM (Fig. 8A), however after transferring to the medium containing BA and GA$_3$, successful shoot elongation was achieved (Fig. 8B). Mean number of shoots per nodule was increased with GA$_3$ concentration and obtained the best at 0.58 µM, with 13.40 shoots per nodule (Table 4). Reversely, the rooting and survival rate of elongated shoots were gradually declined with GA$_3$ concentration, and no significant difference was found between 0.29 and 0.58 µM GA$_3$ treatment (Table 4). Therefore, mWPM + 1.29 µM BA + 0.58 µM GA$_3$ was the optimal medium for shoots elongation, rooting, and further seedling acclimatization. The rooted seedlings were successfully transferred into pots and developed well in the chamber (Fig. 8C, D).
Discussion

To our knowledge, this is the first report of MNs culture system capable of de novo shoot organogenesis in tree peony. MNs have been identified in both direct (Piéron et al. 1993, 1998; Trindade and Pais 2003; Ferreira et al. 2009; Moyo et al. 2009), and indirect morphogenesis pathways through an intervening callus phase (Aitken-Christie et al. 1988; Batista et al. 2000; Fortes and Pais 2000; Xie and Hong 2001). In our studies, the MNs was initiated indirectly form calli derived from cotyledons of *P. ostii* ‘Feng Dan’, which was consistent with previous descriptions in *P. lemoinei* ‘Golden Era’ (Qin et al. 2012) and *P. rockii* (Zhong 2011). Our study demonstrated that organ differentiation from callus was preceded by a MNs stage arise through three developmental phases, including pre-nodular structure, MNs and nodular clusters in sequence. Differentiation occurred only in these nodular areas, suggesting the nodules was prerequisites of de novo shoot organogenesis in tree peony. Similar phenomenon was reported in *Humulus lupulus* that the production of nodules on the callus had been described as a sign of shoot initiation and the development stage of nodules was analogously divided into 3 stages, consisting of pre-nodular structures, MNs and nodular clusters in sequence. Differentiation occurred only in these nodular areas, suggesting the nodules was prerequisites of de novo shoot organogenesis in tree peony. Similar phenomenon was reported in *Humulus lupulus* that the production of nodules on the callus had been described as a sign of shoot initiation and the development stage of nodules was analogously divided into 3 stages, consisting of pre-nodular structures, MNs and ‘polycenter nodules’ (McCown et al. 1988; Batista et al. 1996, 2000).

The acquisition of ECs was a prerequisite for de novo shoot organogenesis. Effect of PGRs on callus induction was dependent on PGRs type, concentration, genotype and explant type. Auxin was necessary for callus induction in tree peony (Du et al. 2020a, b). NAA and 2,4-D are commonly used auxins for callus induction. In this paper, there was no significant difference between the effect of NAA and 2,4-D on the ECs induction rate, the browning rate and

### Table 1

| CPPU (µM) | BA (µM) | TDZ (µM) | The MN induction rate (%) | The leaf cluster differentiation rate (%) |
|-----------|---------|----------|---------------------------|------------------------------------------|
| 1.00      | 0       | 0        | 85.42 ± 3.61<sup>c</sup> | 31.25 ± 0.00<sup>b</sup> |
| 1.00      | 2.57    | 2.27     | 97.92 ± 3.61<sup>a</sup> | 18.75 ± 6.25<sup>c</sup> |
| 2.02      | 0       | 2.27     | 100.00 ± 0.00<sup>a</sup> | 25.00 ± 0.00<sup>de</sup> |
| 2.02      | 5.15    | 0        | 93.75 ± 0.00<sup>b</sup> | 29.17 ± 3.61<sup>bc</sup> |
| 4.04      | 0       | 2.27     | 100.00 ± 0.00<sup>a</sup> | 8.33 ± 3.61<sup>f</sup> |

Different letters within a column show significant differences by Duncan’s multiple range tests (*p*≤0.05). Each data represent mean ± standard error.

### Table 2

| Source of variance | The MN induction rate (%) | The leaf cluster differentiation rate (%) |
|-------------------|---------------------------|------------------------------------------|
|                   | df | *F* test | *p* value | df | *F* test | *p* value |
| CPPU              | 2  | 8.94     | 0.00      | 2  | 21.65    | 0.00      |
| BA                | 2  | 0.12     | 0.89      | 2  | 80.78    | 0.00      |
| TDZ               | 2  | 24.47    | 0.00      | 2  | 8.84     | 0.00      |
| Error             | 20 |          |           | 20 |          |           |

Fig. 7 Effect of callus induction time and plant growth regulators (PGRs) on the meristematic nodule induction rate and leaf cluster differentiation rate in *Paeonia ostii* ‘Feng Dan’. Different letters show significant differences by Duncan’s multiple range tests (*p*≤0.05).
biomass, but previous studies suggested that 2,4-D was not conducive to subsequent callus differentiation and explants status (Shen et al. 2018; Wang et al. 2008). Moreover, combination of auxin and CKs had a better effect on the induction of ECs than auxin alone. Similar results have been extensively concluded in other plants (Du et al. 2020a, b; Ozudogru et al. 2010). Additionally, variations in the optimal PGRs for callus induction among different species and explants were documented in tree peony. In P. ostii ‘Feng Dan’, application of 4.04 µM CPPU + 5.37 µM NAA had the best performance on callus formation from cotyledon explants in our results, but 1.14 µM 2,4-D + 10.3 µM BA showed the most effective with filaments as explants (Du et al. 2020a, b). However, medium containing 2.27 µM 2,4-D was the optimal for callus induction in P. lemoinei ‘Golden Era’ using petiole layers as explants (Qin et al. 2012). This might ascribe to variations of their physiological background.

CKs play a crucial role in in vitro morphogenesis. The appropriate CKs type for MNs induction and differentiation was genotype dependent, for example, BA was recommended for MNs induction of Radiata pine (Aitken-Christie et al. 1988) and Eucalyptus globulus (Trindade and Pais 2003), TDZ for Pelargonium × hortorum, Pelargonium × domesticum (Haensch 2004) and Acacia mangium (Xie and Hong 2001), CPPU for Eucalyptus botryoides (Ito et al. 1996). According to previous studies, high endogenous CKs promoted cell fate transition from callus cells to de novo shoot organogenesis by mediating up-regulated expression of WUS gene (Trifunović-Momčilov et al. 2016; Gordon et al. 2007; Meng et al. 2017; Xiao et al. 2018). On the contrary, some studies had emphasized that a proper auxin/CK ratio was key factor for effective shoot regeneration, instead of sole CK or auxin level (Cheng et al. 2010; Zhang et al. 2008). In addition, the alteration of endogenous hormones content was varied among exogenous CKs with different activity (Ivanova et al. 2006; Yu et al. 2020). Thus, it is speculated that different needs on PGRs for de novo shoot organogenesis among species might ascribe to distinct in their requirement on endogenous hormone level, maybe CKs or auxin/CK ratio.

CPPU, substituted phenylurea compounds, was considerably more effective in promoting in vitro morphogenesis than several other CKs in plants, especially in some recalcitrant species (Huang, et al. 2015; Montalbán et al. 2011). These kinds of plants may need higher level of endogenous CKs or auxin/CK ratio for expression of related gene, thereby starting differentiation. Tree peony probably belong

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**Table 3** Visual analysis of effect of plant growth regulators on the MN induction and leaf cluster differentiation rates in *Paeonia ostii* ‘Feng Dan’

|          | MN induction rate (%) | Leaf cluster differentiation rate (%) |
|----------|-----------------------|---------------------------------------|
|          | CPPU | BA  | TDZ | CPPU | BA  | TDZ |
| k1       | 90.97b | 95.14a | 88.19b | 23.61b | 35.42a | 18.06b |
| k2       | 95.14a | 94.44a | 96.53a | 27.78a | 18.75b | 25.00a |
| k3       | 97.92a | 94.44a | 99.31a | 15.97c | 13.19c | 24.31a |
| R        | 4.87  | 8.34 | 6.94 | 12.5  | 20.84 | 4.17  |

Different letters within a column show significant differences by Duncan’s multiple range tests (p ≤ 0.05).

**Table 4** Effect of GA₃ on shoot elongation, rooting and acclimatization in *Paeonia ostii* ‘Feng Dan’

| GA₃ (µM) | Mean number of shoots per nodule (n) | The rooting rate (%) | The survival rate (%) |
|----------|-------------------------------------|----------------------|----------------------|
| 0.29     | 7.93 ± 0.42d                        | 46.67 ± 3.34a        | 50.00 ± 0.00a        |
| 0.58     | 13.40 ± 0.20a                       | 34.44 ± 5.09b        | 41.67 ± 7.22ab       |
| 0.87     | 10.47 ± 0.31b                       | 27.78 ± 1.92b        | 33.33 ± 7.22b        |

Different letters within a column show significant differences by Duncan’s multiple range tests (p ≤ 0.05). Each data represent mean ± standard error.
to it. Compared with BA, CPPU had great potential in shoot organogenesis induction in *P. rockii* ‘Jing Hong’ (Du et al. 2020a, b). In the present study, CPPU remarkably promoted MNs induction and differentiation from callus, but no efficient organogenesis of tree peony was achieved in previous studies with other CKs, like BA, KT and TDZ (Du et al. 2020a, b; Qin et al. 2012; Zhong 2011). Simultaneously, mixed use of CKs presented better response than single one, which was in accordance to results in *Garcinia mangostana* (Te-chato and Lim 2000). However, studies on *Eucalyptus globulus* showed contradictory results, BA enhanced both node induction and shoot organogenesis, but in the presence of CPPU, explants showed vitrification and no morphogenesis (Trindade and Pais 2003). This might be attributed to the fact that CPPU was far beyond its requirement on CKs activity. In addition, combination of CPPU + NAA and CPPU + GA3 showed negligible effect on differentiation. The endogenous hormone may be changed in different direction and could not achieve the requirements for differentiation with the addition of NAA or GA3. Further in-depth studies about the close connection between endogenous hormone and regeneration need to carry out in the future.

Our results revealed that not all the nodules were able to induce shoots, also, the best efficiency of MNs induction and subsequent differentiation required different PGRs combinations according to variance analysis. Similar result was described by Ito et al. (1996) who found nodules were induced preferentially with CPPU, but shoot organogenesis occurred on the medium with BA. Therefore, a two-step procedure for MNs induction and differentiation in tree peony is a good prospect to further optimize the regeneration system, like scale-up multiplication of MNs in liquid medium and subsequent shoots differentiation in solid medium with different PGRs (Scherer et al. 2013). Additionally, shoots elongation from nodules differentiated with leaf clusters on the MSIM was slowly, whereas a plenty of newly formed shoots were elongated efficiently with GA3 application. The necessity of shoot elongation step in medium supplemented with GA3 had been emphasized in previous findings (Scherer et al. 2013; Kongbangkerd and Wawrosch 2003; Xie and Hong 2001). Concurrently, only after the elongated shoots were excised did the nodules continue mass differentiation.

The inhibition of shoots organogenesis by preexisting shoots had also been reported in Ferreira et al. (2009) where continuous organogenesis of new shoots from nodules of *Populus euphratica* only occurred in regions where previous shoots had been removed. This behavior resembled an apical dominance mechanism, that further shoot organogenesis might be suppressed by the auxins produced in the first shoots (Gurriarán et al. 1999).

Similar morphological response pattern between effect of induction time and subculture days was observed. In detail, the differentiation rate was gradually decreased while the browning rate aggravated with the prolong of time, indicating the decline of organogenic potential. This phenomenon might be result from depletion of hormone content in medium, accumulation and oxidation of phenolic compounds in tissues resulting in the inhibition of growth and a decrease in the regeneration ability of plant cells with long time culture (Jones and Saxena 2013).

**Conclusion**

In the present study, an efficient in vitro regenerative system was developed for the first time via MN culture in *P. ostii* ‘Feng Dan’. The protocol was divided into four consecutive steps, including EC formation, MN induction and leaf cluster differentiation, shoot elongation, rooting and acclimatization. De novo shoot organogenesis and surviving plantlets were realized through screening of optimal culture conditions, which revealed that this protocol is feasible for mass propagation and genetic transformation of tree peony.

**Author contribution** LX conducted the experiments and written the manuscript. FYC and YZ revised the manuscript. All authors read and approved the final manuscript.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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