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Effects of 2,4-D on Callus Formation in Tree Peony (*Paeonia suffruticosa* Andr.) under Different Light Conditions

Haiyun Wang[1,2] • Songlin He[1*] • Michio Tanaka[2] • Pham Thanh Van[3] • Jaime A. Teixeira da Silva[2]**

[1] Faculty of Forestry, Henan Agricultural University, No. 95 Wenhua Road, Zhengzhou City, 450002, China
[2] Department of Applied Biological Science, Faculty of Agriculture and Graduate School, Kagawa University, Miki-cho, Kagawa 761-0795, Japan
[3] The United Graduate School of Agricultural Science, Ehime University, Matsuyama, Ehime 790-8566, Japan

Corresponding author: **hsl213@163.com **jaimetex@yahoo.com**

ABSTRACT

Petioles and leaves of three-month-old *in vitro* tree peony (*Paeonia suffruticosa* Andr. cv. 'Wu Long Peng Sheng') plantlets were used as explants for callus induction. The effects of different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and different light sources and quality were investigated. If no 2,4-D was present in the medium, then no callus formed. To induce maximum callus formation from tree peony, either 1 mg L^{-1} 2,4-D using petiole explants under red or blue cold cathode fluorescent lamps, or the same concentration of 2,4-D using leaf explants in the dark, was required.

Keywords: 2,4-dichlorophenoxyacetic acid, CCFL, light source

INTRODUCTION

Tree peony (*Paeonia suffruticosa* Andr.), a member of the genus *Paeonia*, family *Paenoiaceae*, has a rich history as an important and famous ornamental flower in China. However, conventional culture procedures (seed breeding, plant division, grafting) have numerous problems in actual commercial production. Micropropagation, specifically tissue culture and somatic embryogenesis would be the most effective procedure to standardize the uniformity of tree peony to ensure stable quality plantlets for nurseries and breeding programmes. Callus culture is the first step to achieving somatic embryogenesis.

Even though tissue culture of tree peony can potentially maximize the propagation rate and reduce the breeding period, several production problems *in vitro* remain even though many advances have been made in the micropropagation of this ornamental tree species. Several types of explants have been used to generate callus: buds, leaves, stems, petioles, p*etal*s, filaments, among others (Li *et al*. 1984; Chen *et al*. 2001; Beruto *et al*. 2004; An 2005; Li *et al*. 2005; Shi *et al*. 2005; Niu 2009). Callus formation in tree peony is variable and the percentage of callus formation differs depending on the treatment, the explant source (**Table 1**). Ding *et al*. (2010) showed that by manipulating the light spectral quality of cold cathode fluorescent lamps

Table 1 Main studies in which callus was induced in tree peony.

Explant source	How to induce callus	Effect on callus formation	References
Petioles, leaves	The basal MS medium supplemented with 6-BA (2.0 mg L^{-1}) and NAA (0.1-0.5 mg L^{-1}).	Callus formation (%): rates were leaves (~30%); petioles (70-80%).	Li *et al*. 1984
Buds, leaves and petioles from plantlets *in vitro*; carpels, stamens	Basal MS medium supplemented with 1.5 mg L^{-1} BA and 2.0 mg L^{-1} NAA. All explants cultured in dark for 7-8 days, then transferred under indoor natural light to culture.	100% (buds), 60% (leaves), 30.8% (petioles), 0% (carpels and stamens).	Chen *et al*. 2001
Young leaves	6-BA, 2,4-D, KT, NAA (concentrations unknown).	2,4-D was the key factor for young leaves to induce callus.	Li *et al*. 2005
Phloem	1.0 mg L^{-1} 2,4-D or 1.5 mg L^{-1} NAA was added to MS medium.	Callus induction percentage was about 70-80%.	Shi *et al*. 2005
Filaments, petals	The basal MS medium supplemented with different hormones (0.49 μM NOA; 24.60 μM 2iP and 4.14 μM PIC; 2.27 μM TDZ; 2.27 μM TDZ and 9.05 μM 2,4-D).	Callus formation (% in filaments, petals): NOA (0, 13); 2iP + PIC (90, 100); TDZ (0, 33); TDZ + 2,4-D (95, 100).	Beruto *et al*. 2004
Young leaves, petioles, cotyledons	Different basal media (MS, B_5, N_6, WPM) supplemented with 2.0 mg L^{-1} 6-BA and 0.2 mg L^{-1} NAA.	WPM was the best medium for callus induction, the highest callus formation rates of leaves and petioles was 84%, 96% for cotyledons.	An 2005
Petioles and leaves (from plantlets *in vitro*)	Basal MS medium supplemented with 2,4-D with different concentrations (0, 1, 2, 4, 8 mg L^{-1}). Cultured under 25°C and 16-h photoperiod with different light sources (HFL, dark, 100% blue CCFL and 100% red CCFL), 45 mmol m^{-2} s^{-1}.	Both of leaves and petioles had the highest callus formation (90%) when 1 mg L^{-1} 2,4-D was used. Petioles = highest percentage callus formation under blue or red CCFL. Leaves formed most callus in the dark.	This study

Media: MS = Murashige and Skoog medium; B_5 = Gamborg B5 medium; N_6 = CHU medium; WPM = Woody Plant Medium
Plant growth regulators: 6-BA = 6-benzylaminopurine acid; NAA= 1-naphthaleneacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; TDZ = thidiazuron; NOA = β-naphthoxyacetic acid; 2iP = 6-γ-γ-(dimethylallylamino)-purine; PIC = picloram; KT = 6-furfurylamino purine.
Light sources: HFL = heat fluorescent lights; CCFL= cold cathode fluorescent lamps.

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Research Note

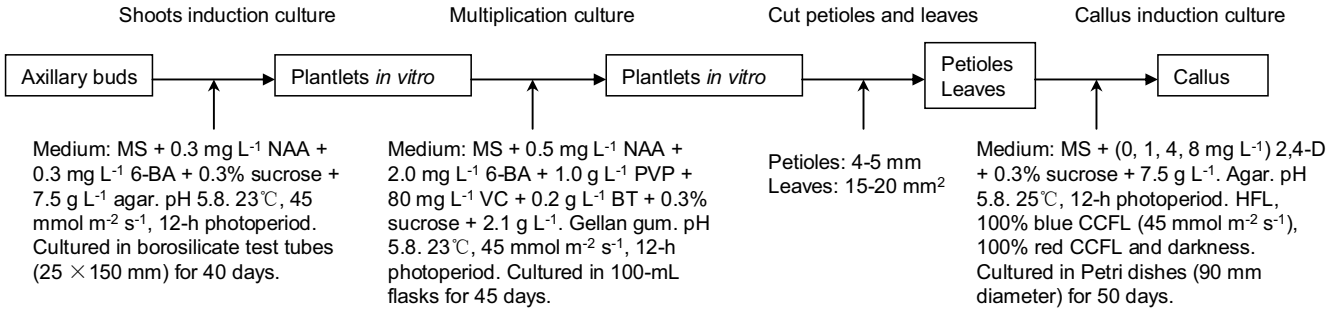

Fig. 1 Flow diagram showing a simple methodology for inducing callus from leaf and petiole explants in tree peony derived from axillary bud culture.

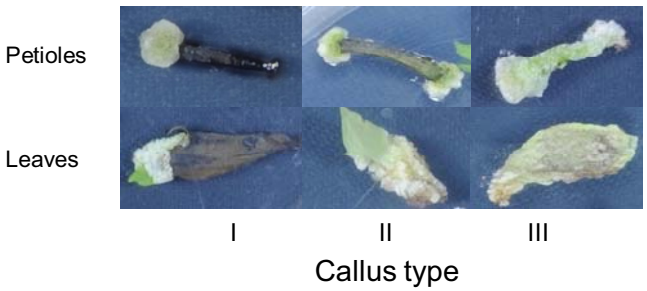

Fig. 2 Three types of callus formed in this experiment. I: poor or little callus; II: mediocre amount of callus; III: a lot of callus.

(CCFLs), shoot production could be increased. However, there is no data on the effect of light conditions and quality on callus formation in tree peony. This is the focus of this experiment.

MATERIALS AND METHODS

Mother plant material

Mother plants were originally derived from 5-year-old *ex vitro* plants that were sterilized and cultured *in vitro* according to the procedures of Yan (2009) and Ding *et al*. (2010). Then, shoot cultures were sub-cultured and maintained according to He (2009), but the concentration of 1-naphthaleneacetic acid (NAA, Nacalai Tesque, Kyoto, Japan) and 6-benzylaminopurine acid (6-BA, Nacalai Tesque) were modified to 0.5 mg L^{-1} and 2.0 mg L^{-1}, respectively. Another important modification of the original protocol was the substitution of agar with 2.1 g L^{-1} Gellan gum (Wako Chemical Industries Ltd., Osaka, Japan). All culture conditions were identical to those employed by He (2009). Petioles and leaves derived from 45-day-old plantlets served as the explants for this experiment and were derived from axillary bud culture as shown in **Fig. 1**.

Petiole and leaf explants were placed on Murashige and Skoog (MS; Murashige and Skoog 1962) basal medium solidified with 7.5 g L^{-1} agar (Wako), supplemented with 30 mg L^{-1} sucrose (Wako) and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; Nacalai Tesque). The culture conditions were 25 ± 1°C and a 16-h photoperiod with different light sources and light quality, explained in detail in *2.2*.

Treatments

Uniform leaf and petiole explants (4-5 mm long and 15-20 mm^2, respectively) were placed on 20 mL of basal MS medium supplemented with four concentrations of 2,4-D: 1, 2, 4 and 8 mg L^{-1} for 50 days in 90-mm diameter Petri dishes (Daiichi Co., Tokushima, Japan). The control was MS basal medium without 2,4-D. Leaf explants were placed abaxial surface down on the medium.

In addition, all explants at each 2,4-D concentration were placed under different light conditions for 50 days: heat fluorescent lights (HFL, Plant Lux; PPFD = 45 mmol m^{-2} s^{-1}, Toshiba Co., Tokyo, Japan), which served as the control; 100% blue CCFLs (PPFD = 45 mmol m^{-2} s^{-1}, NK System, Osaka, Japan); 100% red CCFLs; continuous darkness.

The number of explants that formed callus and the amount of callus that formed on each explant were evaluated 50 days after explants were plated. Regarding the latter parameter, three types were classified: I (poor or little callus), II (a mediocre amount of callus), III (a lot of callus) (**Fig. 2**).

Experimental design and statistical analyses

A completely randomized block design was employed. Means were separated by one-way analysis of variance and significant differences were assessed using Duncan's multiple range test at $P \leq 0.05$ using DPS software version 3.01 (DPS® Data Processing System, China). There were 10 samples for all treatments and each treatment for each explant type was repeated in triplicate.

RESULTS

Effect of 2,4-D concentration on callus formation

When 2,4-D was absent from the medium, no callus formed (see control values in **Table 2**). Maximum callus could be induced from both tree peony leaf and petiole explants

Table 2 Effects of different concentrations of 2,4-D on percentage callus formation of tree peony 'Wu Long Peng Sheng' under different light conditions (n = 30).

Treatment	2,4-D conc. (mg L^{-1})	Petiole		Leaf	
		No. of explants that formed callus	Callus formation rate (%)	No. of explants that formed callus	Callus formation rate (%)
HFL	Control	0	0 c*	0	0 c*
	1	17	56.7 ab	21	70.0 ab
	2	22	73.3 ab	18	60.0 ab
	4	19	63.3 ab	13	43.3 b
	8	21	70 ab	22	73.3 ab
Dark	Control	0	0 c	0	0 c
	1	20	66.7 ab	27	90.0 a
	2	16	53.3 ab	25	83.3 ab
	4	17	56.7 ab	25	83.3 ab
	8	11	36.7 bc	24	80.0 ab
B (CCFL)	Control	0	0 c	0	0 c
	1	27	90.0 a	22	73.3 ab
	2	23	76.7 ab	19	63.3 ab
	4	23	76.7 ab	17	56.7 ab
	8	22	73.3 ab	21	70.0 ab
R (CCFL)	Control	0	0 c	0	0 c
	1	26	86.7 a	16	53.3 ab
	2	22	73.3 ab	14	46.7 b
	4	16	53.3 ab	15	50.0 ab
	8	17	56.7 ab	20	66.7 ab

* Different letters within a column indicate significant differences at $P \leq 0.05$ using DMRT.
HFL = heat fluorescent lights; CCFL = cold cathode fluorescent lamps; B = blue; R = red.

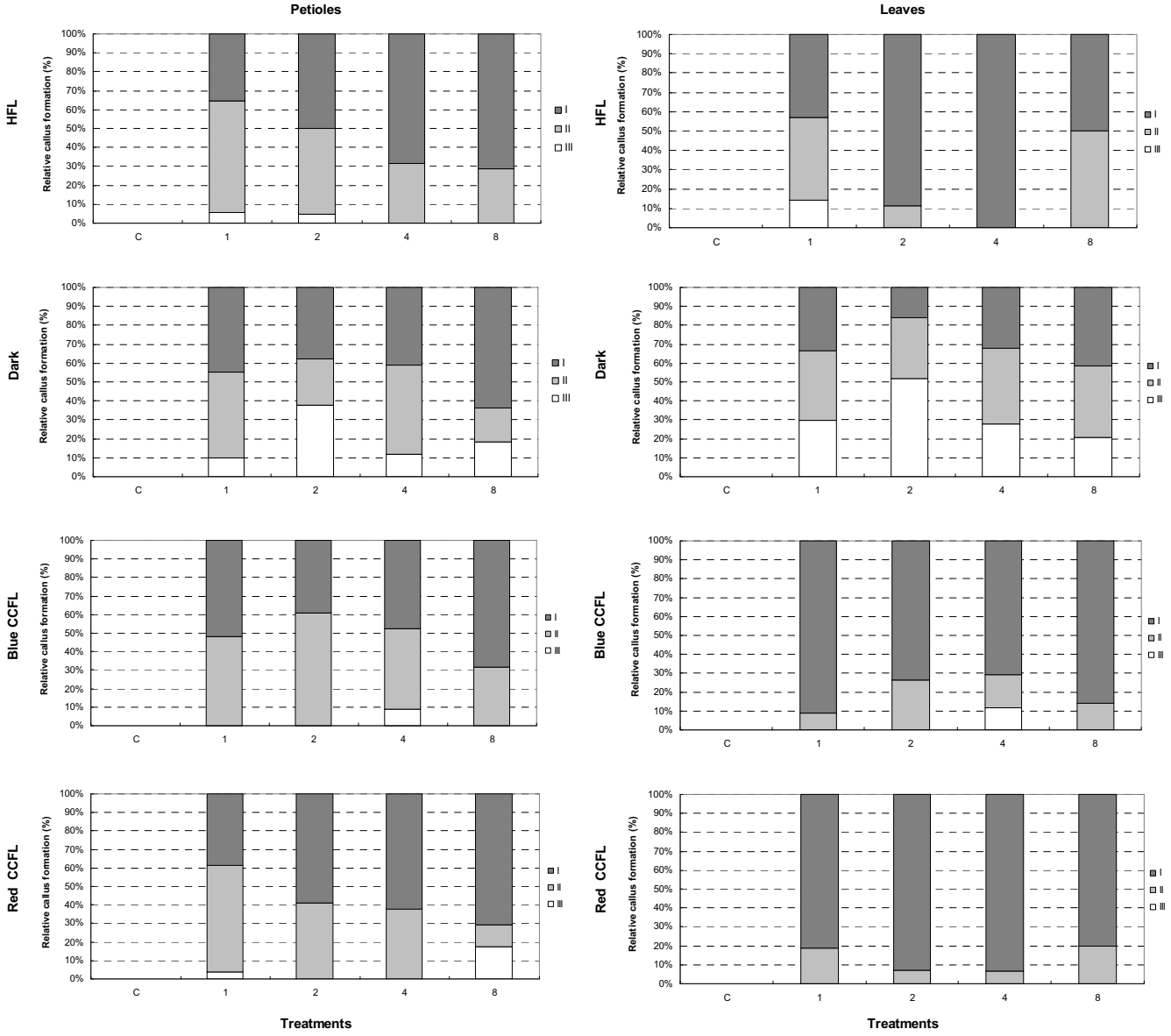

Fig. 3 Quantification of callus by leaf and petiole explants in response to different light sources. Types of callus: I: poor or little callus; II: mediocre amount of callus; III: a lot of callus.

when 1 mg L^{-1} 2,4-D was used, although this optimal concentration depended on the light source and quality, as described next.

Effect of light source and quality on callus formation

The highest percentage of petiole explants formed callus when placed under red or blue CCFLs on MS with 1 mg L^{-1} 2,4-D. Similarly, at the same concentration of 2,4-D, leaf explants formed most callus in the dark. In general, most explants formed little or a mediocre amount of callus (Types I and II; **Fig. 3**). However, a lot of callus (Type III) formed on petioles or leaves cultured in the dark, independent of the concentration of 2,4-D.

DISCUSSION

In this study we were able to successfully induce callus from both petioles and leaves of tree peony under specific conditions. 2,4-D was necessary for callus induction for both explant types, and light quality played a very important role in the amount of callus formed and on the number of explants that were receptive to callus formation.

Callus has, in other studies, been induced from different explants in tree peony under several culture conditions and for different cultivars (**Table 1**). Tissue dedifferentiation is sensitive to different types and concentrations of plant growth regulators (PGRs). Generally, the tissues of nutritive organs (such as buds, leaves, stems, petioles) have a low degree of differentiation, so they are much easier to induce callus than generative organs (such as p*etal*s and filaments), which have tissues with the capacity for a high degree of differentiation (Chen *et al*. 2001).

2,4-D was much more effective than other PGRs (6-benzyladenine, kinetin, 1-naphthaleneacetic acid (NAA), thidiazuron) in the induction of callus from young leaves, filaments and petals of tree peony (Beruto *et al*. 2004; Li *et al*. 2005).

In our study, by using 2,4-D and selecting light conditions, the percentage of callus formation of both petioles and leaves was considerably higher than that reported for other studies that employed BA or NAA (Chen *et al*. 2001; An 2005). A high percentage of callus can increase the chance of inducing somatic embryogenesis.

Niu (2009) studied the effect of CCFLs and light-emitting diodes (LEDs) on the proliferation of tree peony callus induced from petioles using a different basal medium to our study, namely 1/2 MS supplemented with 1.0 mg L^{-1} NAA and 0.3 mg L^{-1} kinetin. In Niu's study, 100% red (R) CCFLs and 100% blue (B) CCFLs were better than other R/B CCFL ratios (60% R + 40% B; 70% R + 30% B; 80% R +

20% B; 100% white). However, callus proliferation of 'Feng Dan Bai' was better under 80% R + 20% B and 70% R + 30% B LEDs.

Yan (2009) and Li (2009) studied the effect of LEDs (PPFD = 50 ± 5 μmol m^{-2} s^{-1}) on the growth of *Gerbera jamesonii* 'Rui Kou' plantlets *in vitro*. Compared with HFL on some growth parameters (plant height, no. of leaves and roots, length of leaves and roots, leaf width), all the different light quality ratios (100% R, 80% R + 20% B, 70% R + 30% B, 50% R + 50% B and 100% B LEDs) were better for *Gerbera* plantlet growth. LEDs (70% R + 30% B) were better than HFL for *in vitro* growth of strawberry cv. 'Akihime', *Eucalyptus citriodora*, and *Cymbidium* Great Katy 'Love Me' plantlets. 80% R + 20% B was the best ratio of LEDs for the plantlet *in vitro* growth of *Phalaenopsis* Gallant Beau 'George Vasquez', Banana cv. 'Nam Dinh' and *Spathiphyllum* cv. 'Merry' (Nhut 2002; Nhut *et al*. 2003, 2006).

Yan (2009) and Ding *et al*. (2010) studied the effect of CCFLs on the growth of tree peony 'Wu Long Peng Sheng' plantlets *in vitro*. All plantlet growth parameters were equal under 70% R + 30% B (the best performing CCFL ratio) and HFL. 100% B significantly inhibited plant height. However, in our study of callus formation 100% B resulted in the greatest number of petioles forming callus in basal medium containing the lowest concentration (1 mg L^{-1}) of 2,4-D when compared with HLF, 100% R or darkness.

In this study, callus was effectively induced from leaves or petioles in an explant-independent manner when 1 mg L^{-1} 2,4-D was used, although the light requirements for each explant differed and could affect the quantitative outcome of callus.

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