Plant Cell, Tissue and Organ Culture (PCTOC) (2021) 145:117–125
https://doi.org/10.1007/s11240-020-01996-y

ORIGINAL ARTICLE

Development of a highly efficient callus induction and plant regeneration system for *Dendrocalamus sinicus* using hypocotyls as explants

Jin Li[1,2] · Chengjie Gao[1] · Yingchun Miao[1] · Zirui Liu[1] · Kai Cui[1]

Received: 11 November 2020 / Accepted: 16 December 2020 / Published online: 2 January 2021

Abstract

Dendrocalamus sinicus is the largest bamboo species in the world. To aid its rapid propagation and explore its desired traits, we identified the optimal medium and culture conditions for callus induction, shoot differentiation, rooting and transplanting using hypocotyls as explants. Comparison of three browning inhibitors indicated that 400 mg/L of citric acid provided the best results. The Box-Behnken model showed that 6-benzyladenine (6-BA), citric acid and 2,4-dichlorophenoxyacetic acid (2,4-D) had decreasing levels of impact on the callus induction rate of *D. sinicus*, with their optimum concentrations being 2.10 mg/L, 420 mg/L and 3.10 mg/L, respectively. Under these conditions, the callus induction rate of *D. sinicus* hypocotyls was predicted to be as high as 89.06%, and was validated as 88.87% in our experiments. The highest frequency of callus formation was on Murashige and Skoog (MS) medium containing 30 g/L sucrose, 6 g/L agar, 500 mg/L casein hydrolysate (CH), 500 mg/L proline (Pro), 500 mg/L glutamine (Gln), 2.10 mg/L 6-BA, 420 mg/L citric acid and 3.10 mg/L 2,4-D. After further callus differentiation, rooting and regeneration, the rooting rate and survival rate of transplanted seedlings reached 91% and 93%, respectively. Our study provides a theoretical basis and a new avenue for the genetic transformation of bamboo plants.

Key message

The main objective of this study was to construct a highly efficient callus induction and plant regeneration system for the largest bamboo species in the world by hypocotyls as explants based on response surface method.

Keywords *Dendrocalamus sinicus* · Callus induction · Browning · Regeneration system

Introduction

Bamboos includes more than 1500 species distributed mainly in tropical and subtropical countries of the world, which is an important raw material for construction, papermaking, handicraft, food and medicine industries (Liese and Khöl 2015). Conventionally, bamboos are propagated through seeds, offsets and culm cuttings. However, propagation through seeds faces several problems including long flowering cycle (Guerreiro 2014), monocarpic nature of plant (Abe and Shibata 2014), poor seed set (Sankhyan et al. 2009), short seed viability (Sarma et al. 2010), highly heterogeneous seedling populations, and consumption of seeds by birds, rodents, and wild animals (Taylor et al. 2004). Similarly, bulkiness and limited availability of propagules, difficulties in transportation over long distances, seasonal dependence, low survival rate and limited rooting of the propagules are the major constraints in bamboo propagation through vegetative methods (Singh et al. 2013).

Tissue culture is expected to be a potential approach to develop bamboo rapidly. So far, some kinds of explants including embryo, tender shoot, small segment of bamboo branch, terminal bud, internode, dormant bud, sheath, anther and inflorescence involved in *Dendrocalamus* (Chambers et al. 1991; Arya et al. 1999), *Bambusa* (Chang and Lan

Communicated by Qiao-Chun Wang.

✉ Kai Cui
cuikai@caf.ac.cn

[1] Research Institute of Resources Insects, Chinese Academy of Forestry, Kunming 650224, People's Republic of China

[2] Nanjing Forestry University, Nanjing 210037, People's Republic of China

1995; Shirin and Rana 2007), *Dendrocalamopsis* (Zeng 2018), *Phyllostachys* (Hassan and Debergh 1987; Ogita 2005) and *Sasa* (Huang et al. 1988) were applied in tissue culture.

Dendrocalamus sinicus is a sympodial bamboo in the genus *Dendrocalamus* and is the largest among 1500 bamboo species worldwide (Ohrnberger 1999). Bamboo shoots of *D. sinicus* began to grow from late May to early June, finishing in late August, and the shoot usually reaches a height of over 30 m, a diameter at breast height of 25–30 cm and a single-plant weight of 100–150 kg within 3 months. To the best of our knowledge, no plant in the world presents such a fast growth rate. Its shoots and timber are good for supplementary health products and raw materials, respectively, and *D. sinicus* thus shows high commercial potential (Yi et al. 2008).

Similar to most bamboo species, *D. sinicus* is sterile with low seed production, which impedes its development. At present, fast propagation of *D. sinicus* is mostly based on the "bud to bud" method (Yang et al. 2004; Li and Hui 2006). Using seeds and spikelets as explants, Geng et al. (2006) successfully induced callus; however, no differentiation or regeneration was obtained. A complete and stable callus induction and plant regeneration system for *D. sinicus* has not been established. Callus regeneration is the basis for genetic transformation and also the premise for genetic investigation and utilization of the giant characteristics of *D. sinicus* stems. Here, we used hypocotyls as explants to screen and optimize a complete system for callus induction, differentiation, rooting and transplanting of *D. sinicus*, providing a theoretical basis for its transgenic breeding.

Materials and methods

Plant material

Seeds of *D. sinicus* were obtained from Menglian county, Yunnan province, P. R. China (22°24′5″ N, 99°38′13″ E) in Febrary 2019. Shells were peeled from plump seeds, which were then rinsed with distilled water. Seeds were surface disinfected in 2.5% (v/v) sodium hypochlorite for 15 min and rinsed three times using distilled water. Seeds were then soaked in 0.5% (v/v) potassium permanganate for 12 h and rinsed three times using distilled water, followed by 10 min of disinfection with 0.1% (v/v) mercury dichloride and rinsing five times with distilled water. Extra moisture on seed surfaces was dried using sterile filter paper before inoculation. Two drops of Tween-20 were added into the disinfection solution as surfactant, and repeated oscillation was applied to ensure the even sterilization of seeds. Seeds were germinated under a 16/8 h light/dark photoperiod at 25 °C for 8–15 days after which hypocotyls 5–7 cm in length were cut into single node sections used as a source of explants.

Optimization of callus induction medium

Murashige and Skoog (MS) medium (Murashige and Skoog 1962) was chosen as basal media. For callus induction, 30 g/L sucrose, 6 g/L agar, and the organic additives [500 mg/L casein hydrolysate (CH), 500 mg/L proline (Pro), 500 mg/L glutamine (Gln)] were added. In addition, considering that the different concentrations of exogenous hormones containing 6-BA, KT and 2,4-D were widely used to induce callus and played an important role in bamboo tissue culture (Rout and Das 1994; Brillas et al. 2000; Liu et al. 2009; Zang et al. 2016), the different concentrations of 6-BA (2 mg/L, 4 mg/L and 8 mg/L), 2,4-D (2 mg/L, 4 mg/L and 6 mg/L) and KT (0.5 mg/L, 1 mg/L and 1.5 mg/L) were used to select the optimal medium for callus induction by orthogonal test. The pH of medium was adjusted to 5.8 ± 0.1. Calli were subcultured every 4 weeks.

Treatment of callus browning inhibitors

To investigate the callus browning during the tissue culture process, three treatments and one control were designed. Three sterilized browning inhibitors citric acid (200 mg/L, 400 mg/L and 600 mg/L), vitamin C (100 mg/L, 200 mg/L and 300 mg/L), activated carbon (400 mg/L, 800 mg/L and 1200 mg/L) were added to calli induction medium, while no browning inhibitor was set as control (CK). Citric acid and vitamin C were sterilized using a 0.22 μm filter, and activated carbon was sterilized by high temperature and pressure. The browning rate and callus induction rate were calculated as follows: (1) browning rate = (number of browning explants/the total number of inoculated explants in each treatment) × 100%; (2) callus induction rate = (number of explants that formed callus/the total number of inoculated explants in each treatment) × 100%.

Callus proliferation and subculture

Callus was subcultured 2–3 times on callus induction medium and then transferred to callus proliferation medium, which was MS medium supplemented with 0.5 mg/L 2,4-D, 500 mg/L Pro, 500 mg/L casein hydrolysate (CH), 500 mg/L Gln, 30 g/L sucrose and 6 g/L agar at pH 5.8. Callus was cultivated at 25 °C without light, and the medium was replaced every 4 weeks.

Differentiation and proliferation of adventitious shoots

Two-month-old, yellowish, and dense callus was transferred onto adventitious shooting medium: MS medium supplemented 30 g/L sucrose, 6 g/L agar, 2.0 mg/L 6-BA, 0.3 mg/L kinetin (KT) and 0.3 mg/L α-naphthaleneacetic acid (NAA) at pH 5.8. The medium was renewed every 4 weeks. When young seedlings reached 2 cm, they were transferred onto shooting proliferation medium, which was 2/3-strength MS supplemented with 2.2 mg/L 6-BA, 1.0 mg/L KT, 30 g/L sucrose and 6 g/L agar at pH 5.8. Tissue was cultured at 25 °C under 16 h light per day. The medium was refreshed every 3 weeks.

Rooting medium, transplanting and acclimatization

Shoots with a height of 3–4 cm and 2–3 buds were transferred onto rooting medium consisting of 1/2-strength MS supplemented with 2.0 mg/L, 4.0 mg/L or 6.0 mg/L indole-3-butyricacid (IBA), 1.0 mg/L NAA, 30 g/L sucrose and 6 g/L agar at pH 5.8. Plant tissue was cultivated under 16 h light per day. The rooting rate was determined 1 month after transfer. Culture bottle caps were loosened when roots reached 3–5 cm. Two days later, seedlings were acclimated under natural light with the cap open for 1 week. The seedlings were then taken out, the roots were rinsed with running water, and transferred to cultivation medium with a nutrient soil: perlite: vermiculite ratio of 1:1:1 (v/v/v). Plant survival rate was calculated after 1 month.

Statistical analysis

Each measurement was repeated three times and mean values were presented. The indices of callus induction were analyzed by ANOVA, and LSD values were calculated at $P=0.05$. Percentage data were transformed by square-root and arcsine methods before the ANOVA. All data analyses were performed using SPSS 17.0. DPS v6.55 was used to analyze orthogonal test. Box-Behnken mode of Design-Expert 12.0 software was used to establish a quadratic model for obtaining the best variable levels via resolving the regression equation and analyzing the response surface. The significance of the model and factors was investigated by F value, and the difference was statistically significant when $P<0.05$.

Results

Selection of callus induction medium

To obtain a highly efficient medium for callus induction from the hypocotyls of *D. sinicus*, we designed an orthogonal test with three factors and three levels to screen media formulations with callus induction rate as the assessment indicator (Table 1). Data indicated that the order of induction effect induced by the three hormones tested was 6-BA > 2,4-D > KT, and their optimal concentrations were 2 mg/L, 4 mg/L and 0.5 mg/L, respectively. Of these, only 6-BA and 2,4-D had a significant difference on callus induction (Table 2).

Table 1 Effect of different medium composition on callus induction from hypocotyl of *D. sinicus*

Orthogonal combination	Factors and levels			Callus formation/%
	6-BA mg/L	2,4-D mg/L	KT mg/L	
1	2	2	0.5	71.32
2	2	4	1	84.16
3	2	6	1.5	76.43
4	4	2	1	57.71
5	4	4	1.5	65.66
6	4	6	0.5	62.93
7	8	2	1.5	53.07
8	8	4	0.5	72.31
9	8	6	1	63.88
X1	77.30	60.70	68.85	
X2	62.10	74.04	68.58	
X3	63.09	67.75	65.05	
R	15.20	13.34	3.80	

Each treatment consisted of three replications and each replication contains 20 explants and the data displayed at table is the mean value of each treatment. X represents value of the test corresponding to a factor in the same level. R = Xmax − Xmin. All data were recorded 8 weeks after inoculated into the callus induction medium

Table 2 Variance analysis of effect of different medium composition on callus induction of *D. sinicus*

Source of variance	Sum of squares	Df	Mean square	F value	Sig
Corrected model	728.55	6	121.43	30.65	0.03
Intercept	41,002.20	1	41,002.20	1.035E4	0.00
6-BA	434.23	2	217.11	54.81	0.02
2,4-D	267.35	2	133.67	33.75	0.03
KT	26.97	2	13.49	3.41	0.23
Error	7.92	2	3.96		

Effect of inhibitors of callus browning

Browning is common during the callus induction process because of the relatively high content of phenolics in *D. sinicus* tissues. Therefore, we added the browning inhibitors citric acid, vitamin C and activated carbon, respectively, into the induction medium. Single factor ANOVA demonstrated

significant callus browning on medium without browning inhibitor during the late growth stage (39.30% in 8-week-old callus). No substantial difference was observed with vitamin C addition, whereas the browning rate was significantly reduced after adding citric acid or activated carbon. The browning rate with citric acid addition (17.59%) was significantly lower than that with activated carbon addition (23.07%). In addition, the concentration of inhibitors had a significant impact; for example, 400 mg/L of citric acid exhibited the best inhibitory effect among three concentrations tested, yielding a browning rate as low as 14.41% (Fig. 1).

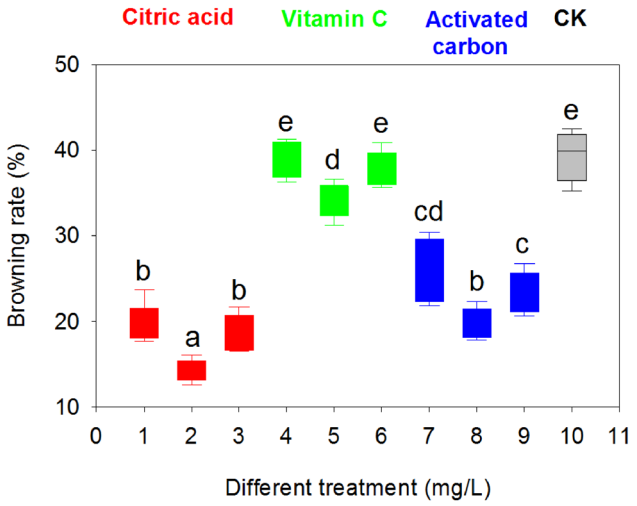

Fig. 1 Inhibition effect of different additives on callus browning. Box with the same color represents the same treatment. The explants were cultured on the MS medium supplemented with 30 g/L sucrose, 6 g/L agar, 2 mg/L 6-BA, 4 mg/L 2,4-D, 500 mg/L CH, 500 mg/L Pro, 500 mg/L Gln, and treated with the different concentrations of browning inhibitor. Different letters in the box indicate differences at $P < 0.05$ based on the LSD (least significant difference) test. Bars represent ST

Optimization of callus induction medium

To identify the optimal callus induction medium with browning resistance, we investigated combinations of the two most effective hormones (6-BA and 2,4-D) and one browning inhibitor (citric acid) (Table 3). Response surface design is a statistical method that uses reasonable experimental design method and obtains certain data through experiments. It uses multiple quadratic regression equation to fit the functional relationship between factors and response values, and through the analysis of regression equation to find the optimal process parameters and solve multivariable problems (Kristensen et al. 2005). Through response surface design implemented by Design-Expert 12.0, stepwise regression fitting and multiple regression analysis were carried out according to the values measured in the Table 3. The following model of

Table 3 Box-Behnken design experiment of important components of callus induction of *D. sinicus*

Treatment no	Factors and levels			Callus formation/%	
	Conc. of 6-BA (mg/L)	Conc. of 2,4-D (mg/L)	Conc. of citric acid (mg/L)	Actual value	Predicted value
1	4	2	600	74.18	75.10
2	4	6	200	75.03	74.01
3	4	4	400	86.35	85.40
4	4	6	600	68.18	68.90
5	8	4	600	65.37	65.60
6	4	4	400	84.23	85.40
7	2	2	400	88.39	89.00
8	2	4	600	82.54	80.70
9	2	6	400	78.16	79.10
10	8	6	400	78.41	77.70
11	4	4	400	85.67	85.40
12	8	2	400	74.33	73.50
13	2	4	200	80.17	80.50
14	4	4	400	83.39	85.40
15	4	2	200	79.08	78.40
16	8	4	200	77.35	78.70
17	4	4	400	87.59	85.40

Each treatment consisted of three replications and each replication contains 20 explants and the data displayed at table is the mean value of each treatment. All data were recorded 8 weeks after inoculated into the callus induction medium

the relationship between callus induction rate and the concentration of 6-BA (A), 2,4-D (B) and citric acid concentration (C) was obtained:

$$\text{Callus induction rate} = 53.04193 - 1.20669A + 4.68113B + 0.164317C + 0.590526AB - 0.005524AC - 0.001219BC - 0.035427A^2 - 0.982937B^2 - 0.000185C^2$$

ANOVA showed that this regression model was extremely significant at the level of $P = 0.0001$ (Table 4), suggesting that the model was appropriately described by the test. By fitting the quadratic polynomial model, a contour plot and response surface plot intuitively reflected the factors and their response values as well as the interactions between factors (Fig. 2). The order of the effect of various factors was as follows: concentration of 6-BA > concentration of citric acid > concentration of 2,4-D. The interaction between the concentrations of 6-BA and citric acid exhibited the most robust effect, followed by the interaction between the concentrations of 6-BA and 2,4-D and the interaction between the concentrations of 2,4-D and citric acid. Significance tests of the three independent variables all reached an extremely significant level, indicating that 6-BA, 2,4-D and citric acid were the main factors for callus production in this model.

As seen in the response surface plot, there was a maximum value in the regression model. The optimal conditions for callus induction were 2.16 mg/L 6-BA, 3.12 mg/L 2,4-D and 420 mg/L citric acid, under which the theoretically predicted maximum induction rate was 89.06%. For ease of operation, the abovementioned conditions were adjusted to 2.10 mg/L 6-BA, 3.10 mg/L 2,4-D and 420 mg/L citric acid, and the resulting callus induction rate was 88.87% in our actual experiment. This was very close to the predicted value, suggesting the accuracy of the model.

Proliferation, differentiation and rooting of callus

The growth rate of the callus was significantly elevated after transferring onto callus proliferation medium (Fig. 3c). After transplanting the pale-yellow, dense callus onto adventitious shooting differentiation medium, green spots emerged on the callus, which gradually developed into adventitious shoots and then seedlings (Fig. 3e–g). The resulting seedlings were further transferred onto rooting medium (Fig. 3h). Considering that IBA concentration has a great impact on the rooting of various plants, the effect of three IBA concentrations on rooting was tested. Root induction rate and root number were significantly higher with 4 mg/L of IBA compared with the other two concentrations, showing a rooting rate as high as 91% (Fig. 4). The seedlings were transplanted into cultivation mixture (nutritional soil: perlite: vermiculite = 1:1:1) after stabilization of the root system (Fig. 3i), and plant survival rate was revealed as 93%, indicating that our scheme from callus induction to full plant growth was feasible.

Discussion

With the complete suspension of commercial harvesting of natural forests in China, bamboo has become a potentially sustainable resource. However, the flowering period of bamboo plants is usually long and hard to predict, and seed acquisition is also difficult, so it is hard to apply traditional breeding approaches for large-scale propagation and the introduction of desired traits, impeding the genetic improvement of bamboo plants. Development of a highly efficient

Table 4 Variance analysis of fitted quadratic polynomial model

Source of variance	Sum of squares	df	Mean square	F value	Sig
Model	668.29	9	74.25	28.22	0.0001
A	142.81	1	142.81	54.28	0.0002
B	32.81	1	32.81	12.47	0.0096
C	57.03	1	57.03	21.68	0.0023
AB	51.19	1	51.19	19.46	0.0031
AC	51.48	1	51.48	19.57	0.0031
BC	0.9506	1	0.9506	0.3613	0.5667
A^2	12.05	1	12.05	4.58	0.0696
B^2	65.09	1	65.09	24.74	0.0016
C^2	230.37	1	230.37	87.56	< 0.0001
Residual	18.42	7	2.63		
Lack of fit	7.25	3	2.42	0.8652	0.5286
Pure error	11.17	4	2.79		
Corrected total	686.70	16			

A conc. of 6-BA, *B* conc. of 2,4-D, *C* conc. of citric acid. $R^2 = 0.97$, Adjusted $R^2 = 0.94$

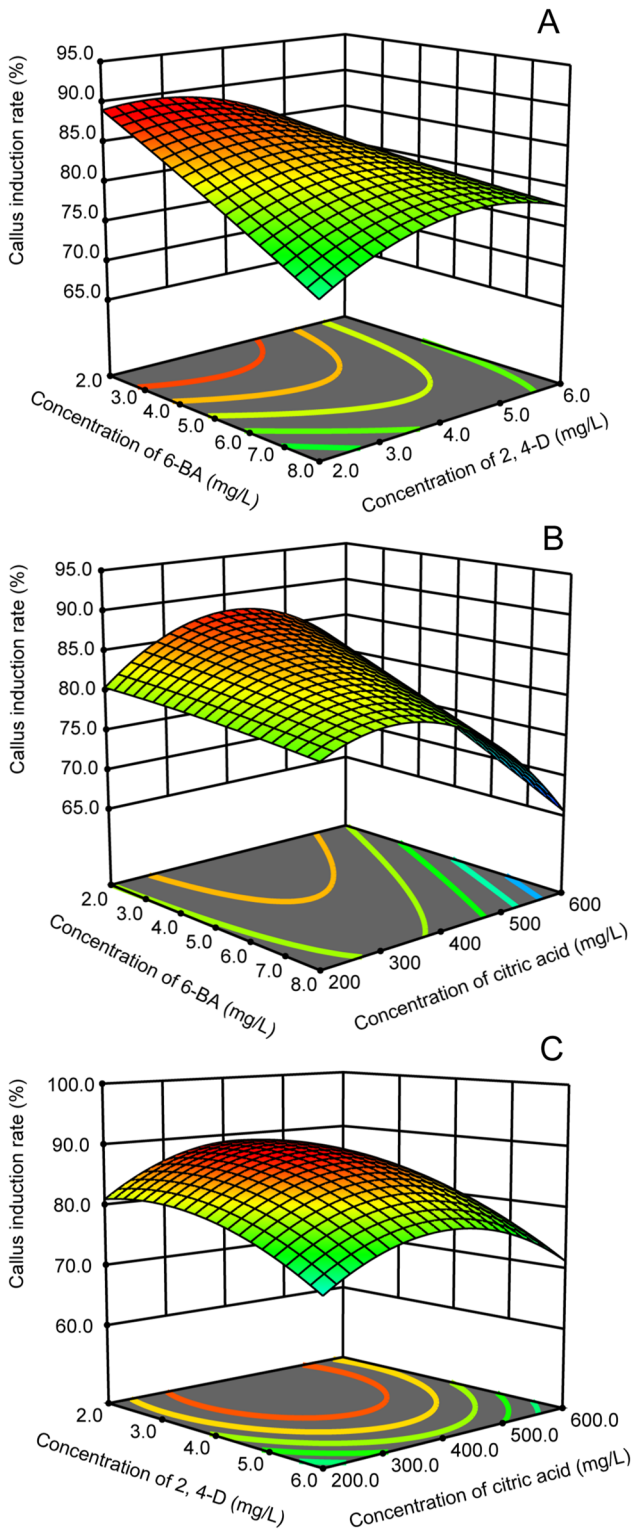

Fig. 2 Effect of interaction of important components in culture medium on *D. sinicus* callus induction rate. The explants were cultured on the MS medium supplemented with 30 g/L sucrose, 6 g/L agar, 500 mg/L CH, 500 mg/L Pro, 500 mg/L Gln, and treated with the different concentrations of 6-BA, 2,4-D and citric acid

genetic transformation system is the foundation of bamboo molecular breeding, and the establishment of a high-quality regeneration system is the most crucial first step.

The majority of previous studies on regeneration of bamboo used either mature or immature zygotic embryos as explants (Yeh and Chang 1987; Saxena and Dhawan 1999; Ning et al. 2010), and the employment of immature inflorescences (Yeh and Chang 1986), anthers (Qiao et al. 2010), spikelets (Yuan et al. 2009), young leaves (Hassan and Debergh 1987), nodes (Rout and Das 1994), roots (Chang and Lan 1995) and shoots (Ogita 2005) as explants has also been reported. According to the report by Chambers et al. (1991), multiple vegetative shoots and flowers emerged from hypocotyl of explants derived from young seedlings of *D. hamiltonii* when cultured on a MS based medium supplemented with the cytokinin 6-BA. Nevertheless, in bamboos, callus induction from hypocotyls has not been reported. In the present study, we used hypocotyls of *D. sinicus* as explants and successfully developed a callus induction and plant regeneration system. Hypocotyls make superior explants compared with other tissues and organs because (1) they are easy to acquire; (2) they are produced under sterile conditions, and thus the subsequent disinfection processes are easy to operate and not affected by external conditions, significantly increasing the induction rate; and (3) their composition is relatively uniform, ensuring the repeatability and stability of the experimental system.

Young shoot of *D. latiflorus* was used as explants to culture, and 53% of callus induction rate and 73% of rooting rate were perforemed, respectively (Ye et al. 2017). In *D. hamiltonii*, shoot tip of explant (Zang et al. 2016) obtained 87% callus induction rate and 95% rooting rate, while mature zygotic embryo of explant (Zhang et al. 2010) obtained 65% callus induction rate and 85% rooting rate. Yuan et al. (2013) used zygotic embryo of *P. heterocycla* as explant and callus induction rate is 50%. Lin et al. (2019) used nodular bud of *Drepanostachyum luodianense* as explant, and callus induction rate and rooting rate were 65% ans 85%, respectively. Geng et al. (2006) performed 70% callus induction rate by explants of seed and spikelet of *D. sinicus.* We accomplished 89% callus induction rate and 91% rooting rate by explant of hypocotyl of *D. sinicus*. Meanwhile, the browning rate of callus decreased significantly. Compared with the abovementioned bamboo species, this presents a high efficient callus induction and plant regeneration system.

The suitable concentration of BA, in some plant species, is beneficial to callus induction and differentiation (Liu and Shi 1996; Liu et al. 2009; Zang et al. 2016). 2,4-D was widely used to induce callus in bamboo, however, a high concentration might inhibit the calli differentiate (Brillas et al. 2000). In this paper, 3.1 mg/L 2,4-D was screened as the best concentration to calli induction. Moreover, some researchers reported that 2,4-D and 6-BA can be used

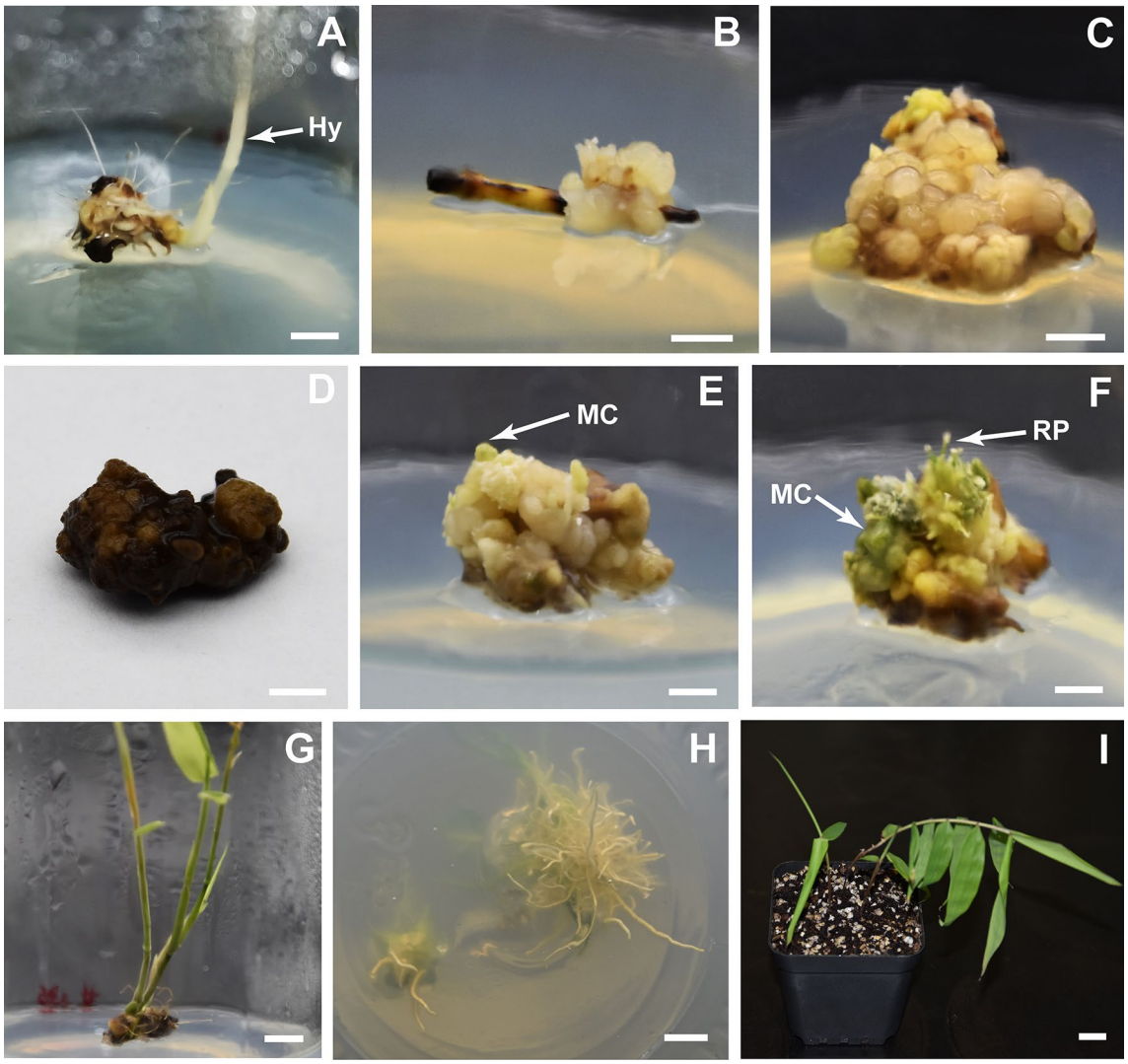

Fig. 3 Callus induction and regeneration from hypocotyl (Hy) of *D. sinicus*. **a** hypocotyl germinated from zygotic embryo; **b** callus induced by hypocotyl; **c** in vitro callus continued to proliferate; **d** callus browning; **e–f** callus differentiation with meristematic center (MC) and root primordia (RP); **g** further differentiation into plantlet; **h** rooting of regenerate plantlet; **i** acclimatization of rooted seedlings. The scale represents 1 cm

together to promote calli induction and plant regeneration in some species (Wang et al. 2007; Siddique and Islam 2015). We performed the combination of 2,4-D and 6-BA to induce calli, and found that the interaction between them had significant effect on callus induction rate (Table 2, Fig. 2a). The precise regulatory mechanism of tissue culture has not yet been fully revealed. There is a viewpoint is generally accepted that callus induction and organ differentiation were mainly determined by hormones balance, which is adjusted by appropriate exogenous auxin and cytokinin (Shirin and Rana 2007). In this study, typical exogenous cytokinins (6-BA and KT) and auxin (2,4-D) were selected to induce calli, and the combination of 2.1 mg/L 6-BA and 3.1 mg/L 2,4-D was optimal.

Browning is the key factor determining the success of plant tissue culture, and its occurrence is usually dependent on plant species and genotype (Fig. 3d). Previous studies have shown that tissue browning is seen more often in woody plants, subtropical plants and medicinal plants compared with herbaceous plants, tropical and temperate plants, and non-medicinal plants (Miller and Murashige 1976; Bhat and Chandel 1991; Hesami et al. 2020). Plants with severe browning often contain relatively high levels of phenolic compounds, including phenolic acids, flavonoids, tannins and lignin (Amin Dalal et al. 1992). Browning phenomena can be classified as enzymatic browning and non-enzymatic browning. Nonenzymatic browning refers to browning that occurs when phenols are oxidized without any enzymes

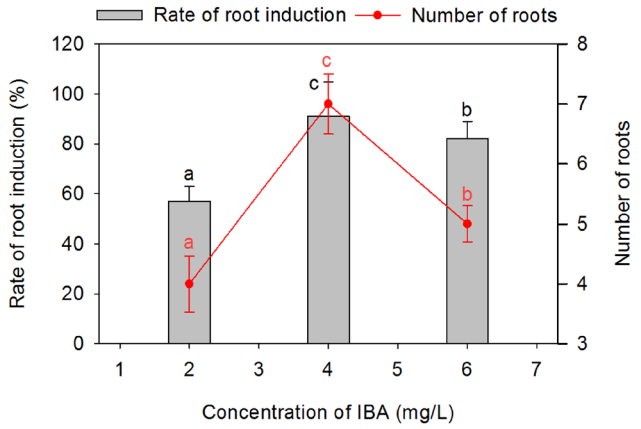

Fig. 4 Effect of different concentrations of IBA on callus rooting. The explants were cultured on the 1/2 MS medium supplemented with 1 mg/L NAA, 30 g/L sucrose, 6 g/L agar, and treated with the different concentrations of IBA. Different letters in the column and broken line indicate differences at $P < 0.05$ based on the LSD test, respectively. Three replications of 20 explants each were used. Bars represent ST

(Pourcel et al. 2007), while the enzymatic browning is the formation of brown quinones and/or their polymers from phenols catalyzed by oxidases. It is generally acknowledged that enzymatic browning is the main pathway of browning during the tissue culture of explants (Saltveit 2000; Huang et al. 2002).

Chabane et al. (2007) indicated that the best antioxidant mixture for inhibiting browning of jujube tree (*Phoenix dactylifera*) explants was 150 mg/L of ascorbic acid with 200 mg/L of citric acid. To control the tissue browning phenomenon, the effects of citric acid and ascorbic acid in callus cultures of *Taxus brevifolia* was evaluated. However, ascorbic acid and citric acid failed to show significant effects on callus growth and browning intensity (Khosroushahi et al. 2011). In the present study, we explored three browning inhibitors: the antioxidants citric acid and vitamin C, and an adsorbent, activated carbon. The addition of citric acid and activated carbon significantly inhibited browning of *D. sinicus* callus, with the best inhibition observed using 400 mg/L of citric acid.

The response surface method is an effective mathematical method for optimizing processing parameters and reaction conditions (Liu et al. 2000; Moghaddam et al. 2010). Based on the classical orthogonal design and using callus induction rate as the indicator, we optimized the components of the callus medium, browning inhibitors, and their concentrations, by employing the response surface method in our study. Compared with the classical uniform design and the orthogonal design, this method is simpler, more scientific and reasonable regarding the stability and workload due to consideration of the interaction among various factors and levels. This study provides some basic data for rapid propagation and genetic breeding of bamboo plants.

Acknowledgements This work was funded by the National Natural Science Foundation of China (32022058 and 31670551), Essential Scientific Research of Chinese National Non-profit Institute (CAFYBB2019ZB006), Yunnan Applied Basic Research Projects (2019FA013) and Training Objects of Technological Innovation Talents in Yunnan Province (2019HB074).

Author contributions KC designed the research; JL conducted the research; YCM and CJG analyzed the data; JL and ZRL wrote the paper. All authors have read and approved the final manuscript.

Compliance with ethical standards

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

References

Abe Y, Shibata S (2014) Seed germination and seedling establishment of the monocarpic dwarf bamboo *Sasa veitchii* var. *hirsuta*. J Forest Res 19:166–173

Amin Dalal M, Sharma BB, Srinivasa Rao M (1992) Studies on stock plant treatment and initiation culture mode in control of oxidative browning in in vitro cultures of grapevine. Sci Hortic-amsterdam 51:35–41

Arya S, Sharma S, Kaur R, Arya ID (1999) Micropropagation of *Dendrocalamus asper* by shoot proliferation using seeds. Plant Cell Rep 18:879–882

Bhat SR, Chandel KPS (1991) A novel technique to overcome browning in tissue culture. Plant Cell Rep 10:358

Brillas E, Calpe JC, Casado J (2000) Mineralization of 2,4-D by advanced electrochemical oxidation processes. Water Res 34:2253–2262

Chabane D, Assani A, Bouguedoura N, Haïcour R, Ducreux G (2007) Induction of callus formation from difficile date palm protoplasts by means of nurse culture. C R Biol 330:392–401

Chambers SM, Heuch JHR, Pirrle A (1991) Micropropagation and *in vitro* flowering of the bamboo *Dendrocalamus hamiltonii* Munro. Plant Cell Tiss Org 27:45–48

Chang WC, Lan TH (1995) Somatic embryogenesis and plant regeneration from roots of bamboo (*Bambusa beecheyana* Munro var. beecheyana). J Plant Physiol 145:535–538

Geng SX, Pu XL, Wang SG (2006) A study on callus induction culture of *Dendrocalamus sinicu*. J W China Forestry Sci (in Chinese, with abstract in English) 35:78–83

Guerreiro C (2014) Flowering cycles of woody bamboos native to southern South America. J Plant Res 127:307–313

Hassan AAE, Debergh P (1987) Embryogenesis and plantlet development in the bamboo *Phyllostachys viridis* (Young) McClure. Plant Cell Tiss Org 10:73–77

Hesami M, Tohidfar M, Alizadeh M, Daneshvar MH (2020) Effects of sodium nitroprusside on callus browning of *Ficus religiosa*: an important medicinal plant. J Forestry Res 31:789–796

Huang L, Chen W, Huang B (1988) Tissue culture investigations of bamboo—II. Liquid suspension cultures of *Bambusa*, *Phyllostachys* and *Sasa* cells. Bot Bull Acad Sin (Taipei) 29:117–182

Huang LC, Lee YL, Huang BL, Kuo CI, Shaw JF (2002) High polyphenol oxidase activity and low titratable acidity in browning bamboo tissue culture. Vitro Cell Dev-pl 38:358–365

Khosroushahi AY, Naderi-Manesh H, Simonsen HT (2011) Effect of antioxidants and carbohydrates in callus cultures of *Taxus brevifolia*: evaluation of browning, callus growth, total phenolics and paclitaxel production. Bioimpacts 1:37

Kristensen JB, Xu XB, Mu HL (2005) Process optimization using response surface design and pilot plant production of dietary diacylglycerols by lipase-catalyzed glycerolysis. J Agr Food Chem 53:7059–7066

Li ZL, Hui CM (2006) Study on tissue culture of *Dendrocalamus sinicu*. Sci Silvae Sinicae (in Chinese, with abstract in English) 42:43–48

Liese W, Khöl M (2015) Bamboo: the plant and its uses. Springer, Heidelberg

Lin S, Liu G, Guo T, Zhang L, Wang S, Ding Y (2019) Shoot proliferation and callus regeneration from nodular buds of *Drepanostachyum luodianense*. J Forestry Res 30:1997–2005

Liu FF, Ang CYW, Springer D (2000) Optimization of extraction conditions for active components in hypericum perforatum using response surface methodology. J Agr Food Chem 48:3364–3371

Liu JH, Shi J (1996) Studies on selection of valuable somaclonal mutants in silage maize. Acta Bot Sin 38:839–842

Liu L, Fan X, Zhang J, Bao YM (2009) Long-term cultured callus and the effect factor of high-frequency plantlet regeneration and somatic embryogenesis maintenance in *Zoysia japonica*. Vitro Cell Dev-Pl 45:673–680

Miller LR, Murashige T (1976) Tissue culture propagation of tropical foliage plants. Vitro Cell Dev-pl 12:797–813

Moghaddam SS, Moghaddam MRA, Arami M (2010) Coagulation/flocculation process for dye removal using sludge from water treatment plant: optimization through response surface methodology. J Hazard Mater 175:651–657

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plantarum 15:473–497

Ogita S (2005) Callus and cell suspension culture of bamboo plant, *Phyllostachys nigra*. Plant Biotechnol-Nar 22:119–125

Ohrnberger D (1999) The bamboos of the world: annotated nomenclature and literature of the species and the higher and lower taxa. Elsevier Science, Amsterdam, pp 12–38

Pourcel L, Routaboul JM, Cheynier V, Lepiniec L, Debeaujon I (2007) Flavonoid oxidation in plants: from biochemical properties to physiological functions. Trends Plant Sci 12:29–36

Qiao G, Li H, Jiang J, Sun Z (2010) Anther culture and plant regeneration of *Dendrocalamus latiflorus*. Chin Bull Bot 45:88–90

Rout GR, Das P (1994) Somatic embryogenesis and in vitro flowering of 3 species of bamboo. Plant Cell Rep 13:683–686

Saltveit ME (2000) Wound induced changes in phenolic metabolism and tissue browning are altered by heat shock. Postharvest Biol Tec 21:61–69

Sankhyan HP, Kumar P, Sehgal RN (2009) Seed Setting in *Dendrocalamus strictus* Nees. (Male Bamboo) in Himachal Pradesh. Environment Ecol 27:1153–1156

Sarma H, Sarma AM, Sarma A, Borah S (2010) A case of gregarious flowering in bamboo, dominated lowland forest of Assam, India: phenology, regeneration, impact on rural economy, and conservation. J Forestry Res 21:409–414

Saxena S, Dhawan V (1999) Regeneration and large-scale propagation of bamboo (*Dendrocalamus strictus* Nees) through somatic embryogenesis. Plant Cell Rep 18:438–443

Shirin F, Rana PK (2007) In vitro plantlet regeneration from nodal explants of field-grown culms in *Bambusa glaucescens* Willd. Plant Biotechnol Rep 1:141–147

Siddique AB, Islam SM (2015) Effect of light and dark on callus induction and regeneration in tobacco (*Nicotiana tabacum* L.). Bangl J Bot 44:643–651

Singh SR, Singh R, Kalia S, Dalal S, Dhawan AK, Kalia RK (2013) Limitations, progress and prospects of application of biotechnological tools in improvement of bamboo—a plant with extraordinary qualities. Physiol Mol Biol Pla 19:21–41

Taylor AH, Jinyan H, Shiqiang Z (2004) Canopy tree development and undergrowth bamboo dynamics in old-growth *Abies-Betula* forests in southwestern China: a 12-year study. Forest Ecol Manag 200:347–360

Wang WG, Wang SH, Wu XA, Jin XY, Chen F (2007) High frequency plantlet regeneration from callus and artificial seed production of rock plant *Pogonatherum paniceum* (Lam.) Hack. (Poaceae). Sci Hortic-amsterdam 113:196–201

Yang BP, Zhang SZ, Hui CM, Zan LM (2004) Tissue culture and rapid propagation of *Dendrocalamus sinicus*. Plant Physiol J 40:346

Ye S, Cai C, Ren H, Wang W, Xiang M, Tang X, Zhu C, Yin T, Zhang L, Zhu Q (2017) An efficient plant regeneration and transformation system of Ma Bamboo (*Dendrocalamus latiflorus* Munro) started from young shoot as explant. Front Plant Sci 8:1298

Yeh ML, Chang WC (1986) Somatic embryogenesis and subsequent plant regeneration from inflorescence callus of *Bambusa beecheyana* Munro var. beecheyana. Plant Cell Rep 5:409–411

Yeh ML, Chang WC (1987) Plant regeneration via somatic embryogenesis in mature embryo-derived callus culture of Sinocalamus latiflora (Munro) McClure. Plant Sci 51:93–96

Yi T, Shi J, Ma L, Wang H, Yang L (2008) Iconographia bambusoidearum sinicarum. Science Press, Beijing, China

Yuan J, Gu X, Li L, Yue J, Yao N, Guo G (2009) Callus induction and plantlet regeneration of *Bambusa multiplex*. Sci Silvae Sinicae (in Chinese, with abstract in English) 45:35–39

Yuan JL, Yue JJ, Wu XL, Gu XP (2013) Protocol for callus induction and somatic embryogenesis in moso bamboo. PLoS ONE 8:e81954

Zang Q, Zhou L, Zhuge F, Yang H, Wang X, Lin X (2016) Callus induction and regeneration via shoot tips of *Dendrocalamus hamiltonii*. Springerplus 5:1799. https://doi.org/10.1186/s40064-016-3520-7

Zeng QM (2018) Study on the tissue culture and rapid propagation technology of *Bambusa pervariabilis* × *Dendrocalamopsis grandis*. Subtropical Soil Water Conserv (in Chinese, with abstract in English) 30:6–8

Zhang N, Fang W, Shi Y, Liu Q, Yang H, Gui R, Lin X (2010) Somatic embryogenesis and organogenesis in *Dendrocalamus hamiltonii*. Plant Cell Tiss Org 103:325–332