ORIGINAL ARTICLE

Assessment of genetic stability and analysis of alkaloids potential in micropropagated plants of *Croomia japonica* Miquel, an endangered, medicinal plant in China and Japan

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Abstract

Croomia japonica (Stemonaceae) is an endangered species both in China and Japan. We have developed an efficient regeneration system through adventitious buds organogenesis in *C. japonica* using rhizome buds as explants. Multiple buds regenerated directly on the explants without calli within 2 months when explants were cultured on Murashige and Skoog's (MS) medium with 8.88 µmol 6-benzylaminopurine (BAP) and 1.07 µmol α-naphthaleneacetic acid (NAA). The adventitious buds of newly forming were proliferated by subsequent subcultures on MS medium with 2.66 µmol BAP and 2.69 µmol NAA. We evaluated the kinds and concentrations of plant growth regulators on adventitious shoot regeneration and root induction and also inspected the adsorbent (polyvinyl pyrrolidone and activated charcoal) and antioxidant (ascorbic acid, AS) on the inhibition of tissue browning. The results showed that soaking the explants with 1.14 mmol AS was the best approach for controlling browning. The maximum number of stout shoots per explant was achieved on MS medium containing 8.88 µmol BAP. In vitro regenerated shoots were rooted on MS medium supplemented with three different concentrations of auxin. The highest rooting rate (84.0 ± 3.6%) was reached on MS medium with 5.71 µmol Indole-3-acetic acid (IAA). One-step culture was developed when the adventitious buds cultured on the medium were supplemented with 2.66 µmol BAP and 0.54–2.69 µmol NAA. Rooted plantlets were acclimatized to the green house and development with a 87% survival rate. Genetic stability assessment of in vitro plants compared to the wild plants was revealed by simple sequence repeat markers. Similarly, flow cytometric analysis confirmed that the ploidy level of in vitro plantlets was stable. Total alkaloid content in wild and in vitro plants was tested by acid dye colorimetric analysis with tuberostemonine as the reference. Our results showed that alkaloids content in 11-week cultures reached 40.7–88.4% of the content of wild plants. The protocol described here could be employed for effective mass propagation of *C. japonica* for commercial and conservational purposes.

Keywords Alkaloids · *Croomia japonica* · Endangered species · Genetic stability · In vitro propagation · Molecular marker · Stemonaceae

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Abbreviations

AC	Activated charcoal
AS	Ascorbic acid
BAP	6-Benzylaminopurine
BCG	Bromocresol green
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	α-Naphthaleneacetic acid
PGR	Plant growth regulator
PVP	Polyvinyl pyrrolidone
SSR	Simple sequence repeat

Introduction

The genus *Croomia* Torr. (Stemonaceae) has three extant species: *C. japonica* Miq., *C. heterosepala* (Bak.) Oku. and *C. pauciflora* (Nutt.) Torr. (Rogers 1982; Li 1952). Of the three species, *C. pauciflora* is native to Southeastern North America, whereas *C. japonica* and *C. heterosepala* are restricted to East Asia (Ji and Duyfjes 2000). Because of their small range size and small number of populations, all three species have been listed as rare and endangered in China, Japan and the United States (Fu 1992; Patrick et al. 1995; Estil and; Cruzan 2001; Kato and Ebihara 2011). Anthropogenic and climate change-induced habitat degradation may have a negative impact on the reproduction of *Croomia* species, which likely contributes to their rarity.

C. japonica, the focal species of the present study, is an erect, perennial herb distributed in Zhejiang, Jiangxi, Fujian, and Anhui provinces in China and on the islands of Kyushu and Shikoku in Japan (Li 1952). As the roots and rhizomes of this species contain the alkaloids such as croomine, didehydrocroomine, pachysamine A and croomionidine (Lin et al. 1993), this species is commonly used in Chinese traditional medicine for detoxifying snake bites (Xi 1993). It occurs in warm-temperate deciduous forests between 800 and 1000 m elevation. The mode of regeneration of *C. japonica* in nature is by seed as well as by rhizome. In many cases, it may produce large colonies by spreading through underground rhizomes (Ji and Duyfjes 2000). Field observations indicate that although the plants flower almost every year, seed production is limited, and seedling establishment is poor. Previous studies have focused primarily on molecular phylogeography (Li et al. 2008), population genetics (Fang et al. 2013), and phytochemistry (Lin et al. 1993). Yet, there is currently little effort towards the development of conservation strategies for this endangered species. One of the most appropriate actions for conservation and sustainable utilization of this species is to improve propagation techniques and to encourage cultivation. The use of in vitro techniques for rapid and mass propagation offers possibilities for recovery of such endangered species. At present, there are few reports dealing with *C. japonica* propagation. The in vitro callus induction and prevention of explants browning was described by Li et al. (2012) and Chen et al. (2012). The callus was derived from the stem explant culturing on MS medium supplemented with 6.97 μmol KT and 2.69 μmol NAA, with the frequency of callus formation was 88.9%. Chen et al. (2012) suggested that the browning inhibitory effect of antioxidants (citric acid, ascorbic acid and dithiothreitol) on explants were worse than that of adsorbents (activated carbon and polyvinyl pyrrolidone). And the browning rate was the lowest when adding activated

carbon with 1500 mg l^{-1} on the medium. Although their conclusions were very meaningful, protocols for regeneration of *C. japonica* have not been yet established.

Field observation indicated that the rhizomes of wild *C. japonica* have one to five dormant buds, of which only one strong bud can sprout to form a new plant. Hence, up to five buds per rhizome can be used for explants. Because the rhizome buds are easily contaminated, the availability of sterile cultures is very difficult.

Plantlets derived from organized shoot meristems are genetically more stable than those regenerated from callus tissues (Pierik 1991). Therefore, utilizing buds from rhizomes as explants, the genetically uniform plantlets will be obtained in a relatively short time. This is very important and essential for conservation of the endangered *C. japonica*.

Maintaining the genetic stability of the donor plant and that of the in vitro plant is indispensable before adopting the protocol for large-scale clonal propagation and for *ex situ* conservation purpose (Jin et al. 2008; Paunescu 2008). With the development of next generation sequencing, it is possible to provide additional means (not just RAPD and ISSR) for assessing genetic stability in plants (Agarwal et al. 2015; Viehmannova et al. 2016). Microsatellites or simple sequence repeats (SSRs) are co-dominant markers and capable of distinguishing homozygous from heterozygous polymorphisms (Haig 1998; Parker et al. 1998). Thus, they have been widely used in assessing population genetic structure and gene flow as well as detecting genetic stability of in vitro plants (Kuroda et al. 2006; Ge et al. 2005; Morais-Lino et al. 2016; Castillo et al. 2010). In view of the medicinal and conservational importance of *C. japonica*, the present study was carried out with the following objectives: (1) to direct shoot organogenesis using adventitious buds originated from the rhizomes as explants, (2) to determine the genetic stability of in vitro regenerated plants by molecular markers (SSRs) and flow cytometry, and (3) to evaluate the total alkaloids contents in both tissue cultured and wild plants.

Materials and methods

Plant material and explant preparation

Plants of *C. japonica* were collected from Tianmu Mountain, Zhejiang Province, in April 2014 and grown for about 3 years in the Botanical Garden of Zhejiang University. The identity of the plants was authenticated by Yingxiong Qiu. Voucher specimens have been deposited in the Herbarium of Zhejiang University (HZU). Adventitious buds, 3–5 mm long, were excised from the donor rhizome and were thoroughly washed under running tap water for 15–20 min, then soaked in 75% (V/V) ethanol for 30 s, followed by thorough washing with distilled water. The explants were finally

treated with 0.1% mercuric chloride for 12 min and subsequently rinsed five times with sterile distilled water.

Direct organogenesis and proliferation on the rhizome bud cultures

The sterile rhizome buds were inoculated in a glass tube (2.5 cm × 9.5 cm, breadth × height) containing 15 ml of medium consisting of Murashige and Skoog (MS; Murashige and Skoog 1962) salts, vitamins, 3% sucrose (w/v), 0.68% agar (w/v) and 0.8 g l^{-1} polyvinyl pyrrolidone (PVP) supplemented with 8.88 µmol 6-benzylaminopurine (BAP) and 1.07 µmol α-naphthaleneacetic acid (NAA) to obtain adventitious buds. The newly formed adventitious buds were divided into single buds and placed on MS medium with 2.67 µmol BAP and 2.69 µmol NAA for rapid propagation to obtain enough buds and rhizomes for subsequent analysis. Direct shoot organogenesis was optimized using the rhizomes and buds as the source of explants. The culture vessels (5.5 cm × 9.5 cm, breadth × height) contained 40 ml of MS medium supplemented with BAP (0–13.32 µmol) alone or BAP at a concentration of 2.67 µmol in combination with NAA (0–5.37 µmol). The pH of the medium was adjusted to 5.8 with 1N KOH or HCl and sterilized by autoclaving at 121 °C for 20 min. The optimum combination of plant growth regulators (PGRs) to promote the proliferative rate of rhizome bud cultures was determined after 6 weeks. The culture vessels were kept under white fluorescent light (35 µmol m^{-2} s^{-1}) for a 16-h photoperiod at 25 ± 2 °C. Each treatment consisted of 15 cultures and the experiment was repeated three times.

In vitro culture of *C. japonica*, the base parts of the plants easily turned brown and caused the death of the culture. Therefore, adsorbents [PVP and activated charcoal (AC)] and antioxidant [ascorbic acid (AS)] were added to the medium or the explants were soaked in combinations of PGRs for studies on bud proliferation.

Rooting and acclimatization

Elongated shoots (3–4 cm) were excised and transferred to MS medium supplemented with different concentrations of indole-3-acetic acid (IAA), NAA or Indole-3-butyric acid (IBA). The frequency of rooting, average number of roots per shoot and root length were recorded after 7 weeks culture.

The regenerated plantlets with well-developed roots were removed from the rooting medium and washed with water to remove traces of agar. The young plantlets were transplanted into pots containing a soil, sand and humus mixture (2:1:1). Plantlets were acclimatized for 4 weeks under greenhouse conditions for a 12 h photoperiod (light 35 µmol m^{-2} s^{-1}; humidity 70–80%; temperature 15–20 °C). The young

plantlets were watered once a week with half-strength MS liquid medium without sucrose. The survival rate (%) of the hardened plantlets was estimated after 2 months of acclimatization.

Genetic stability analysis of regenerated plantlets using SSR markers

Total genomic DNA was extracted from silica-gel dried leaves of donor plants and 39 in vitro regenerants (on media with different plant growth regulators) using the cetyltrimethylammonium bromide (CTAB) method (Doyle 1991). A total of 10 pairs of primers, which have been proved to be polymorphic among the three species (Lu, unpublished data), were used to analyze the genetic stability between the donor plants of *C. japonica* and regenerated plantlets. All the PCR reactions were performed in a 20 µl reaction mixture containing 10 µl 2X master mix (Tsingke, Beijing, China), 8 µl of dd H$_2$O, 0.5 µl of 10 µM forward and reverse primer, and 1 µl of genomic DNA (50 ng). The PCR amplification procedure was as follows: one cycle of 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 50–55 °C for 40 and 30 s at 72 °C with a final extension 72 °C for 10 min, followed by storage at 4 °C in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The successful PCR products were tested for product size and polymorphism using 12% non-denaturing polyacrylamide gels with a 100 bp marker (TaKaRa, Dalian, Liaoning, China) as reference and visualized by silver staining.

Flow cytometry analysis

Intact nuclei suspension was prepared following the protocol developed by Doležel, and Bartoš (2005). The fully developed leaves of the donor plants as well as in vitro regenerated plants were analyzed. The selected leaves were chopped with a sharp razor blade in a plastic petri dish with 2 ml of lysis buffer (Galbraith's buffer) containing 45 mM MgCl$_2$; 30 mM sodium citrate; 20 mM 4-morpholinepropane sulfonate; and 0.1% (w/v) Triton X-100; For a final pH of 7.0. After chopping, the nuclear suspension was filtered through a 40 µm stainless steel mesh filter (East China Pharmaceutical Co., Ltd, Hangzhou, Zhejiang, China) and centrifuged at 5000×*g* rpm for 2 min. Then the precipitate (containing the nuclei) was stained with 50 µg ml^{-1} PI (propidium iodide)/RNase staining buffer (BD-Pharmingen). Estimation of nuclear DNA content was performed within a 15 min period with a BD FACSCalibur flow cytometer (Becton–Dickinson, NJ, USA) equipped with a 15 mW 488 nm air-cooled Argon ion laser. For each sample, at least 10,000 nuclei were tested and histograms were obtained using BD Cell Quest Pro software (version 6.0, BD Bioscience, USA).

Extraction and evaluation of total alkaloids in dried rhizomes and roots from regenerated plants and wild plants

The in vitro rhizomes and roots from the different PGR treatments as well as the wild rhizomes and roots were cut into small pieces and dried in an oven at 50 °C, then ground into powder. The dried powder (0.5 g) was extracted twice with 10 ml methanol (MeOH) under ultrasonic conditions (Elmasonic P120H, Elma, Germany, 37 kHz, 30 °C) for 30 min each time. The MeOH solution was filtered, the filtrates were combined, and the final volume was brought up to 50 ml with MeOH. 2 ml of this solution was transferred to a separatory funnel to which 10 ml of chloroform and 2 ml of bromocresol green (BCG) (0.04%) was added. The mixture was shaken vigorously for 1 min and then kept for 30 min. The layer of chloroform solution was then analyzed.

Standards of tuberostemonine were purchased from (Chengdu PufeiDe Biotech Co., Ltd. Chengdu, Sichuan, China), dissolved in chloroform to a concentration of 0.10 mg ml^{-1} and used for the calibration curves at 6 concentrations. Accurately measured aliquots (0.2, 0.4, 0.6, 0.8, 1 and 1.2 ml) of tuberostemonine solution (0.10 mg ml^{-1}) were diluted with chloroform to 10 ml in a volumetric flask, then each was transferred to different separatory funnels. 2 ml BCG solution (0.04%) was added. The mixture was shaken for 1 min and then kept for 30 min. The absorbance of the complex in chloroform was measured at 416 nm against a blank prepared as mentioned above but without tuberostemonine. The optical density (OD) value for each concentration was calculated and the standard calibration curves were constructed by plotting concentrations against the OD value. The regression equation was: $Y = 61.18X + 0.101$, $r^2 = 0.999$. The results showed that there was a linear relationship in the range of 0.002–0.012 mg ml^{-1}.

Data analysis

Each treatment was repeated three times. The Data were analyzed using one-way analysis of variance (ANOVA) at the 0.05 significance level in SPSS 22.0 (SPSS Inc., Chicago, IL, USA). A test of homogeneity of variances was performed for the data before post hoc multiple comparisons. If equal variances were assumed, Tukey's-b tests were chosen as the post hoc test method; otherwise, Tamhane T2 was chosen.

Results and discussion

Shoot regeneration through direct organogenesis

Rhizome buds (about 3–5 mm in length) (Fig. 1a) were inoculated on MS medium supplemented with 8.88 μmol BAP, 1.07 μmol NAA and 0.8 g l^{-1} PVP. After 2 months of culture, 3–5 adventitious buds were derived from the rhizome buds. The average length of the adventitious buds was 1.41 cm (Fig. 1b). In the next subculture, the adventitious buds were separated into single buds and cultured on MS supplemented with 2.66 μmol BAP, 2.69 μmol NAA and 0.8 g l^{-1} PVP. After 6 weeks' culture, each single bud formed 5–7 adventitious buds that were 3–5 cm long with 5–7 roots in the range of 4–6 cm. Through repeated subculture, many adventitious buds will be further studied to enhance our knowledge about PGRs on bud proliferation.

PVP and PGRs on adventitious buds proliferation

The adventitious buds were induced from the rhizome segments of *C. japonica* within 6 weeks after inoculation of explants on MS medium supplemented with PVP and PGRs. The highest adventitious bud count per rhizome segment was 8.8 ± 2.0 buds on MS medium augmented with 2.22 μmol BAP (Table 1), followed by 2.67 μmol BAP in combination with 2.69 μmol NAA (8.0 ± 1.5). BAP with a concentration of 4.44–13.3 μmol on the medium proved to be beneficial in inducing adventitious bud growth, average range of tall buds was between 3.4 and 3.9, the petiole was stout with light red color and the heights of the plantlet were 4.4–4.8 cm. 2.67 μmol BAP in combination with different concentrations of NAA did

Fig. 1 The wild plant of *C. japonica* and the adventitious buds initiation. **a** Roots and rhizome of the wild plant (arrow showed the adventitious bud), **b** the bud elongated on the MS + BAP 8.88 μmol + NAA 1.07 μmol + PVP 0.8 g l^{-1}. Bars 10 mm
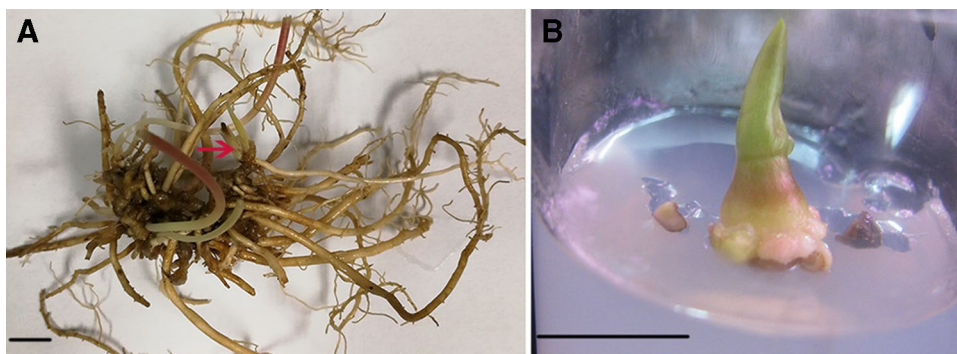
Table 1 Effect of BAP alone or combination with NAA on adventitious buds proliferation of *C. japonica* on the medium with PVP

Plant growth regulators (µmol)		No. of total buds/rhizome segment	No. of tall buds/rhizome segment	Shoot length (cm)	Browning rate (%)	Growth character
BAP	NAA					
0	0	3.8 ± 0.6c	1.4 ± 0.5 d	2.7 ± 0.5b	44 ± 7.5bc	+
0.89	0	5.6 ± 0.5b	2.0 ± 0.7cd	3.1 ± 0.3b	53.3 ± 8.6ab	++
2.22	0	8.8 ± 2.0a	2.5 ± 0.5c	3.7 ± 0.5ab	61.3 ± 6.11a	++
4.44	0	6.8 ± 1.6ab	3.4 ± 0.5ab	4.8 ± 0.5a	26.7 ± 3.8d	++++
8.88	0	7.4 ± 1.6ab	3.9 ± 0.8a	4.6 ± 0.5a	32 ± 3.6cd	++++
13.32	0	6.9 ± 1.2ab	3.4 ± 0.6ab	4.4 ± 0.3a	35.7 ± 5.0cd	++++
2.66	0.54	7.2 ± 2.4ab	2.7 ± 0.7bc	4.7 ± 0.4a	14.3 ± 1.5e	+++
2.66	2.69	8 ± 1.5a	2.6 ± 0.5bc	4.4 ± 0.1a	12.0 ± 2.6e	+++
2.66	5.37	7.3 ± 1.5ab	2.5 ± 0.5c	4.2 ± 0.2a	10.3 ± 2.5e	+++

+ Seedlings grow slowly, the shoots short

++ Seedlings grow slowly, the shoots with moderate height and the petioles thin

+++ Seedlings grow fast, shoots taller

++++ Seedlings also grow fast, shoots taller, leaves big and the petioles stout. Values represent mean ± SD of 15 replicates/treatment and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey's-b test (P ≤ 0.05)

not significantly stimulate adventitious bud proliferation, but with increased concentrations of NAA, the number of tall buds decreased. It was observed that the browning rate decreased with increasing BAP concentration. The average browning rate was about 32.2%, which was significantly decreased to 12.2% when 2.67 µmol BAP in combination with different concentrations of NAA was applied.

Browning is mainly due to the cutting of the explants; the cells near the incision are injured and the phenolic compounds are secreted. The phenolic compounds are converted into quinones by polyphenol oxidase. Browning of tissues is a major problem during culture and effective control is key to the success of plant tissue culture (Jones and Saxena 2013).

In plant tissue culture, several methods were developed to prevent tissue browning: (1) Adding adsorbents such as AC or PVP that bind phenolic compounds to render them less toxic (Thomas 2008; Jones and Saxena 2013). (2) Supplementing antioxidants such as ascorbic acid, citric acid, or melatonin (Uchendu et al. 2011). PVP, an effective antioxidant that prevents phenolic oxidation by adsorption through hydrogen bonding (George and Sherrington 1984), was successful in preventing tissue browning in *Tinospora cordifolia* at 1.0% (Mittal et al. 2017). Shukla et al. (2009) showed that PVP added to proliferation medium induced healthy shoots in *Stereospermum personatum*; however, Panda and Hazra (2010) showed that PVP increased the release of phenolics, and thus resulted in the reduction of sprouting frequency in *Semecarpus anacardium*. In this study, the addition of PVP had some effect on preventing tissue browning with a 67.8% inhibition rate in *C. japonica* at 0.8 g l^{-1}.

AC and PGRs on adventitious bud proliferation

The browning rate was greatly increased when the media was supplemented with AC (Table 2). The average browning rate for all treatments was as high as 40.1%. The regenerated buds were small, thin and the petioles were slender and green. When the BAP concentration was low (0–4.44 µmol), the shoots could hardly grow. The browning rate was extremely high with increased BAP concentration. When the BAP concentration reached 8.88–13.3 µmol, the browning rate was reduced to 34.7–37.7%. The average browning rate was decreased to 26% when 2.67 µmol BAP was used in combination with different concentrations of NAA.

Activated charcoal can serve as an efficient adsorbent of toxic substances (Thomas 2008). However, a recent study showed that AC significantly increased phenolic secretion in transverse thin cell layers of *Rubus hirtus* (Sabooni and Shekafandeh 2017). In our study, it seems that AC may have increased phenolic secretion and caused tissue browning, or AC adsorbed many components (polyphenols and PGRs) and therefore the explants grow poorly and finally withered. Chen et al. (2012) showed that AC with 1500 mg l^{-1} on the medium can prevent browning of *C. japonica* calli, which is inconsistent with our study.

AS and PGRs on proliferation of adventitious buds

The rhizome segments were cut into 1 cm × 1 cm pieces, soaked in AS solution at a concentration of 1.14 mmol for 5 min, then inoculated in MS medium supplemented with BAP alone or in combination with NAA. The regeneration

Table 2 Effect of BAP alone or in combination with NAA on adventitious buds proliferation of *C. japonica* on the medium with AC

Plant growth regulators (μmol)		No. of total buds/ rhizome segment	No. of tall buds/ rhizome segment	Shoot length (cm)	Browning rate (%)	Growth charac-ter
BAP	NAA					
0	0	2.5 ± 0.5b	1.2 ± 0.4c	2.1 ± 0.3c	27 ± 7cde	+
0.89	0	3.6 ± 0.9b	1.4 ± 0.5c	2.3 ± 0.4bc	52.3 ± 4.5b	+
2.22	0	3.5 ± 1.0b	1.5 ± 0.5c	2.5 ± 0.3bc	68 ± 6.6a	+
4.44	0	3.5 ± 1.3b	2.3 ± 0.5abc	2.6 ± 0.4bc	73.7 ± 5.1a	+
8.88	0	4.4 ± 1.1ab	2.8 ± 0.8a	3.5 ± 0.4ab	34.7 ± 4.5cd	++
13.32	0	6.8 ± 1.0a	2.7 ± 0.8ab	3.3 ± 0.7ab	37.7 ± 6.8c	++
2.66	0.54	4.4 ± 1.7ab	1.7 ± 0.7bc	3.7 ± 0.4a	18.3 ± 4.5e	++
2.66	2.69	5.3 ± 0.8ab	2.0 ± 0.7abc	3.4 ± 0.3ab	22.7 ± 4.2de	++
2.66	5.37	4.8 ± 1.0ab	1.6 ± 0.5bc	3.0 ± 0.1b	37 ± 3c	++

+++ Meanings are the same with Table 1

Values represent mean ± SD of 15 replicates/treatment and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey's-b test (P ≤ 0.05)

of adventitious buds and the browning rate was analyzed within 6 weeks culture (Table 3). The browning rate was greatly reduced when the rhizome segments soaked in AS solution. The average browning rate for all treatments was only 12.4%. Adventitious bud induction and bud growth characteristics were similar with those implanted on the PVP media. The highest adventitious bud count per rhizome segment was 9.0 ± 1.6 with 2.22 μmol BAP on the medium, followed by 2.67 μmol BAP in combination with 2.69 μmol NAA (8.6 ± 1.4) (Table 3). BAP (4.44–13.3 μmol) proved to be beneficial for bud growth as the forward PVP treatment. The shoot height on the medium with 2.67 μmol BAP in combination with different concentrations of NAA was

slightly greater than that of MS with 4.44–8.88 μmol BAP, but the petioles were slender and the tall buds on the rhizome segment were less.

In our study, rhizome segments were soaked into AS solution for 5 min and then inoculated in MS medium with different PGRs which seemed to control the tissue browning successfully. Similar findings were observed in *Rubus sanctus* thin cell layer propagation. Explants soaked in 0.34 mmol AS not only had a reduced production of phenolic compounds, but also produced friable, yellow-pale green calluses (Sabooni and Shekafandeh 2017). Guo et al. (2009) showed that with an increase in concentration of AS on the medium, the medium was easily became liquid or

Table 3 Effect of BAP alone or in combination with NAA on adventitious buds proliferation of *C. japonica* when the explants were soaked with 1.14 mmol AS for 5 min

Plant growth regulators (μmol)		No. of total buds/rhizome segment	No. of tall buds/rhizome segment	Shoots length (cm)	Browning rate (%)	Growth character
BAP	NAA					
0	0	6.4 ± 1.2bc	1.5 ± 0.5c	2.8 ± 0.2e	10.0 ± 2ab	++
0.89	0	8.3 ± 1.8a	2.2 ± 0.7bc	3.2 ± 0.4d	13.0 ± 1ab	++
2.22	0	9.0 ± 1.6a	2.6 ± 0.9b	4.0 ± 0.3c	10.7 ± 2.1ab	++
4.44	0	7.6 ± 0.8ab	3.2 ± 0.6ab	4.6 ± 0.4b	14.3 ± 2.5ab	++++
8.88	0	6.3 ± 0.9bc	3.5 ± 0.7ab	5.1 ± 0.6ab	15.3 ± 3.5ab	++++
13.32	0	6.8 ± 1.1bc	3.7 ± 0.7a	4.5 ± 0.6bc	16.7 ± 3.1a	++++
2.66	0.54	5.9 ± 1.4c	3.2 ± 0.4ab	5.2 ± 0.4a	12.0 ± 2.6ab	+++
2.66	2.69	8.6 ± 1.4a	3.4 ± 0.5ab	5.0 ± 0.3ab	10.3 ± 2.5ab	+++
2.66	5.37	8.3 ± 1.3a	3.0 ± 0.7ab	4.8 ± 0.4ab	9.7 ± 1.5b	+++

++, +++, ++++ Meanings are the same with Table 1

Values represent mean ± SD of 15 replicates/treatment and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey's-b test (P ≤ 0.05)

semi-solid and the explants were likely to die. In our experiments, explants were soaked into the AS solution just to inhibit the browning of the tissue and it had no effect on the pH of the medium.

Our results suggested that rhizome segments of *C. japonica* soaked in AS (1.14 mmol, filter sterilization) solution prior to culture in MS medium containing 4.44–8.88 μmol BAP were an effective method for propagation. This not only controlled the tissue browning, but also produced healthy plantlets.

For in vitro propagation of *C. japonica*, cytokinin BAP alone, or in combination with NAA was used. Better results were obtained using BAP at concentrations of 4.44–8.88 μmol. BAP had been reported to be optimal for bud induction in many species of Stemonaceae. Palee et al. (2013) indicated BAP 8.88 μmol was optimal for bud induction and the induction rate was 100%. This was consistent with our results. In some species of *Stemona*, for example *S. curtisii* (Montri and Wawrosch 2007), *S. tuberose* (Biswas et al. 2011), higher concentrations of BAP ranging from 13.32 to 19.97 μmol were more effective in shoot proliferation.

Many studies have shown that a combination of a slightly higher concentration of BAP and a slightly lower concentration of NAA to be optimal for bud induction in many species both in woody plants and herbs, for example, in *Vanilla planifolia* (George and Ravishankar 1997), *Gloriosa superb* (Hassan and Roy 2005), *Tectona grandis* (Shirin et al. 2005), *and Rosa centifolia* (Akhtar et al. 2016). Like the studies, in *C. japonica*, the addition of NAA on the medium lead to an improvement in propagation rate, but the petioles were thin compared with those in the BAP 4.44–8.88 μmol treatment. One advantage of the combination of BAP and NAA was that the rate of browning can be reduced regardless of whether or not the medium supplemented with PVP or rhizome segments was pre-soaked with AS solution. Meenashree et al. (2017) showed that NAA gave healthy calli without browning in *Bacopa monnieri* culture. Chen et al. (1996) showed that calli of *Ginkgo biloba* cultured in medium supplemented with BAP and NAA had the best calli feature (milky yellow, granular) and did not easily brown. This was consistent with our results. Another benefit was that after 7 weeks culture, adventitious roots occurred at the base of the rhizome with a 75% rooting rate, reaching one-step culture to form the seedlings with buds and roots. Shoot proliferation and root induction was more effective on medium with 2.66 μmol BAP combined with 0.54–2.69 μmol NAA. Stimulation of shoot bud and root induction in plant tissue culture is often through a two-step or three-step culture (one step to induce callus, the other step(s) to differentiate shoots and roots simultaneously or respectively) (Hulme et al. 1992; Chen et al. 1987). Yan and Huang (1986) showed that seedlings formed successfully through only one-step culture when anthers of *Tritium aestivum* were cultured in a medium of C17 supplemented with 4.44 μmol BAP and 2.26 μmol 2,4-D. As we found, the one-step culture is as an efficient and often economical, simple and quick method to obtain a large number of plantlets.

Rooting and acclimatization

The shoots (3–4 cm in length) regenerated from rhizome buds were excised individually and cultured on MS medium containing different concentrations of NAA, IAA or IBA for root induction. After 4 weeks, roots only occurred on the base of the shoots cultured in MS medium fortified with IAA and auxin-free medium. In the next 3 weeks incubation, the roots began to appear on the base of the shoots. The highest percentage of rooting (84%) occurred in medium supplemented with 5.71 μmol IAA as described in Table 4, followed by MS medium with 2.85 μmol IAA (82.3%) and MS with auxin-free medium (81.6%). The maximum length of roots (1.7 ± 0.5 cm long) was observed in shoots cultured in MS control medium, followed by MS medium with 2.85 μmol IAA (1.7 ± 0.4 cm) and 5.71 μmol IAA (1.5 ± 0.4 cm). The highest number of roots (6.7 ± 1.3) was found in shoots cultured in MS medium supplemented with 10.74 μmol NAA. The roots were thick and short in all NAA medium. When the medium was supplemented with IBA at a concentration of 2.46–9.84 μmol, the rhizome proliferated greatly and the rooting rate changed from 52 to 75.3%, but the roots were thin and short.

Table 4 Effect of different concentrations of auxins on in vitro rooting from micro-shoots of *C. japonica* after 7 weeks culture

Auxin (μmol)	Rooting (%)	No. of roots per shoot	Length of roots (cm)
Control	81.6 ± 3.5a	4.2 ± 0.6d	1.7 ± 0.5a
IAA			
2.85	82.3 ± 2.5a	4.3 ± 0.9d	1.7 ± 0.4a
5.71	84.0 ± 3.6a	5.4 ± 1.7abcd	1.5 ± 0.4ab
11.42	61.3 ± 3.5b	4.8 ± 1.0cd	1.3 ± 0.4ab
NAA			
2.69	65.7 ± 4.0b	4.8 ± 0.8cd	1.0 ± 0.1b
5.37	77.7 ± 2.5a	6.5 ± 1.0ab	0.9 ± 0.2b
10.74	60.7 ± 4.0b	6.7 ± 1.3a	0.8 ± 0.1b
IBA			
2.46	75.3 ± 4.5a	6.1 ± 1.4abc	1.1 ± 0.4ab
4.92	65.7 ± 4.0b	4.7 ± 1.0cd	1.0 ± 0.3b
9.84	52.0 ± 4.6c	5.0 ± 0.8bcd	0.9 ± 0.2b

Values represent mean ± SD of 15 replicates/treatment and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey's-b test (P ≤ 0.05)

In general, root induction in *C. japonica* needs a long time. The type and concentration of auxin has little effect on rooting and rooting was observed even without auxin. In all three auxins, IAA with 5.71 μmol had a good effect on rooting. Similar findings were observed where IAA was successfully employed for rooting in *Stemona tuberose* (Murthy et al. 2013) and *S. curtisii* (Montri et al. 2007). IAA has also been reported to enhance root formation in many other plant species (Bose et al. 2016; Barpete et al. 2014; Cheruvathur et al. 2012).

After 11 weeks of incubation, the average plantlet height through two-step culture was 5.8 cm (Fig. 2a, b, c, d), while through one-step culture was about 5.4 cm (Fig. 2e). Well-rooted plants were transferred into plastic pots containing a mixture of soil, sand and humus (2:1:1) (Fig. 2f). The acclimatized plantlets were established in the glasshouse with controlled temperature, light and humidity. The plants were then transferred to a natural habitat for cultivation and had a 87% survival rate after 2 months.

Genetic stability of the plantlets using SSR markers

No genetic variation was detected between the plants regenerated from the rhizome buds and a donor plant based on PCR assays using 10 SSR primer sets (Table 5). All primers presented standard single allele patterns, regardless of rhizome buds cultured in mediums with different concentrations of PGRs (see Supplementary online material). Thus, it is possible to obtain large numbers of genetically identical plants in a short period of time.

In recent years, SSRs were utilized to identify somaclonal variation in suspension cell cultures of banana (Morais-Lino et al. 2016) and assess genetic stability of cryopreserved shoot tips of *Rubus* germplasm (Castillo et al. 2010). In our study, in vitro regenerants produced from one-step culture and two-step cultures showed no genetic variation occurrence based on microsatellite marker analysis. This technique has great potential to accelerate the propagation of the endangered plant *C. japonica*.

Fig. 2 The seedlings of *C. japonica* for acclimatization. **a** Seedling after 11 weeks of growth in MS + IAA 2.85 μmol, **b** seedling after 11 weeks of growth in MS + IBA 2.46 μmol, **c** seedling after 11 weeks of growth in MS + NAA 2.69 μmol, **d** seedling after 11 weeks of growth in MS control medium, **e** one-culture seedling after 11 weeks of growth in MS + BAP 2.66 μmol + NAA 0.54 μmol, **f** acclimatized plants in growth chamber after 30 days

Table 5 Simple sequence repeat (SSR) primers used for testing the genetic homogeneity of regenerated plantlets in *C. japonica*

Primer	Repeat unit	Primer sequences (5′–3′)	Annealing temperature (°C)	Size range of amplification product (bp)
Cro10	AG(2*12)	F: CATCAATATCCGAAACTCTCCAG R: CGCCATAAATACTCTCACGAGTC	55	132
Cro21	GAC(3*5)	F: GGGACATTATTGGATCACAACTT R: GTGTATGGGTGGATAGTTTGTGG	55	141
Cro29	GCT(3*6)	F: CTCTTCCCCATCTTGTTCACTC R: GGATAATAATAACGCGAGAAGCC	53	99
Cro37	GGC(3*8)	F: GTCACAGAGCACCCATCTCC R: GACAGAGACTCCGATCATCTCAT	55	127
Cro55	CCG(3*5)	F: CGACCCTCCTCTTCTCCTATTTA R: GTGAGTAAAAGAACGACGCCAT	53	95
Cro60	CAG(3*7)	F: CTCCTTCCATAATTCAGTCTCCC R: GCTGAGGTTGAGAGAGTTGGATA	54	122
Cro76	CAG(3*8)	F: ATCGCTCCACCAACAACAG R: CTGCTAGGGTTTGTCACCATC	53	120
Cro101	CTG(3*8)	F: GCTGCTGTTGATGTAAAGAGAGA R: GGACAACAAAATAGTGTCCCAGA	55	160
Cro109	CGC(3*5)	F: CCTTCGGTTACTACTGCCACAT R: CCTGTTCGCTCTTCTCCTTCT	55	123
Cro121	GAA(3*9)	F: CAACACCGACAACAATAACAATG R: TAGTGGGTTTTGGACACAGAATC	55	146

Flow cytometry of *C. japonica* in vitro regenerants

Genetic stability of in vitro regenerants was additionally assessed with flow cytometry. In all cases, linear histograms of relative nuclear content showed two peaks: the first representing nuclei in the G_0/G_1 phase (Fig. 3M1) of the cell cycle, and the second representing nuclei in the G_2 peak (Fig. 3M2). No significant differences were detected among in vitro regenerants (mean M1 107.39) and donor plants (mean M1 108.37), suggesting that the plants obtained via rhizome buds had an unchanged ploidy level. The results confirmed the ploidy stability of in vitro

regenerants. Previous studies had reported that *C. japonica* was diploid with a chromosome number $2n = 24$ or $2n = 26$ (Oginuma et al. 2001; Li and Xu 1986). As the donor *C. japonica* has been determined to be diploid, hence, the ploidy of the regenerants from in vitro culture can be judged from the relative position of the nucleus peak of the donor plant. From the linear histograms of relative nuclear content, we can determine that the regenerants are also diploid. Wu et al. (2006) also suggested that the ploidy level of an unknown sample can be judged according to the nucleus peak position of the known plant. It was not necessary to know the absolute amount of DNA in each sample, which can save a lot of time and money.

Fig. 3 Flow cytometric analysis of C. *japonica* in vitro regenerants. **a, b** Representative flow cytometric histograms of DAPI stained nuclei obtained from *donor plants* and in vitro regenerants of C. *japonica*. **a** Donor plants, **b** in vitro regenerants. M1 represented G_0/G_1 peak, M2 represented G_2 peak
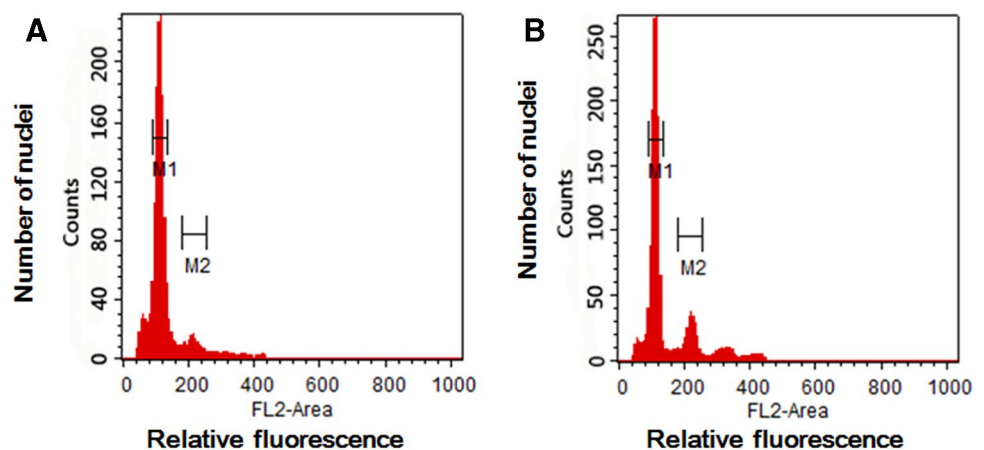
Table 6 Total alkaloids content in wild plants and regenerated plantlets in different plant growth regulators treatment

Plant growth regulators (µmol)	Alkaloids contents (mg g^{-1})
CK	$1.71 \pm 0.01d$
IAA 2.85	$1.39 \pm 0.01f$
IBA2.46	$1.98 \pm 0.02c$
NAA 2.69	$2.67 \pm 0.01b$
BAP 2.66 + NAA 0.54	$1.53 \pm 0.02e$
BAP 2.66 + NAA 2.69	$1.23 \pm 0.01g$
Wild samples	$3.02 \pm 0.01a$

Values represent mean ± SD of 3 replicates/treatment and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey's-b test ($P \leq 0.05$)

Evaluation of total alkaloids in wild plants and regenerants

Eleven-week old rhizomes and roots from one-step culture (solid MS medium containing 2.67 µmol BAP in combination with 0.54–2.69 µmol NAA) and two-step culture (shoots coming from medium with BAP 4.44 µmol and the rhizomes and roots coming from medium with three auxins at a concentration of NAA 2.69 µmol, IAA 2.85 µmol and IBA 2.46 µmol, respectively or control) were analyzed for total alkaloids content by the method of acid dye colorimetric with tuberostemonine as the reference. The total alkaloid content varied significantly among the cultured samples and the wild sample (Table 6). The highest content of total alkaloid was 3.02 mg g^{-1} dry weight in the wild rhizome and roots from Tianmu Mountain, while it was 2.67 mg g^{-1} dry weight in plants cultured in medium with 2.69 µmol NAA, reaching 88.4% that of the wild rhizome and roots. The average content of total alkaloids in rhizomes and roots through one-step culture was respectively 1.53 mg g^{-1} (BAP 2.66 µmol + NAA 0.54 µmol) and 1.23 mg g^{-1} (BAP 2.66 µmol + NAA 2.69 µmol), reached 40.7–50.7% that of the wild sample. The total alkaloid content in the in vitro rhizomes and roots were slightly less than in natural rhizomes and roots. These results may be explained by the effect of root age on alkaloid accumulation because natural roots were grown for more than 3 years and the in vitro rhizomes and roots were only grown for 11 weeks. In our experiment, the alkaloid content in 11-week cultures reached 40.7–88.4% of the content of wild plants. The accumulation of these alkaloids was very fast through in vitro culture. The production of numerous plantlets by this method was found to be faster than natural propagation. This method could also prevent the extinction of *Croomia* plants in nature.

The roots of *C. japonica* have been used for bruises, blood stasis and pain. Until now, only one paper reported the chemical composition in the roots of *C. japonica* (Lin et al. 1993). Due to lack of standards, extraction and preparation of substances from *C. japonica* will deplete a large number of plants, so we adopted an acid dye colorimetric test to determine the total alkaloid content in *C. japonica* regenerants and wild plants with tuberostemonine as the reference. *Stemona* species are closely related to *C. japonica* and have been studied more. Using tissue culture of *Stemona* sp. to produce alkaloids (Chaichana et al. 2011; Palee et al. 2013) and elicitors could enhance the production of *Stemona* alkaloids (Chaichana et al. 2012; Chaichana and Dheeranupattan 2012; Ruangsak and Dheeranupattana 2014; Palee et al. 2016). Therefore, in future studies, we will try to enhance the content of alkaloids in *C. japonica* by adding biological elicitors to the culture medium.

Conclusions

In the present study, we developed an efficient in vitro protocol for adventitious shoot regeneration from *C. japonica* rhizome buds via direct organogenesis. We evaluated several important variables, such as the concentrations of PGRs, adsorbents (PVP and AC) and antioxidant (AS) on adventitious shoot regeneration and tissue browning. Our results showed that one-step culture (rhizome segments which can form seedlings with shoots and roots in the same medium) and two-step culture (one medium for shoots, then transfer to another medium for rooting) are suitable for in vitro propagation of *C. japonica*. In vitro derived plants were found to be genetically stable under in vitro conditions. The rhizomes and roots of *C. japonica* cultured in solid medium with NAA, which can produce high content of alkaloids, would be of potential medicinal usage. The protocol described here could be employed for effective mass propagation of *C. japonica* for commercial and conservational purposes.

Author Contributions WM Jiang conceived the study and wrote the manuscript; SJ Hua conducted culture experiments; XP Zhou performed experiment to measure the total alkaloids; PH Han performed experiment to analyze the ploidy level of regenerated plants; QX Lu performed DNA isolation and SSR analysis; YX Qiu revised the manuscript.

Compliance with ethical standards

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

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