In vitro organogenesis and plant regeneration of *Passiflora xishuangbannaensis*, a species with extremely small populations

Yuan-yuan Meng
  XTBG: Xishuangbanna Tropical Botanical Garden

Shi-jie Song
  University of Chinese academy of sciences

Sven Landrein （✉ sven@xtbg.ac.cn ）
  XTBG: Xishuangbanna Tropical Botanical Garden   https://orcid.org/0000-0003-0028-2450

Abstract

Passiflora xishuangbannaensis (Passifloraceae) is endemic to a few sites of Mengyang nature reserve in Yunnan, Xishuangbanna and less than 40 individuals have been recorded. Nine *Passiflora* species are endemic to Yunnan with most species occurring in South America, making *P. xishuangbannaensis* highly significant and emblematic to the conservation work in the region. This study is designed to provide the first protocol for *in vitro* organogenesis and plant regeneration for *ex situ* conservation and reintroduction for an Asian *Passiflora* species. Using internodes, petioles and tendrils we optimize calli formation and root elongation using several plant growth regulators, individually or in combination. We also assess the genetic stability of regenerated cells. The maximum callus induction and shoot bud differentiation were both achieved on half Murashige and Skoog basal medium supplemented with 4.44 µM 6-Benzylaminopurine and 1.08 µM 1-Naphthaleneacetic acid. The best rooting was achieved from 30 days old, regenerated shoots on half Murashige and Skoog basal medium supplemented with 1.08 µM 1-Naphthaleneacetic acid. Micropropagated plants were subjected to inter simple sequence repeat markers analyses. Collectively, 86 bands were generated from 6 primers of which 12 bands were polymorphic, showing genetic variation between the regenerated plantlets and the original plant. Response to plant growth regulators was more specific than most other studies using South American species, which could be explained by the morphological and physiological differences between South American and Asian *Passiflora* species

Introduction

Passiflora L. is the largest genus in Passifloraceae with more than 600 species (Espinoza et al. 2018). *Passiflora xishuangbannaensis* Krosnick is a rhizomatous species with weak herbaceous stems, belonging to supersection Disemma (Labill.) J.M.MacDougal & Feuillet section Octandranthus Harms (Krosnick 2005). Most *Passiflora* species occur in Central and South America, with only 22 species occurring in Southeast Asia, Australia and the Pacific Islands (Krosnick & Fredeustein 2005). In China, nine species are native to Yunnan, of which three species are endemic to Xishuangbanna and Pu'er alone (*P. altebilobata* Hemsl., *P. xishuangbannaensis* and *P. menghaiensis* X.D.Ma, L.C.Yan & J.Y.Shen, Ma et al. 2019).

During the investigation of this project, we surveyed all known sites of *Passiflora xishuangbannaensis*. A total of 38 individuals were counted from 5 sites of which 2 sites did not have plants anymore. This species is below the Minimum Viable Population (MVP) size, with less than 5000 individuals in total and less than 500 individuals per population and qualify for the Plant Species with Extremely Small Population PSESP (Sun et al. 2019). Furthermore, this species was also reported to be self-incompatible (Krosnick 2005), and rarely sets fruit in the wild, causing a decline in natural population size.

In vitro regeneration and multiplication is a technique that can reproduce plants using organs, tissues or cells with a high success rate, it can also be used to obtain disease-free offspring (González-Benito & Martín 2011). This technique has been used in rare plant conservation strategies and has been studied for *ex situ* conservation of threatened species in many plant families (Sheelavantmath et al. 2000; Ayuso et al. 2019; Rodrigues et al. 2020). *Passiflora xishuangbannaensis* growth was shown to be enhanced by grafting on *P. edulis* Sims. stock (Fu Chuan Wu, personal communication, 2020), however no long-term methods have been developed to propagate this species and no information is known about its *in vitro* regeneration.

Several studies on *Passiflora* tissue culture have been conducted, and many successful cases of regeneration by organogenesis have been reported from a wide range of species (Fernando et al. 2007; Pinto et al. 2010; Silva et al. 2011; Faria et al. 2018). Data from previous studies suggest that many factors could influence the results of *in vitro* propagation, such as explant type, Plant Gowth Regulators PGR, media composition and plant growth regulators. Successful organogenesis protocols have been established from different types of explants, such as leaves, roots, hypocotyls, stem nodes, internode segments or even petioles and tendrils (Mast et al. 2007; Pipino et al. 2010; Ozarowski et al. 2013; Vieira et al. 2014; Shekhawat et al. 2015). Besides explants, PGRs were the most frequently studied factors affecting organogenesis of *Passiflora*. Among all the plant growth regulators used, the synthetic cytokinin 6-benzylaminopurine (benzyl adenine, BA) alone or combined with the synthetic auxin 1-Naphthaleneacetic Acid (NAA) were frequently used in *in vitro* organogenesis and confirmed to be effective (Dias et al.2009; Pipino et al. 2010; Rocha et al. 2012).

Previous studies found that regenerated plants are genetically stable (Venkatachalam et al. 2007; Asthana et al. 2011; Chavan et al. 2015). However, some studies have shown genetic alterations during *in vitro* regeneration (Naseer & Mahmood 2014; Krishna et al. 2016). These alterations might be due to mutagenesis during the breakdown of normal cellular controls (Phillips et al.1994), or accumulation of somatic mutations induced by several factors including the various concentrations and types of PGRs used during the tissue culture period (Martin et al. 2006; Bairu et al. 2011). Molecular markers, such as Simple Sequence Repeat (SSR), Inter Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), Start Codon Targeted (SCoT) polymorphism, Inter-retrotransposon Amplified Polymorphism (IRAP), and Amplified Fragment Length Polymorphism (AFLP), have been confirmed to be informative in studies on genetic diversity and activities related to the conservation of genetic resources (Bornet and Branchard 2001; Campbell et al. 2011; Wang et al. 2013; Rahmani et al. 2015; Al-Qurainy et al. 2018; Rodrigues et al. 2020). Among these molecular markers, ISSR was emphasized because it has good reproducibility and does not require prior knowledge of the primer sequence nor high amounts of DNA (Kumar et al. 2009; Saoza et al. 2015).

The present study aims to establish an efficient *in vitro* organogenesis protocol to produce regenerated plants of *P. xishuangbannaensis*. The differential responses of BA alone or combined with NAA is evaluated for *in vitro* organogenesis, using internodal segments, petioles and tendrils as explants. Different NAA concentrations are also evaluated for *in vitro* rooting of regenerated shoots. Additionally, the genetic stability of regenerated plants from three types of explant is assessed using ISSR markers.

Materials And Methods

Explant preparation

Stems of *Passiflora xishuangbannaensis* were collected from plants cultivated in a greenhouse of Xishuangbanna Tropical Botanical Garden (XTBG; China). In our preliminary experiment, almost all leaf fragments became polluted because of the endophytes in the leaves. Therefore, we discarded leaf fragments in the following experiments. Internodal segments (Ins), petioles (Pts) and tendrils (Tds) were excised from the stems. All explants were surface sterilized with 1% NaClO (w/v) for 8 min and rinsed 3−4 times with sterilized distilled water to remove trace NaClO. After sterilization, the three types of explants were cut into 0.5 cm-long segments.

Callus induction and in vitro shoot regeneration

Half strength (1/2) MS medium (Murashige and Skoog 1962) supplied with 3% (w/v) sucrose, 0.1% (w/v) 0.7% (w/v) agar and activated charcoal were used as the medium for all experiments. To test the effect of BA alone or in combination with NAA on the induction of callus and shoots of different explants, media were supplied with BA at three different concentrations (2.22, 4.44 or 8.88 μM) alone or in combination with NAA at two different concentrations (0.54 or 1.08 μM). Medium without any plant growth regulators was used as control. Each treatment was replicated 10 times, and six explants were placed in each petri dish. Medium was autoclaved at 121°C for 20 min., and PGRs were filtered through a 0.22 μm membrane (Millipore, MA, USA) to remove microorganism before they were added into the medium. Cultures were incubated at 25 ± 2°C with a 12/12-h light/ dark cycle. The status of callus and shoot induction was assessed 45 days after incubation.

In vitro rooting and acclimatization

To test the effect of NAA on the induction of roots from regenerated shoots, 30 days old, regenerated shoots were placed on 1/2 MS medium with NAA at different concentrations (0, 0.54, 1.08 and 1.62 μM). Cultures were incubated at 25 ± 2°C and 80−90% relative humidity, with a 12/12-h light/ dark cycle. Data on the rooting frequency and growth were recorded after 30 days of culture.

Before transferring to a sterilized nutritive soil (Chunnian, China), *In vitro* regenerated and well-rooted plantlets were taken out of the glass bottles and the roots were washed using tap water to remove the medium adhering to them. The plants were left in a laminar flow cabinet to remove the residual water from the root surface, before transferred to the soil and incubated at 25 ± 2°C and 70−80% relative humidity, with a 12/12-h light/ dark cycle.

Genetic Fidelity Assessment

The genomic DNA was isolated from fresh leaf of 9 randomly selected regenerated plants and 1 mother plant using EasyPure Plant Genomic DNA Kit (TransGen Biotech, China). ISSR technique was used to analyze genetic fidelity among regenerated plants (Zietkiewicz et al. 1994). A total of 18 ISSR primers were tested and six were selected in the analysis. The amplification program was as follows: initial DNA denaturation for 5 min at 95°C, followed by 38 cycles at 94°C for 1min, annealing for 1min at 54-61.7°C depending on the primer (Table 1), elongation for 2 min at 72°C followed by final extension at 72°C for 10 min. The amplified fragments of DNA were separated on a 1.7% (w/v) agarose gel. The amplified bands for each primer were counted as present (1) or absent (0) for ISSR analysis. Excel 2016 was used to calculate the total number of bands, the number of polymorphic bands, and the percentage of polymorphic bands.

Table 1
ISSR primers used in the genetic fidelity study.

Primer identification	Sequences (5′–3′)	Tm (°C)	TB	PB
1	ACACACACACACACACCT	58.9	20	4
2	AGAGAGAGAGAGAGAGYA	54.1	16	3
3	GAGAGAGAGAGAGAGAYT	58.9	13	1
4	GAGAGAGAGAGAGAGAGACC	58.9	14	0
5	GAGAGAGAGAGAGAGARC	60.1	14	4
6	ACACACACACACACACG	61.7	9	0
Total			86	12
Tm (°C) annealing temperature, TB total bands, PB polymorphic bands.				

Statistical Analysis

The callus induction and shoot regeneration were analyzed after 45 days of culture using two-way Analysis Of Variance (ANOVA), and root induction after one month from 30-d-old regenerated shoots was analyzed using one-way ANOVA. We used the Tukey's tests for multiple comparisons among treatments. All data presented are means ± standard deviation. All statistical analyses were performed in R software environment (version 3.3.3). The alpha-type I error was fixed at 0.05 (thus, all non-significant differences have $P > 0.05$).

Results

Callus and shoot induction

In this experiment, calli cannot be induced from any of the three types of explants (Ins, Pts and Tds) on ½ MS medium or ½ MS medium supplied with BA alone (from 2.22 to 8.88 μM). By contrast, calli are achieved from all explant types cultured in ½ MS medium supplied with 4.44 μM BA and 0.54 μM NAA or with 4.44 μM BA and 1.08 μM NAA (Fig. 1a,b,c; Table 2). According to our observations, calli started to form after 7 days of culture in these two treatments. After 45 days of culture, the best results of calli induction are seen with Ins, Pts and Tds on ½ MS medium supplemented with 4.44 μM BA and 1.08 μM NAA, resulting in a callus induction frequency of 91.67 ± 11.7%, 90.00 ± 14.05% and 88.33 ± 11.25%, respectively (Table 2). When we did multiple comparisons analysis among treatments and explant types, we found no differences in callus induction among the three explant types or the two treatments (Fig. 2a). Optimal growth of callus is achieved with ½ MS medium supplemented with 4.44 μM BA and 1.08 μM NAA, resulting in a fresh weight of callus of 1.58 ± 0.22, 1.17 ± 0.11 and 0.71 ± 0.11g, respectively (Table 2). And the highest callus fresh weight occurs with Ins, which is significantly higher than Pts and Tds (Fig. 2b).

Table 2
Effect of BA alone or its combination with NAA on the induction of callus and shoots of different explants after 45 days of incubation.

Treatment ½MS + PGR (µM)	Internodal segments (Ins)			Petiole segments (Pts)			Tendril segments (Tds)		
	Callus (%)	Callus FW (g)	No Shoots/ Explant	Callus (%)	Callus FW (g)	No Shoots/ Explant	Callus(%)	No Shoots/ Explant	Callus FW (g)
Without PGR	0 ± 0[b]			0 ± 0[b]			0 ± 0[b]		
BA 2.22	0 ± 0[b]			0 ± 0[b]			0 ± 0[b]		
BA 2.22 + NAA 0.54	0 ± 0[b]			0 ± 0[b]			0 ± 0[b]		
BA 2.22 + NAA 1.08	0 ± 0[b]			0 ± 0[b]			0 ± 0[b]		
BA 4.44	0 ± 0[b]			0 ± 0[b]			0 ± 0[b]		
BA 4.44 + NAA 0.54	78.3 ± 19.3[a]	1.4 ± 0.1[a]	11.0 ± 5.0[a]	80.0 ± 20.5[a]	1.1 ± 0.1[a]	11.0 ± 5.1[a]	76.7 ± 22.5[a]	10.8 ± 5.7[a]	0.7 ± 0.1[a]
BA 4.44 + NAA 1.08	91.7 ± 11.8[a]	1.6 ± 0.2[a]	12.1 ± 6.2[a]	90.0 ± 14.1[a]	1.2 ± 0.1[a]	12.7 ± 6.0[a]	88.3 ± 11.3[a]	10.7 ± 6.7[a]	0.7 ± 0.1[a]
BA 8.88	0 ± 0[b]			0 ± 0[b]			0 ± 0[b]		
BA 8.88 + NAA 0.54	0 ± 0[b]			0 ± 0[b]			0 ± 0[b]		
BA 8.88 + NAA 1.08	0 ± 0[b]			0 ± 0[b]			0 ± 0[b]		

Shoots can be induced directly after 30 days from the calli in media supplied with 4.44 µM BA and 0.54 µM NAA or with 4.44 µM BA and 1.08 µM NAA, the latter is the best for shoot induction for all explant types with a shoot number of 12.09 ± 6.17 for Ins, 12.69 ± 5.97 for Pts and 10.71 ± 6.67 for Tds (Table 2). But no significant differences in the number of shoots are observed among the three explant types (Fig. 2c).

In vitro rooting

Healthy roots can be formed after 30 days of transferring to a rooting medium. Among all treatments (0, 0.54, 1.08 and 1.62 µM NAA), best rooting is achieved by transferring the shoots onto a rooting medium containing 1.08 µM NAA (Fig. 1h), with the average root number of 5.25 ± 1.94 (Fig. 3a), and the average root length of 3.10 ± 0.79 mm (Fig. 3b), although the root length is not significantly longer than with addition of 0.54 µM NAA (2.38 ± 1.56).\

Genetic fidelity

Initially, 18 ISSR primers were tested for amplification. Of these, 6 primers give clear, unambiguous amplified DNA bands among mother plants and regenerated plants (Fig. 4). A total of 86 amplified bands ranging from 200bp to 4000bp are scored, with an average of 14 bands per primer. Among the 86 amplified bands, 12 are polymorphic, resulting in 13.95% genetic variation among mother pants and regenerated plants (Table 1).

Discussion

Leaves, roots, hypocotyls, internodes, petioles and nodal segments were frequently used explants in *Passiflora* organogenesis (Ozarowski and Thiem 2013 Mast et al. 2007; Lombardi et al. 2007). A few studies used leaves from plants grown in a greenhouse (Pinto et al. 2010), but in our preliminary experiment, almost all leaf fragments collected from a greenhouse became polluted because of the endophytes in the leaves, even if Plant Preservation Mixture (PPM) was used (data not shown). Leaves of some *Passiflora* species are confirmed to be inhabited by a microbial community of endophytes (Henrique et al. 2020).

In our study, we used internodes, petioles and tendrils as explants and all of them produced abundant organogenic calli and shoots under proper treatments (Fig. 1c). Tendrils of *Passiflora* were rarely used in organogenesis, however, *In vitro* regeneration was achieved in *P. trifasciata*, *P. x* 'Guglielmo Betto' and *P.* 'Manta' using young tendrils (Pipino et al. 2010). In our present study, tendrils produced abundant organogenic calli and adventitious shoots, which were not significantly different from the other two types of explant except for the fresh weight of callus (Fig. 2).

Besides explants, organogenesis during micropropagation is influenced by many other factors, such as plant growth regulator and their concentration, media composition, and most importantly endogenous cytokinin levels of explants (Gentile et al. 2014). Plant regeneration by organogenesis was successfully established in *Passiflora* using BA alone and at a wide range of concentrations, most successful cases occurred at low concentration of BA (2.2–8.87 µM) (Ozarowski and Thiem 2013). For example, 76% of leaf segments of *P. cincinnata* formed buds on MS medium supplied with 4.4 µM and 5.87 µM BA, and 54% of leaf segments formed buds with 2.2 µM BA (Lombardi et al. 2007). A greater number of shoots of *P. edulis* were obtained on MS medium supplied with 4.4 µM and 6.65 µM BA (Prammanee et al. 2011). However, this is different to the findings presented here as organogenesis did not occur with BA alone at any concentration (2.22, 4.44 or 8.88 µM) (Table 2). A possible explanation could be that *P. Xishuangbannaensis* already has high endogenous cytokinin level. The requirement of auxin in combination with cytokinin may regulate the endogenous cytokinin level by the inhibition of cytokinin biosynthesis (Javed et al. 2013). The best callus and shoot induction was achieved in medium supplied with BA (4.44 µM) in combination with NAA (1.08 µM), in the present study. These results agree with several other findings in *Passiflora* and other plant species (Komathi et al. 2011; Perveen et al. 2015; Naaz et al. 2019). However, callus and shoot induction failed when a higher dose of BA (8.88 µM) in combination with 1.08 µM NAA were used.

Various studies have shown that rooting of regenerated *Passiflora* shoots was observed on 1/2 MS or MS medium without supplementation of plant growth regulators (Pinto et al. 2010; Garcia et al. 2011; Pacheco et al. 2012). However, in the present study, best rooting was observed by transferring the shoots onto a rooting medium containing auxins (1.08 µM NAA). Several previous studies have also demonstrated that low doses of auxins were needed for the rooting of regenerated shoots of *Passiflora*, such as *P. foetida, P. edulis and P.*

edulis f. *flavicarpa* (Nhut et al. 2007; Prammanee et al. 2011; Ragavendran et al. 2012). When regenerated shoots were kept on media supplied with 4.44 μM BA and 1.08 μM NAA and after 3 months, no roots formed (data not shown). It could indicate that BA has impeded rooting of regenerated shoots (Gentile et al. 2014).

In contrast to many previous reports on the genetic stability among the micro-propagated plants and mother plants (Venkatachalam et al. 2007; Asthana et al. 2011; Chavan et al. 2015) the present study shows a relatively high level of genetic variation between the mother plants and regenerated tissue. Several reports have shown low percentage of genetic variation of regenerated plants in different species, such as *Spilanthes calva* (Razaq et al. 2013) and *Ansellia africana* (Bhattacharyya et al. 2017). High level of genetic variation has been observed in other regeneration systems (Rathore et al. 2011; Rawat et al. 2013). These results indicate the need to test the genetic variability of regenerated plantlets before actual conservation practices.

Conclusions

The present study was designed to establish a protocol for plant regeneration by organogenesis of *P. xishuangbannaensis.* The maximum callus induction and shoot bud differentiation were both achieved on ½ MS medium supplemented with 4.44 μM BA and 1.08 μM NAA. The best rooting was achieved from 30-d-old, regenerated shoots on ½ MS medium supplemented with NAA 1.08 μM. Assessment of genetic fidelity of ten regenerated plantlets using ISSR primers revealed a relatively high genetic variation among mother and regenerated plantlets but due to the endangered status of *P. xishuangbannaensis*, this procedure provides a reliable method for its *ex situ* conservation.

Declarations

Conflicts of interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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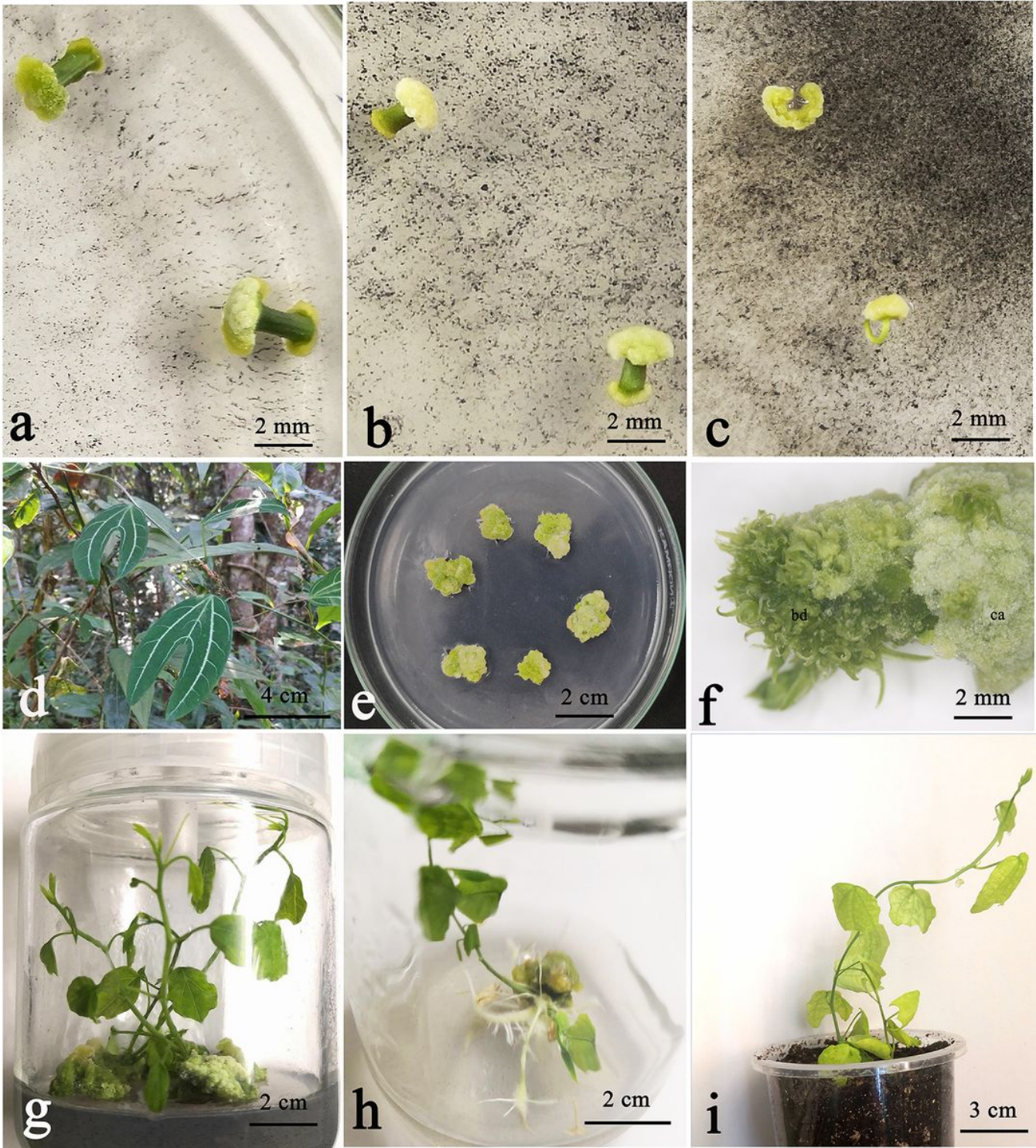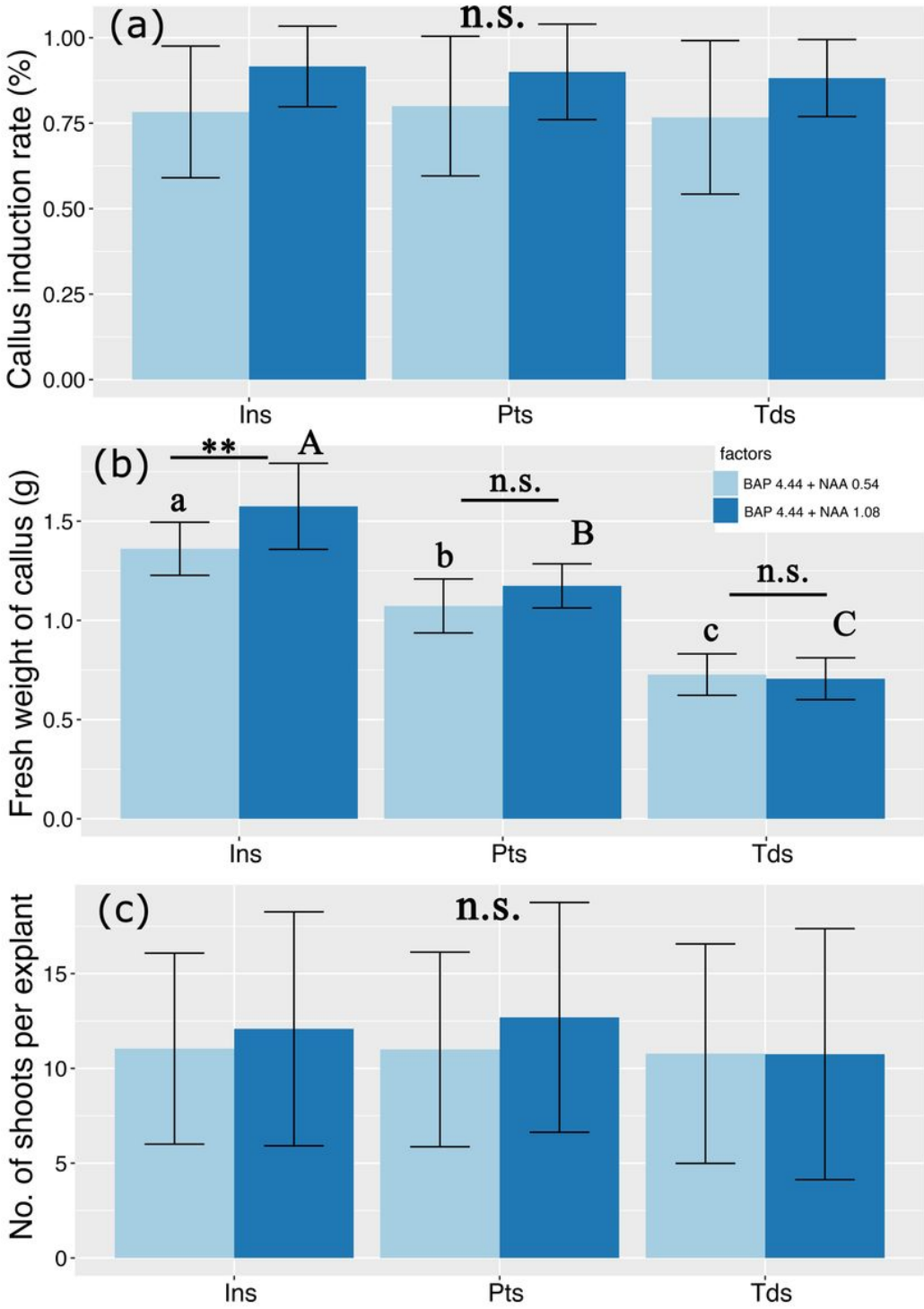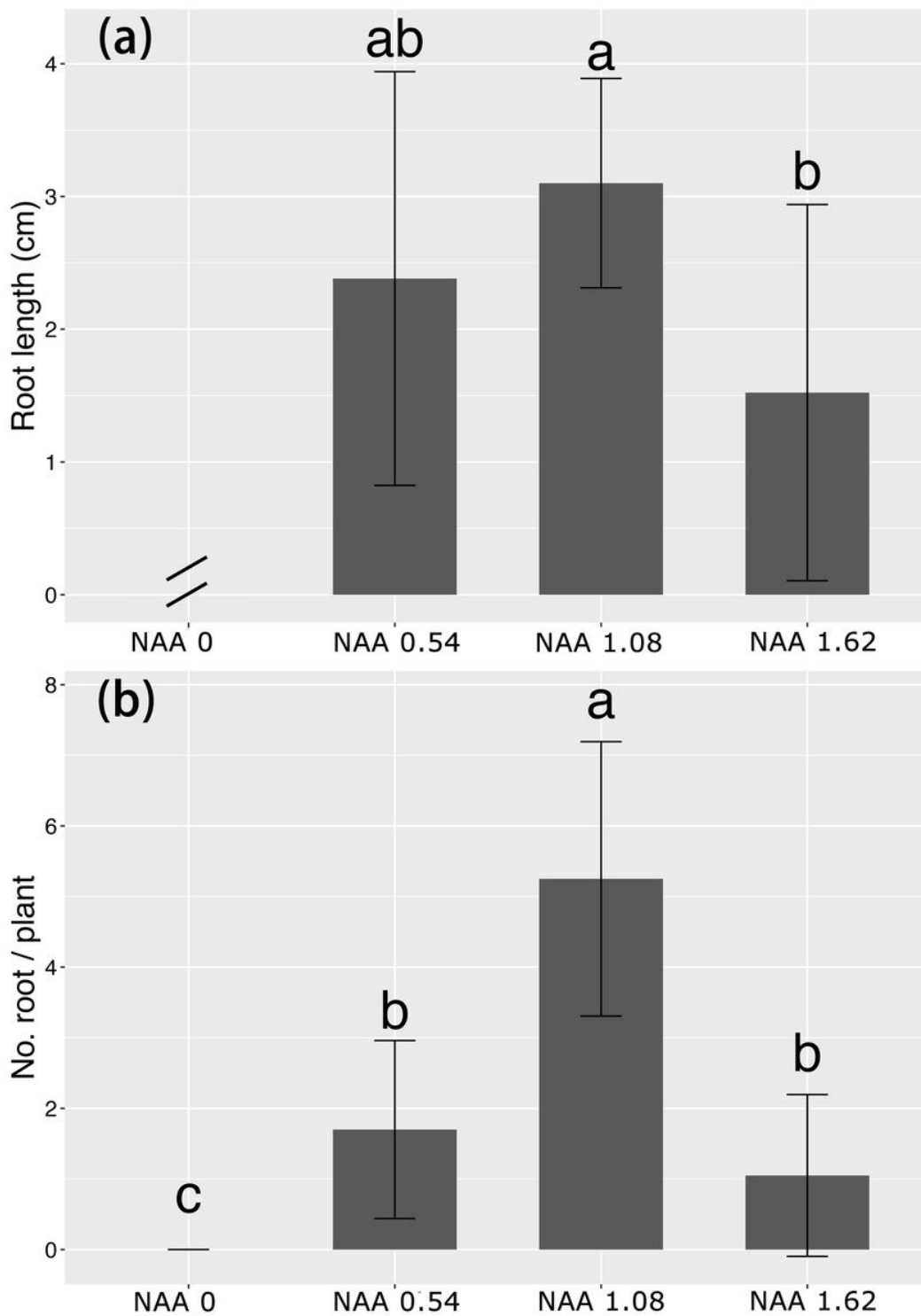
Figures

Figure 1

(a-c) Calli induced from internodal segments (Ins), petioles segments (Pts). and tendrils segments (Tds), respectively. (d) A mature plant of Passiflora xishuangbannaensis. (e) Calli and buds induced from internodal segments explants (Ins) on 1/2 Murashige and Skoog medium (MS) with 6-benzylaminopurine (BA) 4.44 µM and naphthaleneacetic acid (NAA) 1.08 µM, after 45 days of incubation. (f) Details of Figure e showing multiple buds from regenerated callus. Bud (bd); callus (ca). (g) Multiple shoots from Ins on basal medium with BA 4.44 µM +NAA 1.08 µM, after 30 days of subculture. (h) Root formation after 30 days of transferring to a rooting medium, i.e. 1/2 MS medium with NAA 1.08 µM. (i) Acclimatized plants under greenhouse conditions.

Figure 2

Effect of different explants on callus induction and shoot-bud regeneration from 1/2 Murashige and Skoog (MS) medium with BA 4.44 μM + NAA 0.54 μM, or MS with BA 4.44 μM +NAA 1.08 μM after 45 days of incubation. Values are mean ± SE. ** highly significant (P < 0.01). n.s. non-significant (P > 0.05).

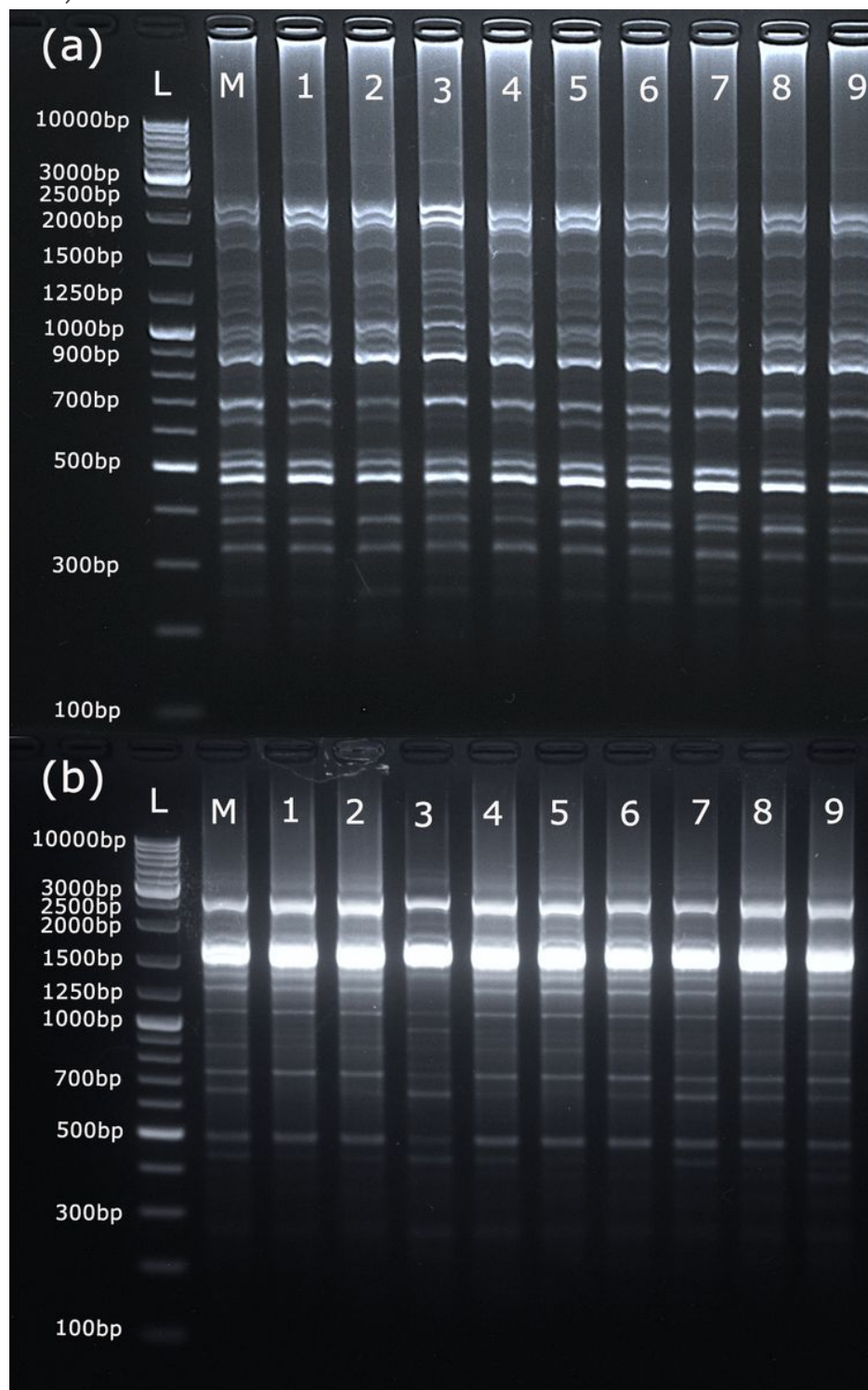## Figure 3

Effects of naphthaleneacetic acid (NAA) on root induction from 30-d-old regenerated shoots after 30 days of incubation. Values are mean ± SE. Different letters indicate significant differences among treatments (P <

0.05).

Figure 4

ISSR amplification products produced using the primer 1 (a) and 3 (b), and separated on a 1.7% agarose gel. L: 2-log ladder, M: mother plant, 1 to 9: in vitro raised plantlets.