STIMULATION OF SHOOT REGENERATION THROUGH LEAF THIN CELL LAYER CULTURE OF PASSIFLORA EDULIS SIMS.

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Received: 17.7.2018
Accepted: 30.10.2018

SUMMARY

Passiflora edulis Sims. belonged to the genus Passiflora, is one of the important economic crops of the world as well as Vietnam. Nowadays, the commercial P. edulis is mainly propagated by seeds, cuttings and grafting; however, these methods still have some limitations such as genetic degradation and heterogeneity and the spread of pathogenic viruses. Micro-propagation has been used for clonal breeding and disease-free plant breeding, as well as providing a source of materials for Passiflora breeding. In this study, leaf explants of P. edulis Sims. (2.0-month-old) excised from the in vitro culture of ex vitro axillary buds cut longitudinally and transversally into thin cell layers (lTCL and tTCL) were used as plant materials to evaluate the shoot regeneration. In addition, the effects of explant age and lighting condition on shoot regeneration were also investigated. After 8 weeks of culture, the results showed that shoot regeneration rate (100%) and shoot multiplication coefficient (13.33) of the in vitro leaf-lTCL-4 were higher than those of other treatments and control. The shoot regeneration rate of P. edulis Sims. also varied with the change of explant age. The highest shoot regeneration rate (100%) was obtained from leaf explants of 1.5-month-old shoots after 8 weeks of culture. Moreover, the light (fluorescent lamps with photoperiod of 16 hours/day and lighting intensity of 40 - 45 µmol.m-2.s-1) improved not only morphogenesis rate, but also shoot regeneration rate (100%) of leaf explants after 8 weeks of culture. This study provided a novel method for rapid micro-propagation of P. edulis Sims.

Keywords: leaf explant, Passiflora edulis, shoot regeneration, thin cell layer

INTRODUCTION

Passiflora edulis Sims. is the most important economic plant of Passiflora, the largest genus in the Passifloraceae family. It has been extensively planted in subtropical and tropical areas to provide fresh fruits and material source for juice production. Moreover, the leaves of P. edulis have been commonly used in folk remedies for the treatment of alcohol intoxication, anxiety, migraine, and insomnia (Li et al., 2011). Therefore, the plants are now grown on a large scale in many countries like Brazil, Peru, Australia, Ecuador, and Vietnam.

P. edulis is a perennial crop that can be propagated by seeds, cuttings, and grafting (traditional breeding methods). Nowadays, commercial P. edulis is mainly propagated by seeds because it is the easiest way. However, this method still has some limitations such as genetic degradation and heterogeneity. Propagation by cuttings and grafting is sometimes useful, but these methods have the potential to infect the pathogenic viruses (Nakasone, Paull, 1998). Thus, in vitro culture used for clone breeding and disease-free plant breeding, as well as providing a source of breeding materials for Passiflora.

Studies related to Passiflora genotypes began in the 1960s and since then, several culturing techniques have been established for different Passiflora species (Vieira, Carneiro, 2004; Vieira et al., 2005; Zerbini et al., 2008; Alexandre et al., 2009). In particular for P. edulis, there have been
many reports of regeneration and in vitro propagation involving the use of different sources of explants such as shoot tip (Faria, Segura, 1997), axillary bud (Kantharajah, Dodd, 1990), shoot (Biasi et al., 2000), leaf (Otahola 2000; Becerra et al., 2004; Trevisan, Mendes, 2005), hypocotyl (Fernando et al., 2007; Dias et al., 2009), and root (Silva et al., 2011). Although there have been several studies of in vitro culture of P. edulis published, no study has used the thin cell layer (TCL) culture technique for regeneration and propagation of this species. In this study, the effectiveness of shoot regeneration from leaf explants using TCL techniques of Passiflora edulis Sims. was evaluated.

MATERIALS AND METHODS

Materials

Plant materials

The leaves excised from in vitro shoots (2.0-month-old) of Passiflora edulis Sims. were used as materials in this study. The shoots were obtained from in vitro culture of ex vitro axillary buds from the greenhouse of Tay Nguyen Institute for Scientific Research.

Culture medium

The shoot regeneration medium used in the experiments was MS basal medium (Murashige, Skoog, 1962) supplemented with 1 mg.l⁻¹ benzyl adenine (BA), 30 g.l⁻¹ sucrose, and 8 g.l⁻¹ agar (Trevisan, Mendes, 2005). The medium was adjusted to pH 5.7 - 5.8 then sterilized (autoclaved for 30 min at 121°C and 1 atm).

Methods

Effect of TCL culture on shoot regeneration

The in vitro leaves were cut longitudinally and transversally into thin cell layers (ITCL and tTCL) according to the method described in table 1 and figure 1.

Figure 1. Diagram of establishing a thin-layer culture system for shoot regeneration of P. edulis Sims. (1): Cut leaves into square pieces (10 mm x 10 mm); (2): Cut in different ways and sizes; (3): Transfer leaf-TCL to shoot regeneration medium; (4): Shoots were recorded after 8 weeks of culture.
 Shoot multiplication coefficients were compared by: $Sc = \frac{Rs(\%)}{S} \times N$

Where: $Sc$: Shoot multiplication coefficient; $Rs$: Shoot regeneration rate (%); $S$: Number of leaf fragments cut longitudinally or transversally; $N$: Average of shoots/explant.

**Effect of explant age on shoot regeneration**

Leaf-TCL obtained from the second pair of leaves from the shoot tip of the plantlet at different explant ages (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0-month-old) were cut into optimal leaf explants for shoot regeneration. The purpose of this experiment was to determine the appropriate age of leaf explants for shoot regeneration.

**Effect of light condition on shoot regeneration**

Leaf-TCL was cultured on the shoot regeneration medium and placed under different light conditions (fluorescent lamps, FL) with photoperiod of 16 hours/day and lighting intensity of 40 - 45 µmol·m⁻²·s⁻¹, and darkness) to compare the shoot regeneration rate.

**Anatomical observations**

Samples were taken at each developmental stage of *in vitro* leaf explant culture. The shoots obtained in the process were fixed in 10% sodium hypochlorite for 15 minutes. Then, the samples were washed with sterile distilled water, fixed for 15 minutes in 45% acetic acid, rinsed off, and soaked in iodine carmine for 5 minutes. Finally, they were washed with sterile distilled water, placed on a glass slide, covered with a slip. Photographic records of the sample were obtained with an optical microscope at 10× and 40× magnifications.

**Culture conditions and statistical analysis**

Leaf-TCL were cultured at 25 ± 2°C with humidity of 55 - 60% and photoperiod of 16 hours/day under the light of FL (40 - 45 µmol·m⁻²·s⁻¹) or in the darkness depending on each experiment purpose.

Each treatment was replicated 3 times and each replicate were 10 culture vessels. Data was recorded after 8 weeks of culture and analysis of variance was performed. The mean values were compared by LSD and Duncan’s multiple range test using SPSS (Version 20.0) at $\alpha = 0.05$ (Duncan, 1995).

**RESULTS AND DISCUSSION**

**Effect of TCL culture on shoot regeneration**

After 8 weeks of culture, the shoot regeneration rates and shoot multiplication coefficients of leaf-TCL and ITCL culture were recorded (Table 2, Figure 2, 3).

The results showed that TCL techniques affected the shoot regeneration rate of *P. edulis* Sims. The shoot regeneration rates of tTCL-3 and tTCL-4 were the highest (100%), which showed significant differences with the results of other treatment conditions and control (82.22%). Meanwhile, the number of shoots per explant was the highest (4.33 shoots) in control (Table 2, Figure 3a). However, the height of shoot was the lowest (0.13 cm) in the control treatment, which was significantly lower than 1.27 cm in the tTCL-4 treatment (Table 2, Figure 3b). Although the number of shoots per explant was the highest, the control shoots were not optimal for propagation because only shoots longer than 1 cm have significant implications for propagation. In addition, tTCL-4 gave the highest shoot multiplication coefficient (13.33) and it was 4 times higher than that of control (3.55).
Table 2. Effect of different types of TCL culture on shoot regeneration of *P. edulis* Sims. after 8 weeks of culture.

| Leaf explants | Shoot regeneration rate (%) | No. of shoots/explant | Shoot height (cm) |
|---------------|-----------------------------|-----------------------|-------------------|
| Control       | 82.22 c                     | 4.33 a                | 0.13 d            |
| lTCL-2        | 74.44 d                     | 2.67 b                | 0.83 c            |
| lTCL-3        | 91.11 b                     | 2.33 bc               | 1.03 b            |
| lTCL-4        | 78.89 c                     | 3.00 b                | 0.87 c            |
| lTCL-5        | 100 a                       | 3.33 b                | 1.33 a            |
| tTCL-4        | 100 a                       | 3.33 b                | 1.27 a            |
| tTCL-5        | 22.22 e                     | 1.67 c                | 0.10 d            |

Note: Different letters shown in the same column represent significant differences at α = 0.05 in Duncan’s multiple range test.

Figure 2. Shoot multiplication coefficient of TCL culture of *P. edulis* Sims. after 8 weeks of culture.

Figure 3. Shoot regeneration of *P. edulis* Sims. from leaf explants after 8 weeks of culture. a: Control (leaf was cut off the edges – 10 mm × 10 mm); b: tTCL-4 (10 mm × 2.5 mm).
Gattuso et al. (2003) indicated that regeneration is an extremely complex process, which affected by multiple qualitative and quantitative factors including genotype, culture medium, plant growth regulators (cytokinins and auxins), agar, and explant type, size, and age. The results of this study showed that TCL culture techniques had a positive influence on the shoot regeneration rate as well as the shoot multiplication coefficient. Shoot regeneration rate (100%) and shoot multiplication coefficient (20) were higher than those of *P. caerulea* L. *in vitro* shoot regeneration via cotyledonary node and shoot tip explants (Jafari et al., 2017). Cotyledonary node explants cultured on MS medium supplemented with 1.5 mg.l\(^{-1}\) BA and 0.15 mg.l\(^{-1}\) indole-3-butyric acid (IBA) gave the regeneration frequency of 90% and the number of shoots of 8.86 and shoot tip explants cultured on the above-mentioned medium gave higher regeneration rate (96.66%) and number of shoots (9.86 shoots/explant).

The technique of TCL culture was successfully used in regeneration and propagation of various plant species such as *Panax ginseng* (Ahn et al., 1996), *Lilium* (Nhut et al., 2001), *Chrysanthemum* (Teixeira da Silva, Fukai, 2003), and *Panax vietnamensis* Ha et Grushv. (Nhut et al., 2012). However, there has been no study on TCL culture of *Passiflora* in the world as well as in Vietnam. The results of this study are considered as a new orientation in shoot regeneration by using TCL technique. This method could improve the regeneration rate as well as shoot multiplication efficiency during *in vitro* culture, which is very important in improving the producibility of commercial seedlings.

**Effect of explant age on shoot regeneration**

Explant age was an important factor influencing to the regeneration of *P. edulis* Sims. The regeneration rate of shoot increased in proportion with the increase in age of explants from 0.5 to 1.5-month-old. The highest shoot regeneration rate (100%) was showed in leaf explants of 1.5-month-old shoots (Table 3).

| Explant age | Shoot regeneration rate (%) | No. of shoots/explant | Shoot height (cm) |
|-------------|-----------------------------|----------------------|------------------|
| 0.5-month-old | 36.67 d\(^{a}\) | 5.67 a | 0.17 d |
| 1.0-month-old | 84.44 c | 4.33 b | 0.53 c |
| 1.5-month-old | 100 a | 4.00 bc | 1.43 a |
| 2.0-month-old | 86.67 b | 3.67 bc | 0.73 b |
| 2.5-month-old | 44.44 c | 3.33 c | 0.20 d |
| 3.0-month-old | 16.67 e | 2.00 d | 0.10 d |

Note: \(^{a}\) Different letters shown in the same column represent significant differences with \(\alpha = 0.05\) in Duncan’s multiple range test.

**Figure 4.** Effect of explant age on shoot regeneration of *P. edulis* Sims. from *in vitro* leaf-TCL after 8 weeks of culture. a: Leaves from *in vitro* shoots with different explant ages; b: Shoot regeneration after 8 weeks of culture. Bar: 1 cm.
The rate of shoot regeneration decreased with the increase of explant age (from 2 to 3-month-old) and become the lowest (16.67%) in 3-month-old explants. The number of shoots per explant decreased proportionally with the increase of explant age and it was the highest at 0.5-month-old treatment (5.67 shoots/explant) (Table 3).

Up to now, some studies have examined the effect of explant age on plant regeneration ability. The results of this study were similar to that of Becerra et al. (2004) in the shoot number per explant which followed an inverse linear tendency in relation to the explant age.

**Effect of light condition on shoot regeneration**

In this study, light condition affected not only morphogenesis rate, but also affected direct shoot regeneration from leaf explant culture and indirect shoot regeneration via callus after 8 weeks of culture (Table 4, Figure 5).

The direct shoot regeneration rate of leaf explants cultured on MS medium supplemented with 1 mg.l⁻¹ BA, 30 g.l⁻¹ sucrose and 8 g.l⁻¹ agar under FL was 100% after 8 weeks of culture. Callus formation (4.44%) was also observed under FL condition although this was not significant (Table 4).

In contrast, *in vitro* leaf-TCL culture performed under darkness condition showed 100% callus formation with 71.11% of them regenerated shoots after 8 weeks of culture (Table 4).

In addition, the number of shoots per explant as well as the shoot height under FL condition were also higher than those in the darkness (4.33 shoots and 1.13 cm compared with 3.33 shoots and 0.47 cm, respectively) (Table 4).

![Figure 5. Effect of lighting condition (FL and darkness) on shoot regeneration of *P. edulis* Sims. after 8 weeks of culture. a, a₁: Direct shoot regeneration; b, b₁: Indirect shoot regeneration; c₁: Initial structure of bud; c₂: Complete structure of bud; d₁: Callus formation after 2 weeks; d₂: Shoot formation after 4 weeks; d₃: Shoot formation after 6 weeks; d₄: Shoot formation after 8 weeks. Bars: 1 cm (a, a₁, b, b₁); 100 µm (c₁, c₂); 65 µm (d₁, d₂, d₃, d₄).](image-url)
The effect of light on organogenesis and shoot regeneration as well as callus formation, and embryogenesis are enormous. Light plays an important role on the photosynthetic pathway of plant through affecting on photosensitive receptors (Kendrick and Kronenberg, 1994). The results of this study showed that the impact of lighting condition on shoot regeneration through or not through callogenesis was significant.

Different methods for in vitro shoot regeneration have been successfully developed and they mostly depended on indirect organogenesis pathways, which were relatively troublesome and time consuming (Cai et al., 2015). Direct organ regeneration of *P. edulis* Sims. has not been reported so far.

Up to now, there have been very few studies on both direct and indirect shoot regeneration and the shoot regeneration rate of *P. edulis* Sims. was performed. In the present study, the regeneration rate was 100%, which was potential for micropropagation.

This study has successfully used the leaf-tTCL explants for shoot regeneration. In addition, the leaf explants of 1.5-month-old shoots and FL condition had a significant impact on the direct shoot regeneration from leaf-tTCL explants. The results of this study provide a new way for the micropropagation of *P. edulis* Sims.

Acknowledgements: This research was supported by Molecular Biology and Plant Breeding Department, Tay Nguyen Institute for Scientific Research.

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Table 4. Effect of light condition (FL and darkness) on shoot regeneration of *P. edulis* Sims. after 8 weeks of culture.

| Light condition | Morphogenesis rate (%) | No. of shoots/explant | Shoot height (cm) | Morphogenesis                  |
|-----------------|------------------------|-----------------------|-------------------|-------------------------------|
|                 | Shoots                 | Calli                 |                   |                               |
| FL              | 100 ± 0.0*             | 4.44 ± 1.11           | 4.33 ± 0.33       | 1.13 ± 0.33                   | Regenerating shoot directly from leaf explant |
| Darkness        | 71.11 ± 2.22           | 100 ± 0.0             | 3.33 ± 0.33       | 0.47 ± 0.33                   | Regenerating shoot indirectly via callus      |

Note: *The values represent the mean ± SE (t-test treatment in Duncan with statistically significant 95%).
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**KÍCH THỊCH SỰ TÁI SINH CHỞI THÔNG QUA NUÔI CÁY LỚP MỌNG TỂ BẢO LÂM CÁY CHANCHI ĐÁY TÌM (***PASSIFLORA EDULIS SIMS***)

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Tóm tắt

*Passiflora edulis* Sims. Việc chèn *Passiflora*, là một loại cây trồng kinh tế quan trọng ở trên thế giới cũng như ở Việt Nam. Ngày nay, giống chèn đầy tím thường mới chủ yếu được nhân giống bằng hạt, hom và ghép; tuy nhiên, các phương pháp này vẫn tồn tại một số hạn chế như sự thoát hóa giống, tính không đồng nhất về mặt di truyền, sự lây lan của virus gây bệnh... Vì nhàn giống có thể là phương pháp hiệu ức cho nhân giống vô tính và tạo giống cây sạch bẻn cũng như cung cấp nguồn giống đối với *Passiflora*. Trong nghiên cứu này, mẫu là *in vitro* của *P. edulis* Sims. (2 tháng tuổi) được thuần hóa trực tiếp từ nuôi cây đó trên mang chất ních ex *in vitro* sử dụng lam vật liệu thực vật [cắt lỗ tế bào mong theo chiều dài (TCL)] và theo chiều ngang (TCL) để đánh giá sự tài sinh chởi. Ngoài ra, ảnh hưởng của mẫu và điều kiện anh sáng đối với quá trình tài sinh chởi cũng đã được khảo sát. Sau 8 tuần nuôi cây, kết quả cho thấy tỷ lệ tài sinh chởi (100%) và hệ số nhân chởi (13,33) của mẫu là TCL-4 cao hơn so với các nghiệm thức khác và đối chứng. Túi của mẫu cùng ảnh hưởng điều kiện vào sự tài sinh chởi của *P. edulis* Sims. Tỷ lệ tài sinh chởi cao nhất (100%) đã được ghi nhận ở mẫu là của chởi 1,5 tháng tuổi sau 8 tuần nuôi cây. Hơn nữa, điều kiện chiều ních (đơn huỳnh quang với cường với cường độ 16 giờ/ngày ở cường độ ánh sáng 40 - 45 μmol.m-2.s-1) không chỉ ảnh hưởng tích cực đến tỷ
lệ phát sinh hình thái mà còn ảnh hưởng đến tỷ lệ tái sinh chính thức tiếp (100%) từ mẫu lá sau 8 tuần nuôi cây.
Kết quả của nghiên cứu này đã cung cấp phương pháp mới trong việc nhân giống giống chanh dây tim.

Từ khóa: lặp mống tế bào, mẫu lá, Passiflora edulis, tái sinh chính