

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

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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 (ITCL 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-tTCL-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 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) 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.

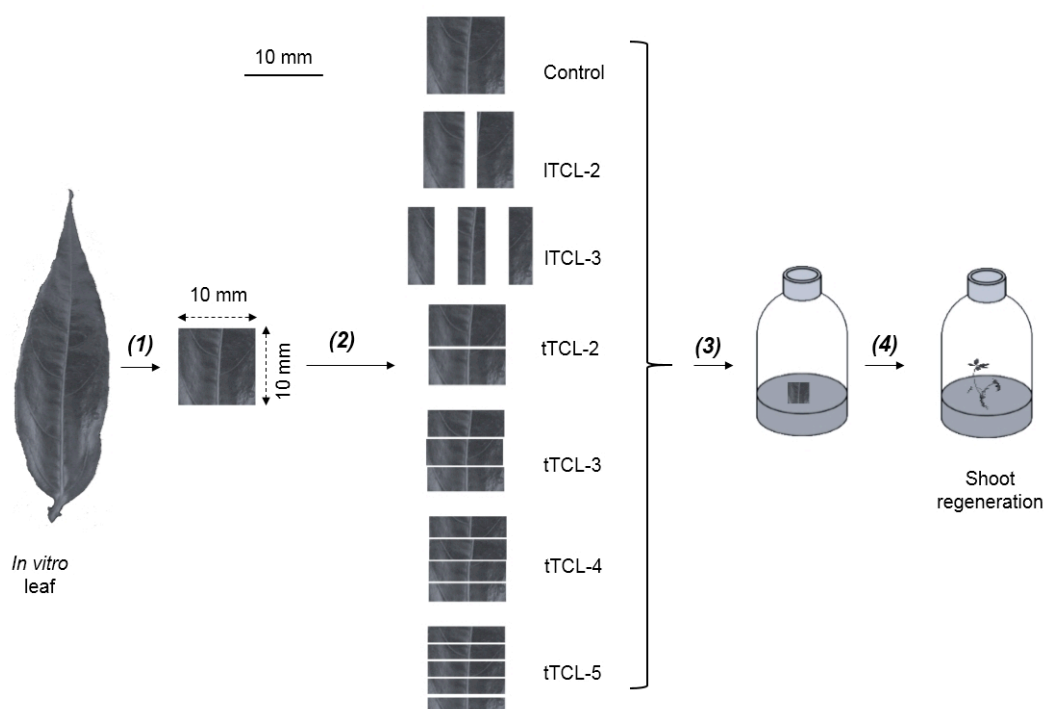


Figure 1. Diagram of establishing a thin-layer culture system for shoot regeneration of *P. edulis* Sims. (1): Cut leaves into square pieces (10 mm x 10 mm); (2): Cut in different ways and sizes; (3): Transfer leaf-TCL to shoot regeneration medium; (4): Shoots were recorded after 8 weeks of culture.

Table 1. Establishment of TCL culture for shoot regeneration of *P. edulis* Sims.

Treatment	Cutting style	Size ^a (mm × mm)
Control	Leaves were cut off the edges	10 × 10
ITCL-2	Square pieces of leaves were cut longitudinally along the ribs into 2 pieces	5 × 10
ITCL-3	Square pieces of leaves were cut longitudinally into 3 pieces	3.33 × 10
tTCL-2	Square pieces of leaves were cut transversally perpendicular to the ribs into 2 pieces	10 × 5
tTCL-3	Square pieces of leaves were cut transversally into 3 pieces	10 × 3.33
tTCL-4	Square pieces of leaves were cut transversally into 4 pieces	10 × 2.5
tTCL-5	Square pieces of leaves were cut transversally into 5 pieces	10 × 2

Note: ^a: length × width

Shoot multiplication coefficients were compared by: $S_c = R_s(\%) \times S \times N$

Where: **S_c**: Shoot multiplication coefficient; **R_s**: 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 plantlets at different explant ages (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0-month-old) were cut into optimal leaf-TCL in the above experiment. 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 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, 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 $25 \pm 2^\circ\text{C}$ with humidity of 55 - 60% and photoperiod of 16 hours/day under the light of FL ($40 - 45 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) 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-tTCL and ITCL culture were recorded (Table 2, Figure 2, 3).

The results showed that TCL techniques affected to 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
ITCL-2	74.44 d	2.67 b	0.83 c
ITCL-3	91.11 b	2.33 bc	1.03 b
tTCL-2	78.89 c	3.00 b	0.87 c
tTCL-3	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 $\alpha = 0.05$ in Duncan's multiple range test.

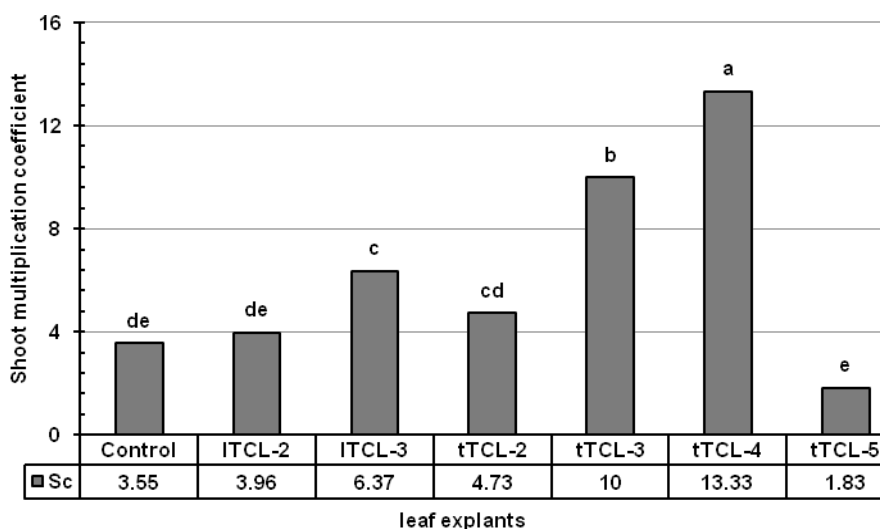


Figure 2. Shoot multiplication coefficient of TCL culture of *P. edulis* Sims. after 8 weeks of culture.

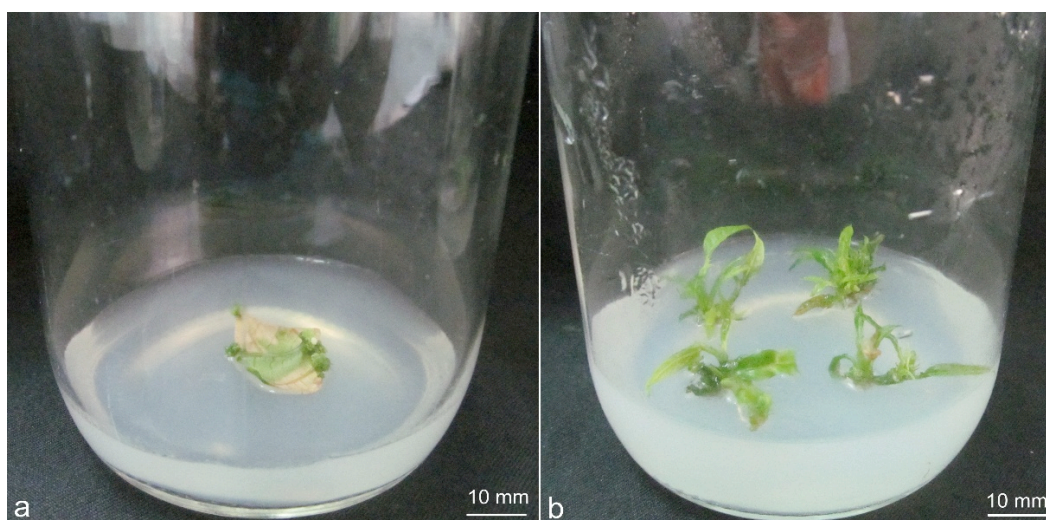


Figure 3. Shoot regeneration of *P. edulis* Sims. from leaf explants after 8 weeks of culture. **a:** Control (leaf was cut off the edges – 10 mm × 10 mm); **b:** tTCL-4 (10 mm × 2.5 mm).

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).

Table 3. Effect of explant age on shoot regeneration of *P. edulis* Sims. after 8 weeks of culture.

Explant age	Shoot regeneration rate (%)	No. of shoots/explant	Shoot height (cm)
0.5-month-old	36.67 d ^x	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: ^x 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 became 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).

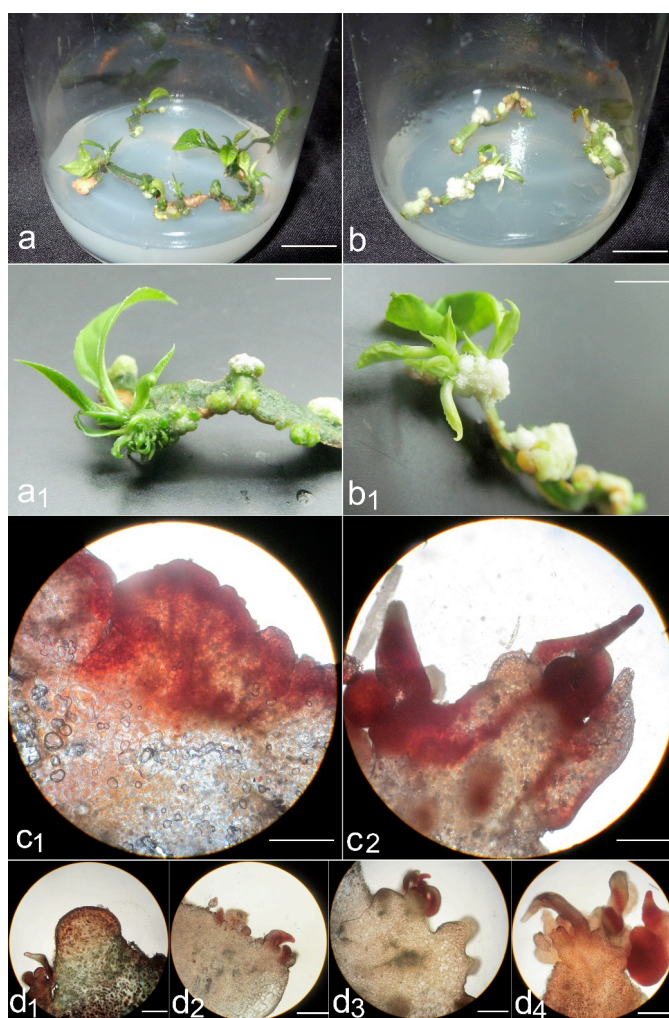


Figure 5. Effect of lighting condition (FL and darkness) on shoot regeneration of *P. edulis* Sims. after 8 weeks of culture. **a, a₁**: Direct shoot regeneration; **b, b₁**: Indirect shoot regeneration; **c₁**: Initial structure of bud; **c₂**: Complete structure of bud; **d₁**: Callus formation after 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).

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 ^y	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: ^y The values represent the mean ± SE (t-test treatment in Duncan with statistically significant 95%).

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 micro-propagation. CONCLUSION

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 micro-propagation of *P. edulis* Sims.

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KÍCH THÍCH SỰ TÁI SINH CHỒI THÔNG QUA NUÔI CÂY LỚP MÔNG TẾ BÀO LÁ CÂY CHANH DÂY TÍM (*PASSIFLORA EDULIS* SIMS.)

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TÓM TẮT

Passiflora edulis Sims. thuộc chi *Passiflora*, là một loại cây trồng kinh tế quan trọng ở trên thế giới cũng như ở Việt Nam. Ngày nay, giống chanh dây tím thương mại 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 thoái hóa giống, tính không đồng nhất về mặt di truyền, sự lây lan của virus gây bệnh... Vì nhân giống có thể là phương pháp hữu ích cho nhân giống vô tính và tạo giống cây sạch bệnh cũng như cung cấp nguồn giống đối với *Passiflora*. Trong nghiên cứu này, mẫu lá *in vitro* của *P. edulis* Sims. (2 tháng tuổi) được thu nhận trực tiếp từ nuôi cấy đoạn thân mang chồi nách *ex vitro* được sử dụng làm vật liệu thực vật [cắt lớp tế bào mỏng theo chiều dọc (ITCL) và theo chiều ngang (tTCL)] để đánh giá sự tái sinh chồi. Ngoài ra, ảnh hưởng tuổi của mẫu và điều kiện ánh sáng đối với quá trình tái sinh chồi cũng đã được khảo sát. Sau 8 tuần nuôi cấy, kết quả cho thấy tỷ lệ tái sinh chồi (100%) và hệ số nhân chồi (13,33) của mẫu lá tTCL-4 cao hơn so với các nghiệm thức khác và đối chứng. Tuổi của mẫu cũng ảnh hưởng hiệu quả đến sự tái sinh chồi của *P. edulis* Sims. Tỷ lệ tái sinh chồi cao nhất (100%) đã được ghi nhận ở mẫu lá của chồi 1,5 tháng tuổi sau 8 tuần nuôi cấy. Hơn nữa, điều kiện chiếu sáng (đèn huỳnh quang với quang chu kỳ 16 giờ/ngày ở cường độ ánh sáng 40 - 45 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) không chỉ ảnh hưởng tích cực đến tỷ

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lệ phát sinh hình thái mà còn ảnh hưởng đến tỷ lệ tái sinh chồi trực tiếp (100%) từ mẫu lá sau 8 tuần nuôi cấy. Kết quả của nghiên cứu này đã cung cấp phương pháp mới trong vi nhân giống giống chanh dây tím.

Từ khóa: *lớp mỏng tế bào, mẫu lá, Passiflora edulis, tái sinh chồi*