Scanning Electron Microscopy Studies and *In Vitro* Regeneration of *Passiflora edulis* Sims var. *edulis* for Conservation

Rubashiny Veeramohan, Noorma Wati Haron, and Rosna Mat Taha

***Abstract*—In this study, different combinations of benzylaminopurine (BAP) and naphthalene acetic acid (NAA) were used to establish an efficient *in vitro* regeneration in two different types of explants: stem and shoot tip of seedlings obtained from *in vitro* seed germination of *Passiflora edulis* Sims var. *edulis*. The results obtained showed that BAP induced shoots while NAA induced roots. Scanning Electron Microscopy (SEM) studies were carried out to observe and differentiate the morphology of *in vitro* and *in vivo* grown *Passiflora edulis* Sims var. *edulis*. SEM techniques were done to observe the stomata and microcharacters of both the abaxial and adaxial surfaces of *in vitro* and *in vivo* leaves of *Passiflora edulis* Sims var. *edulis*.**

***Index Terms*—*In vitro*, *in vivo*, *Passiflora edulis* Sims var. *edulis*, regeneration, Scanning Electron Microscopy (SEM).**

I. Introduction

The family Passifloraceae consists of 18 genera and about 630 species, most of which are tendril climbing vines native to warm regions of the world [1]. In America, the family is represented by four genera (*Ancistrothyrsus*, *Dilkea*, *Mitostemma* and *Passiflora*), of which *Passiflora* is numerically and economically the most important genus of the family. There are about 500 species of *Passiflora* worldwide, but only less than 10 species could be found in Malaysia. *Passiflora edulis* Sims is considered as the most important species of the genus *Passiflora*, mainly because of its botanical and commercial value, as well as for crop breeding and genomic programs.

Passiflora edulis Sims var. *edulis* is also known as purple passion fruit and is a vigorous perennial woody climber. It is originated in the Amazon region of Brazil, but has been commercially cultivated in Hawaii, Australia, New Zealand, Fiji, South Africa and Kenya [2]-[4]. In Malaysia, it is widely distributed in the northern, east coast, and central regions. The leaves are green and alternate, turning into 3 lobed leaves when they mature. The bisexual solitary flower is borne at leaf base of new growth. It is about 4 to 5 cm in diameter and very conspicuous, colourful and fragrant. It has five whitish petals and two purplish rows of thread like rays called corona. The pistil consists of an ovary tripartite style, each branch terminating in a sticky stigma. The fruit is round or oval, about 4 to 5 cm long and greenish yellow or purple when ripe and smooth [2]. They grow swiftly about 15 to 20 feet per year [4].

Passiflora edulis Sims var. *edulis* is widely grown in the tropics for its edible fruits as they are good sources of Vitamins A and C. The fruit has anticarcinogenic effects and the pulp acts as a stimulant and tonic. The flower extract of *Passiflora edulis* has sedative and hypnotic effects. Glycosides, phenols and alkaloids are the major constituents in *Passiflora edulis* [3], [4]. The identified constituents in *Passiflora edulis* includes anthocyanins, carotenoids, γ-lactones, l-ascorbic acid, flavour components, volatile oil constituents, minerals, amino acids, carbohydrates, the cytoplasmic enzyme pyruvate kinase, cycloartane triterpenes, cyclo passiloic acids A-D, and their saponins, cyclopassi-flosides I-VI [4], [5].

Tissue culture studies of *Passiflora* started as early as 1966 and since then many reports on tissue culture based methods applied to the genus have been published [6]. Most of the *in vitro* culture techniques for *Passiflora* were developed using *Passiflora edulis* Sims f. *flavicarpa* Deg. explants and have been used for micropropagation [7], [8], organogenesis [9], somatic hybridization [10], [11], and genetic transformation [9] of the species. Organogenesis-based plant regeneration system is currently being established in passion fruit [6], [11]. However, reports on *in vitro* plant regeneration and taxonomic studies of *Passiflora edulis* Sims var. *edulis* are scanty especially on the micromorphological aspects. Therefore, the objectives of this study were to study the influence of different combinations of BAP and NAA hormones on *in vitro* regeneration, to observe and differentiate the characteristics of *Passiflora edulis* Sims var. *edulis* grown *in vivo* and *in vitro* via Scanning Electron Microscopy (SEM), and to increase micromorphological taxonomic information of *Passiflora edulis* Sims var. *edulis*.

II. Materials and Methods

A. Seed Germination

Seeds of *Passiflora edulis* Sims var. *edulis* were obtained from ripe fruits collected from the Institute of Biological Sciences garden in University of Malaya, Kuala Lumpur. Seeds were germinated via *in vitro* and *in vivo* techniques. The pulps were removed completely to be cultured *in vitro* and *in vivo* using aseptic techniques to obtain explants from seedlings. The seeds were then washed under running tap water for 30 minutes and treated with 70%, 50%, and 20% (v/v) commercial bleach for 10 minutes at each concentration. Next, seeds were rinsed in distilled water for 2 minutes and

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transferred to the laminar flow chamber. Surface sterilization of seeds was done using 70% (v/v) of ethanol for 30 seconds, followed by three rinses in sterile distilled water and left to air-dry in the laminar flow chamber. Sterilized seeds were germinated in jam jars containing Murashige and Skoog (MS) salt and vitamins supplemented with 3% sucrose and 0.8% agar for *in vitro* germination. The pH of the media was adjusted to 5.8±0.1 before autoclaving at 121°C for 20 minutes. Twenty replicates were used for each experiment and maintained at 25±2°C with 16 hours of light and 8 hours of dark. Whereas, for *in vivo* germination, seeds of *Passiflora edulis* Sims var. *edulis* were germinated on the soil in a small pot and watered daily. *In vivo* grown plants were then used for micromorphological comparison with *in vitro* plantlets.

B. Scanning Electron Microscopy (SEM) Studies

Passiflora edulis Sims var. *edulis* leaf specimens were collected from the Institute of Biological Sciences garden in University of Malaya, Kuala Lumpur. Leaves were cut in square shape with measurement of approximately 3 mm x 3 mm and soaked in a mixed solution of 8% Glutaraldehyde and Sorencen's Buffered Phosphate with a ratio of 1:1 for an hour. The leaf specimens were washed with Sorencen's Buffered Phosphate solution and distilled water with a ratio of 1:1 for 5 minutes. Specimens were then soaked in a mixed solution of 4% Osmium and distilled water with a ratio of 1:1. After about 14 hours at low temperature, the dehydration process was conducted in vessel fumes. The concentration of ethanol was increased for every 15 minutes, from 10% until it reaches 100%. Specimens were soaked in mixture of 100% ethanol and 100% acetone with ratios of 3:1, 1:1, and 1:3 for 20 minutes respectively. Right after that, the leaf specimens were then soaked in 100% acetone solution for 20 minutes, repeating them for four times. Next, the method of Critical Point Drying (CPD) was conducted using Bal-Tec CPD 030 Critical Point Dryer. Specimens were mounted on aluminium with diameter of 12.5 mm using Conducting Carbon Cement (LEIT-C), kept in a drying jar, and coated with a thin layer of gold (40-60 nm) by using BIO-Rod SEM Coating System. Structures such as stomata and the abaxial and adaxial surfaces structure of the leaves were observed under the scanning electron microscope and recorded.

C. In Vitro Regeneration

After 60 days, stem (1 cm) and shoot tip (1 cm) explants were excised into specific measurements from seedlings to be inoculated in culture tubes containing MS medium [12] supplemented with agar and 0.5 mg/L BAP + 0.5-2.0 mg/L NAA and 0.5 mg/L NAA + 0.5-2.0 mg/L BAP. The pH of the medium was adjusted to 5.8±0.1 prior to autoclaving (20 minutes at 120°C). The cultures were then incubated in a culture room at 25±2°C under a regular cycle of 16 hours of light and 8 hours of dark. Observations were made and the data obtained was subjected to statistical analysis for computation of the standard error of the mean (SE). A completely randomized design with 20 replications for each explant was used. Observations of each explant were made after 45 days of culture. Data obtained were analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same row differ significantly at p = 0.05.

III. Results

A. Scanning Electron Microscopy (SEM) Analysis

Figs. 1(a) & 2(b) shows that *Passiflora edulis* Sims has papillose epidermal surface, with undulating anticlinal wall on the abaxial surface (Fig. 2(b)). Fig. 2(c) shows the absence of stomata and irregularly shaped polygonal epidermal on the adaxial surface. Epicuticular wax is sparsely distributed on the surface. Referring to Figs. 1(b) and 2(b), the paracytic stoma of an *in vitro* species has stomata measurement of 11.0 µm by length and 8.82 µm by width, while the *in vivo Passiflora edulis* Sims leaf measures 13.4 µm by length and 10.1 µm by width. Stomata were absent on the adaxial surfaces of both *in vitro* and *in vivo* species (Figs. 1(c) & 2(c)).

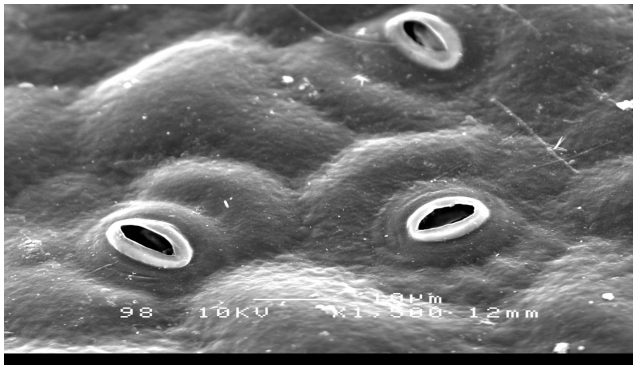

Fig. 1(a). Abaxial surface of an *in vivo Passiflora edulis* Sims leaf.

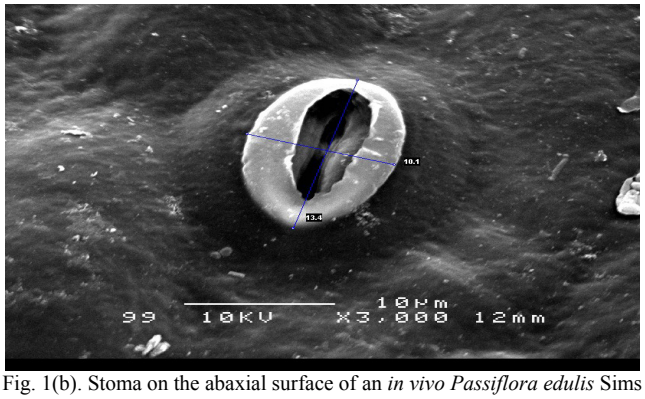

Fig. 1(b). Stoma on the abaxial surface of an *in vivo Passiflora edulis* Sims leaf.

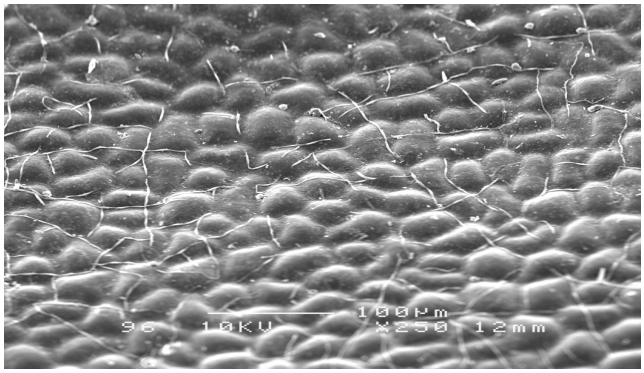

Fig. 1(c). Adaxial surface of an *in vivo Passiflora edulis* Sims leaf.

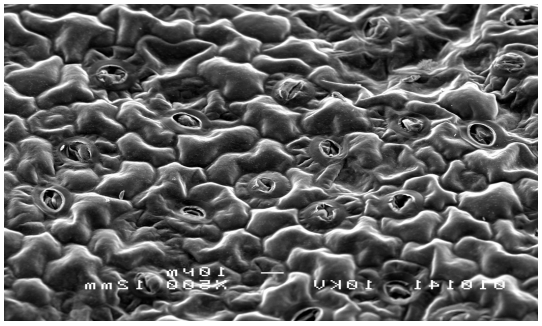

Fig. 2(a). Abaxial surface of an *in vitro Passiflora edulis* Sims leaf.

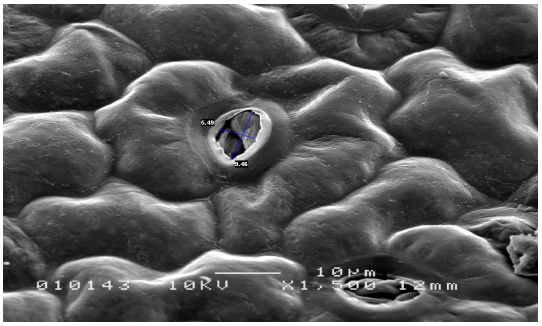

Fig. 2(b). Stomata on the abaxial surface of an *in vitro Passiflora edulis* Sims leaf.

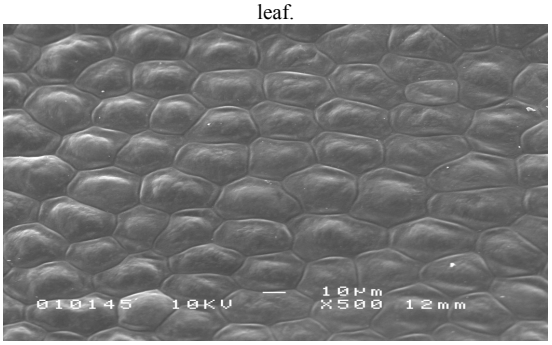

Fig. 2(c). Adaxial surface of an *in vitro Passiflora edulis* Sims leaf.

B. In Vitro Regeneration

Passiflora edulis Sims var. *edulis* seeds that were germinated *in vitro* in MS media without hormone supplement gave 90% response of healthy seedlings within 60 days. Table I shows that the highest average shoot number was exhibited by shoot tip explants cultured on MS media supplemented with 0.5 mg/L NAA + 2.0 mg/L BAP which produced 2.70 ± 0.22 average shoot (Fig. 3). The highest average shoot number produced was significantly different from all plant growth regulator combinations, except 0.5 mg/L NAA + 1.0-1.5 mg/L BAP. Stem explants cultured on MS media fortified with 0.5 mg/L BAP + 0.5 mg/L NAA and 0.5 mg/L NAA + 2.0 mg/L BAP which showed the highest average shoot number of 1.25 ± 0.29 and 1.25 ± 0.30 respectively, were significantly different from all the other plant growth regulator combinations except for 0.5 mg/L NAA + 1.0 mg/L BAP, which resulted in an average shoot number of 1.05 ± 0.19. Stem explants produced an average root number of 4.90 ± 2.42 and shoot explants produced an average root number of 5.10 ± 2.28 in MS media supplemented with 0.5 mg/L BAP + 1.5 mg/L NAA. Stem explants cultured on MS media supplemented with 0.5 mg/L BAP + 1.5-2.0 mg/L NAA which produced an average root number of 4.90 ± 2.42 and 1.65 ± 0.47, respectively, were significantly different from responses exhibited by other plant growth regulator combinations. The average number of roots produced by shoot tip explants (5.10 ± 2.28) in MS media fortified with 0.5 mg/L BAP + 1.5 mg/L NAA was significantly different from responses showed by other plant growth regulator concentrations.

IV. Discussion

Based on the SEM observation of Figs. 1(a) & 2(b), the abaxial anticlinal wall of *in vitro* species appears to be more undulated compared to the *in vivo* species. The smaller size of an *in vitro Passiflora edulis* Sims var. *edulis* stomata compared to the *in vivo* species stomata may be due to the age differences between the *in vivo* and *in vitro* species. It is also observed that the stomata of *in vitro* species were sunken compared to the stomata of *in vivo* species. This may be due to the low relative humidity and high CO_2 concentration in the culture vessel during the photoperiod [13].

In this work, shoot formation was observed in shoot tips explants for all treatments, including the control. On the other hand, stem explants formed shoots on all treatments, except for the control. Root formation was absent in stem explants cultured in MS media supplemented with 0.5 mg/L BAP + 0.5 mg/L NAA and the control treatment, whereas shoot tip explants showed root formation only on MS media supplemented with 0.5 mg/L BAP + 1.5 mg/L NAA (Table I).

TABLE I: The Influences of MS Media Supplemented With Various Hormone Combinations of BAP And NAA on Shoot And Root Regeneration from Stem And Shoot Tip Explants of *Passiflora edulis* After 45 Days in Culture

PGR Concentration (mg/L)		Stem explants		Shoot tip explants	
BAP	NAA	Average shoot no. (±SE)	Average root no. (±SE)	Average shoot no. (±SE)	Average root no. (±SE)
0	0	0.05±0.05d	0.00±0.00b	0.00±0.00d	0.00±0.00b
0.5	0.5	1.25±0.29a	0.00±0.00b	1.35±0.22b	0.00±0.00b
0.5	1	0.70±0.16bc	0.55±0.20b	0.20±0.09d	0.00±0.00b
0.5	1.5	0.40±0.11cd	4.90±2.42a	0.40±0.11cd	5.10±2.28a
0.5	2	0.50±0.12cd	1.65±0.47a	0.75±0.10c	0.00±0.00b
1	0.5	1.05±0.19ab	0.00±0.00b	2.40±0.11a	0.00±0.00b
1.5	0.5	0.75±0.23bc	0.00±0.00b	2.60±0.21a	0.00±0.00b
2	0.5	1.25±0.30a	0.00±0.00b	2.70±0.22a	0.00±0.00b

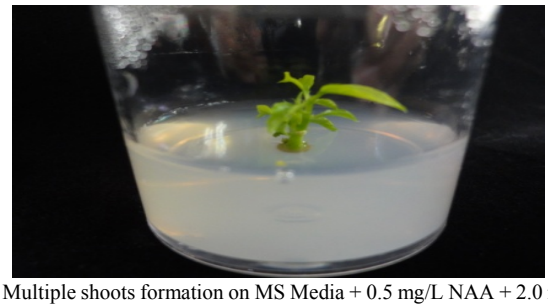

Fig. 3. Multiple shoots formation on MS Media + 0.5 mg/L NAA + 2.0 mg/L BAP

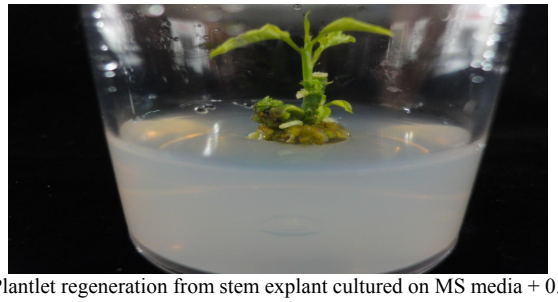

Fig. 4. Plantlet regeneration from stem explant cultured on MS media + 0.5 mg/L BAP + 1.5 mg/L NAA.

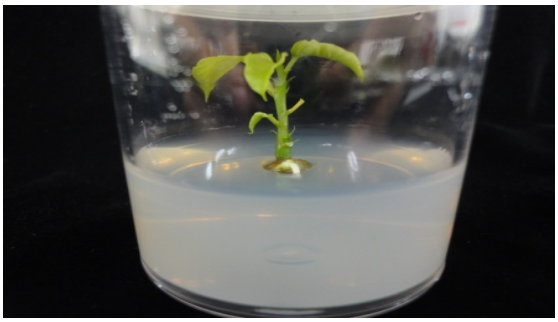

Fig. 5. Plantlet regeneration from shoot tip explant cultured on MS media + 0.5 mg/L BAP + 1.5 mg/L NAA.

There are a number of plant regeneration reports available on *Passiflora* using various explants and plant growth regulators combinations [14], [15]. Reference [16] reported plant regeneration through leaf discs of passion fruit using kinetin, BAP, and NAA. They also discussed that callus and roots could be induced in leaf discs of *Passiflora edulis* Sims f. *flavicarpa* Deg. by altering the concentration of cytokinin and auxin. Roots were obtained when NAA was used but no roots were obtained with the use of indoleacetic acid (IAA) or indolebutyric acid (IBA). Based on this work, roots were obtained from stem and shoot tip explants of *Passiflora edulis* Sim var. *edulis* in combinations of both BAP and NAA plant growth regulators. Reference [17] found that lower concentration of NAA induced lower root formation. Proportionally, higher concentration of NAA induced higher root formation in this research. Indirect plant regeneration was observed from stem and shoot tip explants cultured on MS + 0.5 mg/L BAP + 1.5 mg/L NAA (Figs. 4 & 5), which shows a great potential to carry out mass propagation of this plant. These *in vitro* grown complete plantlets could be transferred from the culture room to pots outside through a process of acclimatization.

V. Conclusion

From the present study, it was found that the abaxial anticlinal wall of *in vitro Passiflora edulis* Sims var. *edulis* leaf is more undulated compared to the *in vivo* species. The stomata of *in vitro Passiflora edulis* Sims var. *edulis* are smaller in size compared to the stomata size of *in vivo* grown plant which may be due to the age differences between the *in vivo* and *in vitro* grown plant. This study has also added new taxonomic information especially on the micromorphological aspects of the species.

Stem and shoot tip explants cultured on MS media supplemented with 0.5 mg/L BAP and 0.5-2.0 mg/L NAA and MS media fortified with 0.5 mg/L NAA and 0.5-2.0 mg/L BAP showed different responses of indirect organogenesis. Generally, MS media supplemented with 0.5 mg/L BAP and 1.5 mg/L NAA was optimum for plant regeneration of *Passiflora edulis* Sims var. *edulis*. Further research should be conducted to study the possibilities of inducing mass micropropagation through *in vitro* regeneration of *Passiflora edulis* Sims var. *edulis*.

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