Tissue Culture Studies of *Sinningia speciosa*

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ABSTRACT

Diseases were a frequent problem that occurred in the natural environment. Sinningia speciosa may develop root rot from overwatering. Leaves may be damaged by excessive watering and therefore watering must be avoided from the foliage part. Mites and trips were the insect and pest that related to this species. By the aid of tissue culture technique, all of these problems can be avoided. The objective of this study was to evaluate the best proliferation of different explants of Sinningia speciosa on various combinations of plant growth regulators. This project also aimed to select the best shoot proliferation and rooting medium to produce complete plantlets of Sinningia speciosa via in vitro culture technique. Generally, the aim of this study was to develop a micropropagation protocol that could maintain a mass production and continuous supply of Sinningia speciosa propagules to fulfill the market demand. In this study, leaves had been recognized to be the best explants in micropropagation of Sinningia speciosa. This explant showed an indirect regeneration pathway by obtaining shoots from the intermediary callus. From the micromorphological study using scanning electron microscope (SEM), the stomata of in vivo leaves were larger than in vitro leaves. There were also more trichomes located on in vivo plants compared to in vitro leaves and their sizes were longer. The plants propagated through tissue culture techniques did not show any morphological abnormalities when compared to the original or parent plants. The best medium for shoot proliferation of this ornamental plant was MS basal medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP. The highest number of shoots per explants was obtained from leaves explants by using this optimal medium (6.3 ± 1.3). The healthy young shoots were induced to form roots in hormone-free MS basal media. Complete in vitro plantlets were acclimatized successfully in the natural environment.

Keywords: *Sinningia speciosa, plant regeneration, hormones (plant growth regulators)*

Introduction

Sinningia speciosa, commonly known in the horticultural trade as "Gloxinia", is a tuberous member of the flowering plant family Gesneriaceae. The plants produce large, velvety, brightly colored flowers and are popular houseplants. *Sinningia speciosa* can be grown from seed, tubers, or leaf or stem cuttings. Producing flowering plants from seeds takes about six months, depending on the seasons involved (Chautems *et al.* 2000; Zaitlin and Pierce, 2010).

Some species of *Sinningia speciosa* have been reported as being used in rural medicine, but the importance of the family Gesneriaceae lies in its cultivated ornamentals. Other than that, *Sinningia speciosa* can offer a better potential model species for ornamental plant genomics. Flow cytometry of leaf cell nuclei indicated that the genome of *S. speciosa* is small for an angiosperm, larger than that of *Arabidopsis thaliana* (~157Mb) but smaller than the ~420Mb genome of rice (*Oryza sativa*). The small genome of *S. speciosa* opens the way for the isolation of genes involved in processes of ecological significance (tuberization, floral development, pollinator preference). Genomic resources developed for *S. speciosa* would also enable comparative studies within *Sinningia*, a diverse genus of 70 species and, in a larger context, the Gesneriaceae (Zaitlin & Pierce, 2010).

S. speciosa are extremely sensitive to their environment. They simply cannot tolerate stress well. Anything that restricts their root growth or top growth will reduce both plant size and the number of flowers initiated. Bright, indirect light is necessary to keep *S. speciosa* in flower. *S. speciosa* leaves are somewhat brittle, which is made worse by temperatures lower than those recommended. For best growth, the night temperature should be 18-20°C and 24°C during the day. Proper ventilation and relative humidity is kept in balance to reduce condensation and prevent diseases (Kessler, 1999). *S. speciosa* may develop root rot from overwatering. Leaves may be damaged by cold water and therefore watering must be avoided from the foliage part. Mites and trips were the most insect and pest that related to this species. Apart from that, they were easily infected by foliar nematodes (Lehman, 1991).

In vitro propagation was frequently used as an option to solve this problem, mainly due to its well known features; a sterile and rapid plant producing procedure. To date, one successful result was obtained on node culture of *S. speciosa* reported by Nhut *et al.* (2006) with a limited quantity of about 10 regenerated shoots per explant. Thus, enhancing shoot multiplication rate was a significant work in *S. speciosa* micropropagation that helped to supply the increasing demand of commercial ornamental plants as well as plant materials for many studies of *in vitro* plant tissue culture. The objective of this study is to evaluate the best proliferation of different explants of *S. speciosa* at various combinations of plant growth regulators. This project also aimed to select the best shoot proliferation and rooting medium for producing complete plantlets of *Sinningia speciosa* via *in vitro* culture technique. Other than that, the macromorphological and micromorphological characteristics of *in vivo* and *in vitro* plants were investigated in order to observe any differences in morphology of *S. speciosa* in both *in vivo* and *in vitro* condition.

Materials and Methods

Plant materials

The seeds of *S. speciosa* were bought from a hardware store in Mid Valley Shopping Center, Kuala Lumpur. Other than seeds, intact plants were also bought at a garden center in Sungai Buluh. Only vigorous, young and healthy plants were chosen for this study. Leaves, petiole and peduncle were taken from the intact plant as source of explants for this study. Flowers could not be used in this study as the flower of this plant is fragile and survival rate towards sterilization process is low (Xu *et al.* 2009).

In vitro culture

The seeds were cultured on MS (Murashige and Skoog, 1962) media without any addition of plant growth regulator (hormones). While for explants, there were 4 types of hormones used in this study; Naphthaleneacetic acid (NAA), Benzylaminopurine (BAP), 2,4–Dichlorophenoxyacetic acid (2,4–D) and Thidiazuron (TDZ) and all of the hormones were exploited at different concentration in order to achieve best proliferating medium.

The seeds and explants were washed thoroughly under running tap water for 1-2 hours. The plant material were then transferred to the laminar air flow cabinet and were rinsed again with distilled water for 3 times, then surface sterilized by immersing in ethanol 70% for 3 min, followed by continuous shaking for 15 minutes in 50% solution of sodium hypochlorite (NaOCl) with the addition of 0.1% Tween 20 and subsequently rinsed 5 times in sterile distilled water.

The seeds and explants were placed on 25 ml agar-solidified culture medium in a sterile plastic vials. The basal medium consisted of salts and vitamins of MS medium and solidified with 0.7% (w/v) agar technical (Agar No.3). The medium was adjusted to pH 5.8 before adding agar and then sterilized by autoclaving at 121°C for 20 minutes. The cultures were then placed in growth chamber at 25°C ± 1°C under standard cool white fluorescent tubes with flux rate of 50 µmol $m^{-2}s^{-1}$ and a 16-h photoperiod. The performance of cultures was examined by direct observation.

Rooting and acclimatization of regeneration plants

Well developed shoots from proliferating shoot cultures were separated and rooted on MS media without addition of hormone. Ten to twelve weeks old regenerated plantlets which had few leaves, 5 cm of roots length and 4-5 cm height were taken out from culture tubes and any excess agar was removed to prevent from microorganism infection. The complete plantlets were soaked in 0.5% (w/v) benlate, an antifungal agent for 2-3 minutes. The *in vitro* rooted plantlets were then transferred to sterile soil with added nutrient solution and covered with transparent plastic bags to maintain relative humidity to the plants. The plants were place in growth chamber with a 16-h photoperiod, and a night/day temperature 18/20°C for 2 weeks. The hardened plants were then transferred to the green house.

Morphological study

Morphological study involved the study of macromorphology and micromorphology of *S. speciosa* in the *in vivo* and *in vitro* condition. The macmorphology study was carried out by observing the external structures of the plant via naked eyes. While, the micromorphology study was performed using SEM (Scanning Electron Microscopy) to observe the microscopic structures of *in vivo* and *in vitro* leaves of *S. speciosa*.

Results and Discussion

***In vitro* seeds culture**

The purpose of culturing seeds is to obtain aseptic seedlings that later can be used for source of explants to culture on different types of media. This provides a better technique as aseptic seedlings had fewer risk of contamination compared to explants that were obtained from intact plants. However, regeneration of aseptic seedlings in *in-vitro* culture was not successful. After few weeks of culturing, there was no response observed from the seeds culture. These might due to the viability of the seed used for culture. The probabilities that might caused the low viability of seeds were mainly due to high concentration of sodium hypochlorite used and long exposure to disinfecting agents during sterilization of the seeds. From observation, the color of the seeds was changed from dark brown to white in color due to bleaching.

***In vitro* explants culture**

Effect of various combinations of NAA and BAP

In general, leaves explants were observed to give a higher response than petioles and peduncles explants when cultured on MS media supplemented with NAA and BAP. These findings were concurrent to many tissue culture studies that obtained high shoot proliferation using leaves as explants (Xu *et al.* 2009; Nhut *et al.* 2006). Green shoot primordia developed on explants after 6-7 weeks of culture after the formation of intermediary callus. Eight weeks after initial cultures numerous adventitious shoot formations took place readily from these nodules callus. The numbers of adventitious shoots varied depend on the concentrations of NAA and BAP used. The highest number of shoot produced per explants was obtained on leaves explants cultured on media supplemented with 1 mg/l NAA and 1.5 mg/l BAP (6.3 ± 1.3).

Other than that, media supplemented with NAA (2.0, 2.5 and 3.0 mg/l) and 1.0 BAP, NAA (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) and 1.5 BAP and NAA (0.5, 1.0 and 2.0 mg/l) and 2.0 BAP also produced shoots indirectly after formation of intermediary callus. While, root formation was observed in MS media supplemented with 1.0 mg/l NAA and 2.5 mg/l BAP. This medium generated the highest number of roots per explants grow from the intermediary callus (5.2 ± 0.8). The other combinations of NAA and BAP only produced light green nodules callus.

It was well documented regarding the balance of auxin (NAA) and cytokinin (BAP) played a major role in producing high morphogenic responses (Park *et al.* 2012; Biddington, 1992). However, some studies have achieved high shoot proliferation using BAP alone. Naz *et al.* (2001) obtained high shoot proliferation in media supplemented with 3 mg/l BAP using newly formed leaves as explants. Other studies also demonstrated BAP strongly enhanced regeneration of adventitious shoot in petioles explants of carnation (Messeguer *et al.* 1993), gerbera (Nongmanee & Kanchanapoom, 1995) and African violet (Sunpui & Kanchanapoom, 1995).

Effect of different concentrations of 2,4–D

The response of the explants towards MS media supplemented with 2,4–D were increased as the level of 2,4-D concentration increased. However, there were no shoots formation observed from this media. The leaves, petioles and peduncles explants cultured on this media only developed into callus. It showed a linear relationship between the response of the explants to increasing 2,4-D concentration. The biggest callus was obtained on media with highest concentration of 2,4–D.

The result obtained from this study is similar to the results obtained from previous studies. Using this hormone alone will only induce callus formation rather than shoot proliferation. The study reported by Ma *et al.* (2010), leaf explants cultured on medium supplemented with 5.0 µM 2,4-D produced callus after 8 - 9 weeks and the callus was granular and did not exhibit somatic embryogenesis or adventitious shoot formation. However, when these callused explants were transferred to media containing 5.0 µM TDZ or 5.0 µM BAP (or combinations of these) somatic embryos or adventitious shoot formation could be induced.

Effect of different concentration of TDZ

In MS media supplemented with TDZ, most leaf explants were induced to form good callus on the MS media enriched with 0.5, 1.0, and 1.5 mg/l TDZ. In the beginning, callus growth was rather slow and after 4 weeks, the speed of callus formation was improving and small green nodules could be seen. The same result obtained for peduncles and petioles but they were often yield callus in a small size compared to the leaves.

High activities of TDZ in plant regeneration were reported in other ornamental plants such as carnation (Nakano *et al.* 1994) and roses (Hsia & Korban, 1996). However, there were no shoots obtained using this hormones in this study. Observation from previous study indicated the optimal concentration of this hormone to induce shoots formation is between 0.1 – 0.5 mg/l. The study conducted by Nhut *et al.* (2006) using tube-shaped nylon film culture system containing MS media supplemented with 0.2 mg/l TDZ has achieved highest number of regenerated shoots (18.8 shoots/ explants). The result demonstrated a practical use of tube-shaped nylon film culture system in *Sinningia speciosa* shoot regeneration.

Rooting and acclimatization of regeneration plants

Shoots obtained from the shoot induction media did not produced roots hence single shoots of 1.5-2 cm in length were used for *in vitro* rooting to MS basal medium without any addition of hormone. The regenerated shoots were separated manually from their clusters and transferred on a rooting medium. The generated shoots were rooted after 3 weeks. The healthy develop plantlets were taken out from the vessels for acclimatization. The development of acclimatized *S. speciosa* could not be observed as the time provided for this study is limited. From previous study, substantial number of micropropagated plants did not survive in natural environment. The green house and field have substantially lower relative humidity, higher light level and septic environment that are stressful to the acclimatized plants (Pospisilova & Haisel, 1999). Many studies were carried out to standardize the hardening medium for *Sinningia speciosa*. The best protocol yet is by Kasyap & Dhiman (2011) using cocopeat + perlite (3:1) and was found to be the best hardening medium which gave a survival percentage of 79.02. The total duration cycle from leaf explants to complete plantlets was 13 weeks; 8 weeks for shoot initiation and multiplication, 3 weeks for root induction and development and 2 weeks for hardening.

Morphological study

Macromorphological study

The macromorphology study was performed by observing external structures of the plants. There was a significant difference between the size of *in vitro* and *in vivo* plants. The size of *in vitro S. speciosa* was smaller compared to *in vivo* plants. But, this was a normal observation obtained from all *in vitro* plants which might due to several chemical and physical factors.

Result found that the leaves of *S. speciosa* were green in color and it also had a velvety surface. The leaves were 3-13 cm of length and the width is 4-8 cm. The similar characteristics observed in *in vitro* plants but only differed in their size. The size of *in vitro* leaves was 2-6cm and 3-5 cm for length and width respectively. The petioles of *in vivo* plants were short and grew near to the soil surface. The petioles were light green in color, herbaceous and had some tiny hairs surrounding it. Similar characteristics observed on *in vitro* plants but the petioles still undeveloped and it were shorter that intact plants. The root of *in vivo* plants is tiny fibrous roots that were brown in color and the length is measured to 10-15 cm. While, for *in vitro* plants, the roots were tiny and light yellow in color. The length of the roots was approximately 3-5 cm. The flowers of intact plants have 5 petals and were purple in color. It also had a velvety surface and fragile to touch. The sepals were green in color. The peduncles that support the flower formation were light green in color and the length was about 4-10 cm. It

also had tiny hairs surrounding it. These characteristics could not be compared to *in vitro* plants as the flower and peduncles do not develop in cultures.

In general, the size of plants of *in vitro* condition was smaller than *in vivo* condition. These observations were common for all in-vitro plants since their growth is hindered by the size and volume of culture vessels (Conner and Thomas, 1982; Robinson *et al.* 2009).

Micromorphological study

The micromorphology study was conducted to observe the differences in leaves morphology of *in vivo* and *in vitro* plants by the aid of SEM (Scanning Electron Microscopy). From the SEM study conducted on *S. speciosa* leaves, the size of stomata in *in vitro* plant was smaller than *in vivo* plant. This observation was comparable to morphological studies performed on *Murraya paniculata* where they noticed reduced size of stomata in *in vitro* leaves and the stomata were elevated above epidermis but in *in vivo* leaves, the stomata were found to be leveled with epidermis (Mat Taha and Haron, 2008).

Other than stoma, trichomes were also compared between *in vivo* and *in vitro* leaves. From our observation, the upper surface of *in vivo* leaves was densely packed with trichome and fewer amount of trichome observed at the lower part of the leaves. The size of trichomes in *in vivo* leaves was longer consisted of 5 nodes long. While, for *in vitro* leaves, the upper surface of the leaves were covered with trichome but the amount of trichome was slightly fewer than *in vivo* leaves and the amount of trichomes at the lower surface of the leaves was not as much as in *in vivo* leaves. The size of trichome in *in vitro* leaves consisted of 2 to 4 nodes long.

There were many altered characteristics of the *in vitro* regenerated plants have been reported (Hempel 1989). Scanning electron microscopy studies have revealed a considerable reduction or absence of epicuticular waxes on the leaves of plantlets produced in culture compared with greenhouse grown plants (Mat Taha and Haron, 2008). A comparative study of the histological traits of *in vitro* and *in vivo* grown *Dianthus callizonus* plants has revealed numerous anatomical differences, such as reduced deposition of epicuticular waxes, blocked and abnormally positioned stomatal apparatus, abnormally shaped cell epidermis and altered trichome morphology (Paunescu, 2008). In this study, the trichomes of *in vitro* plants were shorter and less dense compared to *in vivo* plants. This was an expected observation since *in vitro* plantlets were grown in very high humidity environment thus eliminating the need of trichome to control transpiration.

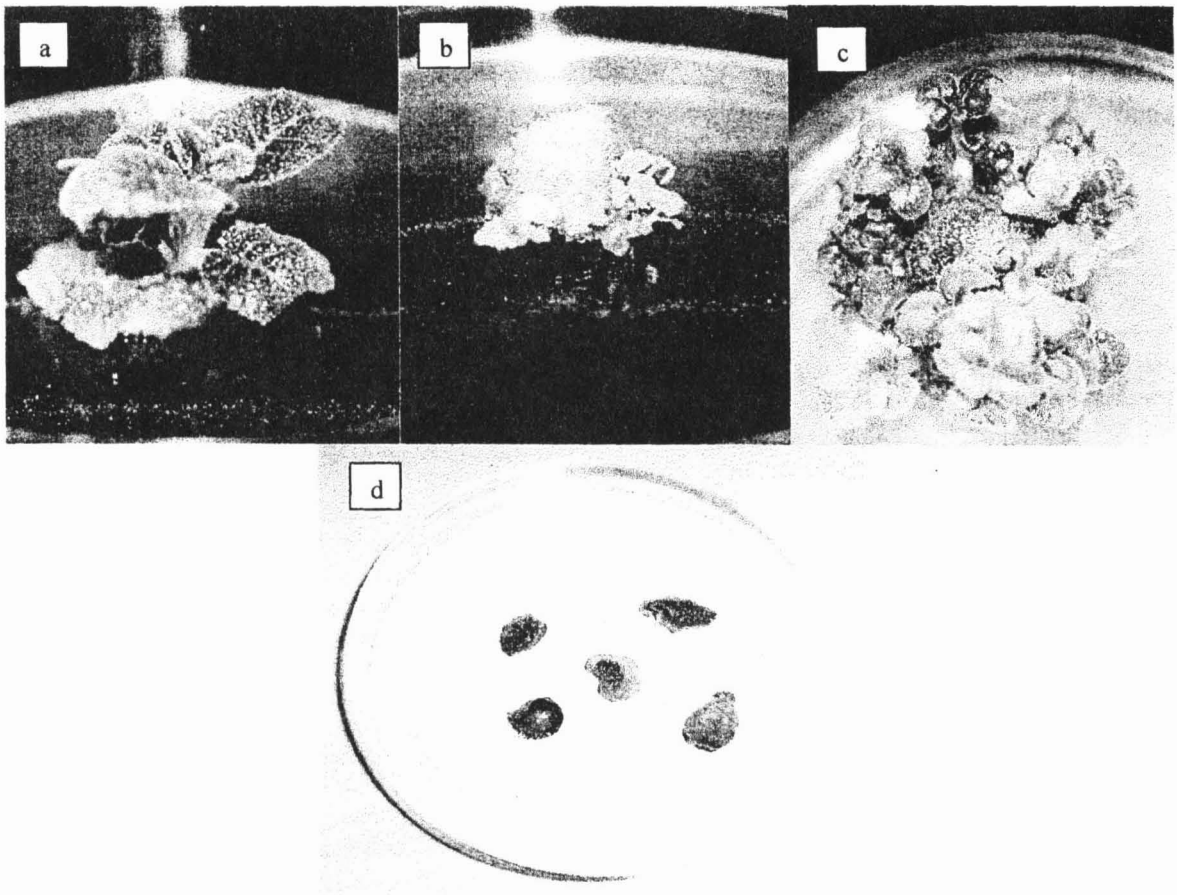

Figure 1: The shoot formation obtained from; (a) leaf explant on MS media supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP, (b) petiole explant on MS media supplemented with 1.0 mg/l NAA and 2.0 mg/l BAP, (c) peduncle explant on MS media supplemented with 2.0 mg/l NAA and 2.0 mg/l BAP, (d) Several callus formation from leaves, petioles and peduncles explants obtained on MS media supplemented with different concentration of 2,4–D and TDZ.

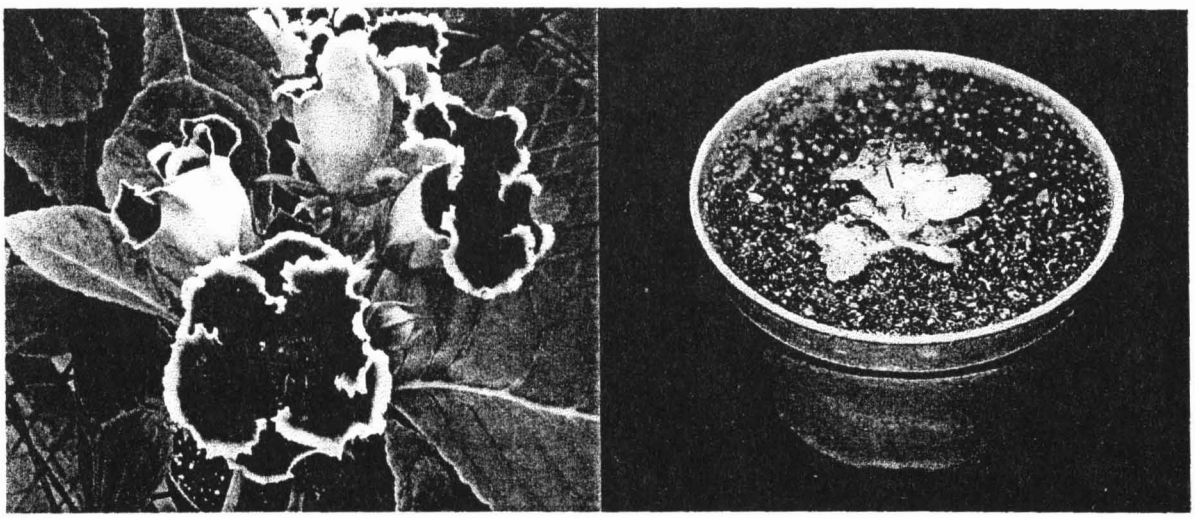

Figure 2: (a) The macromorphology of *in vivo* plant, (b) The macromorphology of *in vitro* plant after acclimatization.

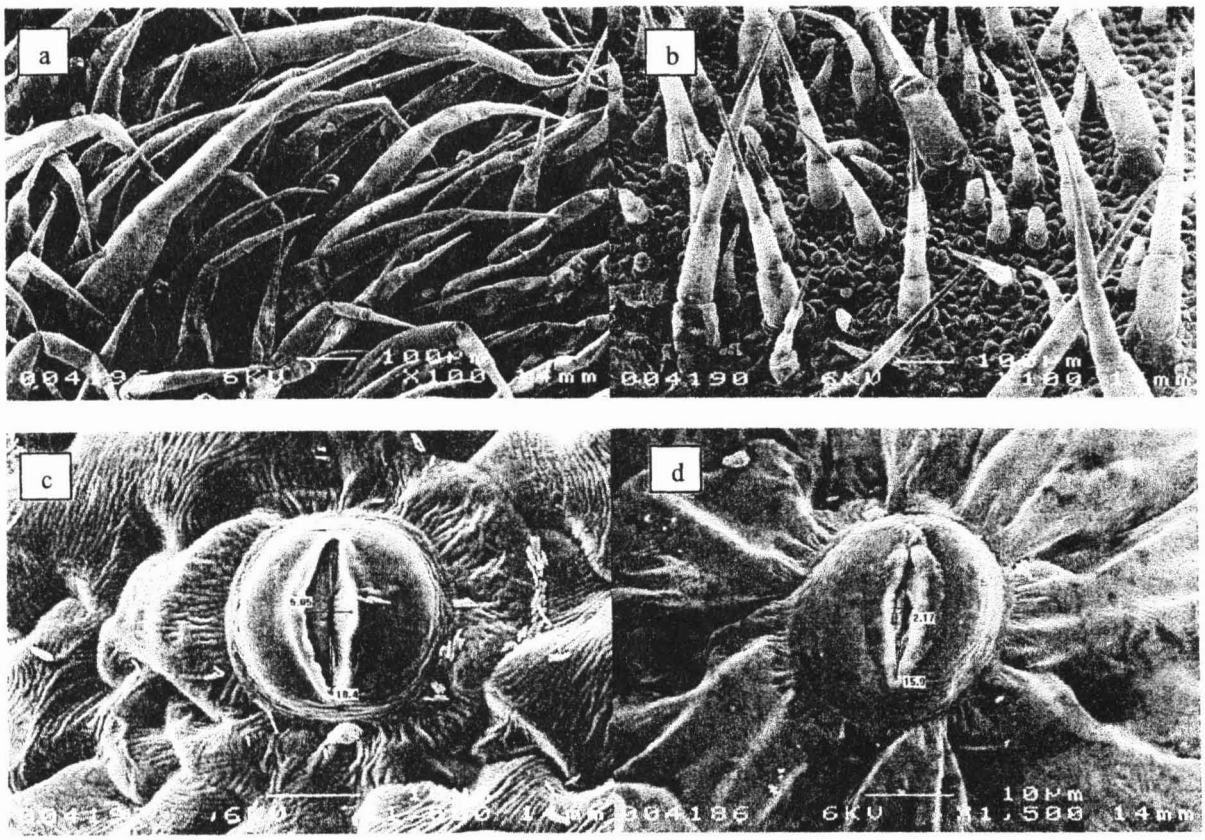

Figure 3: The micromorphology of leaf structures. The structure of trichomes located on the leaf of *in vivo* plants (a) and *in vitro* plants (b). The structure of stoma located on *in vivo* leaf (c) and *in vitro* leaf (d).

Conclusion and Recommendations

In this study, leaves had been recognized to be the best explants in micropropagation of *S. speciosa*. The regeneration of this explant exhibited indirect regeneration pathway in which the shoots proliferated from the intermediary callus. The best medium for shoot proliferation was MS basal medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP with highest number of shoots per explants (6.3 ± 1.3). The healthy young shoots were induced to form roots in hormone-free MS basal media. Complete *in vitro* plantlets were acclimatized successfully under the natural environment. The plants propagated through tissue culture did not show any morphological abnormalities when compared with the parent plants.

For future studies of this plant, some modification can be made to improve the regeneration of *S. speciosa*. Nhut *et al.* (2006) indicated that conventional vessels had some considerable disadvantages negatively affecting the growth and development of plantlets *in vitro*. Hence the used of polyethylene (PE), commonly termed nylon, for making the tissue culture system more progressive. Having the property of good ventilation (high gaseous exchange) and low price made it applicable in commercial micropropagation with some advantages for growth and development compared to conventional micropropagation systems.

Other than that, plants growing *in vitro* in closed vessels have been reported to generate ethylene during cell division thus accumulation of ethylene occurs in the vessel. Ethylene is a gas hormone that is known to play an important role in plant growth and cell division (Kumar *et al.* 1998) and has certain positive effects of callus culture and root growth but largely functions to inhibit growth of shoots. Hence, for enhancing shoot formation ethylene inhibitors can be added to plant media such as $AgNO_3$, aminoethoxyvinylglycine (AVG) and $CoCl_2$ (Biddington, 1992). A study carried out by Park *et al.* (2012) show a significant increase in shoot production of

S. speciosa when $AgNO_3$ added in the MS media. An improved method for the regeneration of intact plant from tissue culture is essential for establishing a multiple micropropagation system and a genetic transformation protocol.

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