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In vitro Propagation of *Anthurium andraeanum* Linn. (White) via Indirect Organogenesis through the Use of Leaf Lamina and Petiole Explants

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Abstract

This research discribes an efficient protocol for the micropropagation of Anthurium andraeanum Linn. with the use of plant growth regulators namely; 2,4-D and BAP through indirect organogenesis of callus formation. Callus induction, callus proliferation, shoot initiation, shoot multiplication and root formation from regenerated shoots were investigated during this research. The multiple segments of youngest and newest leaf laminas and petioles from the adult plant were used as explants in current experiment. Both explant segments gave regenerated plantlets when they were exposed to plant hormones previously mentioned in basal MS full strength medium. The highest callus formation was found in MS medium supplemented with 6mg/L 2,4-D among other concentrations of 2,4-D such as 0.4, 0.5, 0.6, 0.7 and 0.8 mg/L. For multiplication purpose, BAP 3mg/L was the best among other treatments (BAP 1, 1.5, 2, 2.5 mg/L) in the same basal MS medium. Leaving well-developed shoots in the same medium without changing into the fresh medium caused rooting from these shoots. After about three months, the regenerated Anthurium plantlets were successfully transferred to clay pots containing the ratio of (3:2:1) coconut husks, broken bricks and charcoals.

Keywords: *Anthurium andreaeanum*, BAP, 2,4-D, MS medium, callus, leaf lamina, petiole.

INTRODUCTION

Anthurium belongs to the plant family Araceae and order Spathiflorae, and it is one of the most important ornamental plants which comprise of 108 genera and approximately 3750 monocotyledonous species (herbaceous and creepers). *Anthurium* is a perennial herbaceous plant and has been widely cultivated for its long-lasting ability, unusual very attractive heart shaped inflorescence and luxurious and exotic foliage. This inflorescence is composed of heart shaped spathe and finger like structure of spadix protruding from a long flower stalk [1]. The *Anthurium* flower is actually a modified leaf comprising of numerous small botanical flowers on a pencil-like protrusion called 'spadix' and also it has a long vase life of 14-28 days. *Anthurium* plant has been widely grown as one of the popular potted flowering plants among numerous species of such kinds of potted flowers in the tropical and subtropical countries all over the world. Moreover, the potted *Anthurium* flowers are generally valued and traded in the global market due to the attractive resembles renounced and respected status of their beauty [2]. They have been produced for several purposes including cut flowers, potted flowers, interior and exterior decorations and also landscaping.

It is being a tropical flower, *Anthurium* grows best in the day temperature of 25-32˚C and night temperature of 21-24˚C [3]. Among various kinds of other *Anthurium* species, *Anthurium andraeanum* is commercially exploited widely as ornamental plants in many countries throughout the world. They may be cultivated conventionally in three basic propagation methods such as seed propagation, traditional vegetative propagation and plant tissue culture technique. *Anthurium* can be propagated conventionally vegetative way by separating the newly developing plants. But it is time consuming process and it also takes years to develop into a certain amount of commercial quantities of elite clones. Hybrid propagation is also a very difficult aspect because there is a very low number of a new plants development from the base. Seed propagation is also not a very useful technique due to there will be cross pollination and the resulting progenies could be heterozygous. And also *Anthurium* plants are such kind of very poor germination rate plants and the seeds are very low liability as well. They may be propagated either sexually or asexually and most of the cultivated *Anthurium* varieties are found to be self-sterile and always remains as a rare species. Micropropagation technique is an attractive alternative for mass production of highly-prized

cultivars at a faster rate than traditional propagation methods. *Anthurium* has been micropropagated using various tissues as explants such as leaf, petiole, spadix, spathe, seed, lateral bud and shoot tip in recent years [4]. And also, micropropagation is the only mean to get true-to-type plants involved in exploitation as a major trade worldwide. The purpose of this investigation was to develop a simple and successful way of *in vitro* micropropagation protocol for the *Anthurium andraeanum* using leaf lamina and petioles as explant materials for its conservation and future sustainable use.

MATERIALS AND METHODS

A. Source of Explants

Leaf lamina and petioles segments of Anthurium plant were used as the sources of explant materials for this experiment and these materials were obtained from the nursery of Plant Tissue Culture lab, Biotechnology Research Department, Kyaukse Township, Mandalay Region, Myanmar.

B. Explant Collection and Surface Sterilization

The healthy young leaf laminas and petioles were collected and then thoroughly washed with running water first. Plant materials are alive which have been thrived in the natural environment and therefore there may be natural contaminations on their surfaces (sometimes even interiors) with some kinds of microorganisms. So, surface sterilization of explant materials with the aid of certain chemical solutions is vitally important aspect usually using with alcohol, sodium or potassium hypochlorite solution. In most of explants, there is a universally adopted surface sterilization system which use of 70% (v/v) ethanol for 1 minute followed by 0.1% mercuric chlorite solution for 5-10 minutes and then rinsing with sterilized distilled water or treating with 1-10 % sodium hypochlorite solution for 10-20 minutes in order to disinfect the explant materials. Gantait *et al*., reported that the shoot tip explants of *Anthurium andraeanum* varieties were surface sterilized with the use of antifungal solution cetrimide for 5 minutes followed by the use of sodium hypochlorite and 0.1% mercuric chlorite solution [5]. Jahan and his colleagues also reported that 70% (v/v) ethanol for 1 minutes and 1.5% sodium chloride solution for 8 minutes adding with 0.01% Tween-20 also could be used as an effective sterilization procedure [6].

Then, Atak and Celik also found that an effective surface sterilization protocol with the use of 70% (v/v) ethanol for 1 minutes, gentamicin solution for 30 minutes and then 20% (v/v) sodium hypochlorite solution for 12 minutes could diminish the contamination of fungus, endogenous and exogenous bacteria [7].

Here, in this research, the explants were surface sterilized using various kinds of sterilants even including fungicide, antibiotics, etc. and the amounts and time durations for each sterilant were as shown in Table (1).

Table 1: Different concentrations and time durations of sterilizing agents for surface sterilization of *Anthurium* explants

Sterilizing agents	Concentration	Time duration
Detergent	2 ml/L	a few mins
Bavistin	0.6 ml/L	40 mins
Amoxicilin	250 mg/L	30 mins
NaOCl	10 ml/L	10 mins
Ethanol	70 ml/L	1 min

After each step, the explants were washed three times with sterilized distilled water. And then they were cut into appropriate sizes to inoculate into the media.

C. Sterilization of Glassware and Instruments

All the instruments such as scalpel handles, forceps and plates were rubbed with 70% ethanol and then packed with paper covering by plastic bag. The culture bottles were washed with tap water and then soaked with detergent. After a while, they were washed with tap water and rinsed until all the detergent bubbles gone. The glass bottles were dried in the basket till all the water droplets completely gone.

Finally, all the culture bottles and instruments were placed in the autoclave and then sterilized at 121°C for 20 minutes to get rid of any possible contamination.

D. Media Preparation

The basal MS media fortified with four different

concentrations of one kind of auxin hormone, 2,4-D (2,4-Dichlorophenoxy acidic acid) 0, 0.4, 0.5, 0.6, 0.7 and 0.8 mg/L with the supplementation of 3% sucrose and 0.6 % agar were used for callus formation experiment. For the shoot induction, full strength MS media supplemented with various concentrations of cytokinin hormone namely; BAP (Benzyl amino purine) 0, 1, 1.5, 2, 2.5, 3 mg/L, 3% sucrose and 0.6 % agar were used in this study. All the media were adjusted to pH 5.8 using drop by drop of 1N NaOH and 1N HCl solution with the aid of bench top pH meter.

E. Callus Formation

The sterilized leaf lamina and petiole pieces were carefully excised into 0.5 to 1cm segments in the laminar air flow cabinet. The excised explant materials were then inoculated into the culture bottles containing basal full strength MS (Murashige and Skoog) media supplemented with various concentrations of 2,4-D (0.4,0.5,0.6,0.7 and 0.8 mg/L) and without any 2,4-D as a control containing 3% sucrose and 0.6% agar [8]. Two to three explant segments were put into each culture vessel and then they were incubated in the dark side of the growth room. The calli were observed from the excised portions of the explant materials after about six months of the culture period. It took too long to form the callus from the wounded tissues of the explants but after the callus induction period, callus proliferation, somatic embryogenesis and shoot proliferation were very fast in a short period of time. The induced calli were transferred to shoot induction media; MS media containing different concentrations of BAP.

F. Shoot Induction and Shoot Proliferation

The proembryogenic calli were transferred to the MS

media supplemented with various concentrations of BAP, 30 g/L sucrose and 6 g/L agar. The inoculated culture bottles were incubated under continuous fluorescent tube light. After about four to five weeks of culture incubation, the proembryogenic calli developed into numerous shoots. They were transferred into the fresh media respectively every four to five weeks of culture intervals. At this stage, the shoots produced very vigorously and the numbers of the new shoots were nearly double to triple amount from each mother callus.

G. Root Formation

The well developed and elongated shoots produced

roots simultaneously by themselves in the previous shoot formation media containing various concentrations of BAP without addition of any rooting hormone such as NAA, IBA, etc. This condition should be due to the auxin hormones produced by the young shoot tips of the plantlets themselves. Auxins are powerful plant growth hormones naturally produced by plants themselves. They are found in plant organs such as shoot and root tips and they can promote cell division, stem and root developments. So, even the *in vitro* plantlets can produce this kind of plant hormone when it has well developed leaves. Vargas *et al*., previously observed that all plants simultaneously rooted nearly forty days after the emergence of first shoot and there was no particular medium fortified with any kind of plant growth regulator needed [9]. And also rooting occurs more easily without addition of any plant growth promoting substance found out by Somaya *et al*., but their efforts showed

that the supplementation of 0.25 mg/L NAA (Naphthelene acetic acid) into the media could increase the quality and the amount of root produced [10]. In this case, the plantlets bearing fully grown leaves were only transferred to the fresh shoot formation media fortified with BAP and then left about four to six weeks to trigger root development. After rooting appeared on the developing plantlets, they were transplanted into the pots containing certain hardening mixtures for acclimatization stage so far.

H. Culture Condition

Callus formation was carried out under the dark condition at the temperature range of (27˚C to 29˚C). And shoot formation, proliferation and root formation were carried out under the continuous fluorescent tube light at the same temperature above mentioned in callus formation stage. In the primary hardening stage, the well-developed plantlets were kept in the growth chamber of temperature (27˚C), relative humidity (60%) and light intensity (40%).

I. Hardening

For primary hardening period, the culture bottles of

well-developed *Anthurium* plantlets were left open inside the plant growth chamber for about two days before transplantation. Then, the agar from the fully developed plantlets was removed carefully without causing any damage to the soft roots of juvenile plantlets. The plantlets were transferred into the earthen pots containing sterilized coconut husk, broken brick and charcoal pieces (3:2:1) and then they were kept inside the growth chamber in the laboratory controlling the temperature at 27°C, relative humidity at 60% and the illumination at 40% for about two months. For secondary hardening process, the primary acclimatized plantlets were transferred into the nursery which was actually a shade house covering with some kind of plant material products and also kept in this place for a long time because *Anthurium* is one kind of shade loving house plant and also it does not like direct sunlight.

RESULTS AND DISCUSSION

An efficient surface sterilization protocol was achieved for the tissue culture of *Anthurium andraenum* Lin. using the explant materials of leaf lamina and petiole segments from this research. The recent studies had shown that conventional vegetative propagation methods for various *Anthurium* spp. were very difficult to get the new offspring. Furthermore, high contamination aspect could also be a greatest problem to establish a successful method using the application of plant tissue culture techniques [11]. In the present work, only a few contamination and explant dead could be observed when the explants were treated with Bavistin as fungicidal solution, Amoxicilin as antibacterial agent, other disinfactants such as ethanol and sodium hypochloride solution (NaOCl). So the surface sterilization protocol used in this experiment was a very effective method to obtain a successful starting explant material for *Anthurium* tissue culture.

A largest amount of callus formation achieved on basal full strength MS supplemented with 0.6mg/L 2,4-D for both types of explant materials; leaf lamina and petiole segments (95.5% for petiole and 82.9% for leaf lamina) but only a few callus formation observed in concentrations of 2,4-D (0.7 and 0.8 g/L). There was no callus formation in control and a few callus formation in the concentrations of 2,4-D (0.4 and 0.5 mg/L), it may be due to no or very little amount of 2,4-D in the media which could not trigger the initiation of callus.

After the callus development reached a certain amount, they were cut into suitable pieces not too big nor too small, and then transferred into the shoot initiation media MS media containing different concentrations of BAP (1, 1.5, 2, 2.5, 3 mg/L) and without BAP as a control. At that time in the shoot induction media, the callus turned green from creamy yellowish white, and it seemed the development of somatic embryogenesis. Transferring the calli from the callus formation into the shoot media caused shoot development and depression of callus formation. After about four to five weeks of culture incubation in shoot media, the very first shoots were observed in MS media supplemented with all concentrations of BAP. Among them, BAP 3mg/L gave the best result in the frequency of shoot development (4 shoots per plant) and also in the number of leaf produced (9.5 leaves per plant) as compare to the rest of the remaining treatments (Fig. 1). But the longest shoot formation (2.4 cm) could be observed in the MS media fortified with BAP 1.5 mg/L (Fig. 2). The shortest shoot formation and the lowest number of shoot could be found in the concentration of BAP 1mg/L (Fig. 1 and 2).

Maintaining in the shoot media caused simultaneously root formation without any other special auxin like hormone in full strength media. The resulting fully grown shoots bearing roots were separately transferred into the full strength MS media without any plant growth regulator. It could trigger the plantlets to form steadily root development and also made the plantlets strong and healthy. Geier (1986) observed that leaf explants of *Anthurium scherzeranium* produced root relating with the extension of shoots and leaflet development, the larger shoots with more prominent leaflets forming roots quicker [12]. In this experiment, *Anthurium andraeanum* also gave the quicker root on the larger shoots with very prominent leaflets. After about six to seven weeks in the MS media, the micropropagules were transferred into the earthen pots containing coconut husks, broken bricks and charcoal pieces (3:2:1) for acclimatization process. In this case, the tissue culture *Anthurium* plants we got had been never used any kind of chemical-fertilizer or bio-fertilizer and blooming substances. After about one year later, the *Anthurium* plantlet was fully grown up into the adult plant stage and it produced the very unique, elegant white colored spathe protruding pink colored spadix as shown in Figure (4).

Table 2: Embrogenic callus formation of two types of *Anthurium* explant materials

Explant materials	Percentage of Embryogenic Calli	Type of Calli
Leaf lamina	82.9%	Yellowish white, compact
Petiole	95.5%	Yellowish white, compact,

The data was based on four replicates for each type of explant.

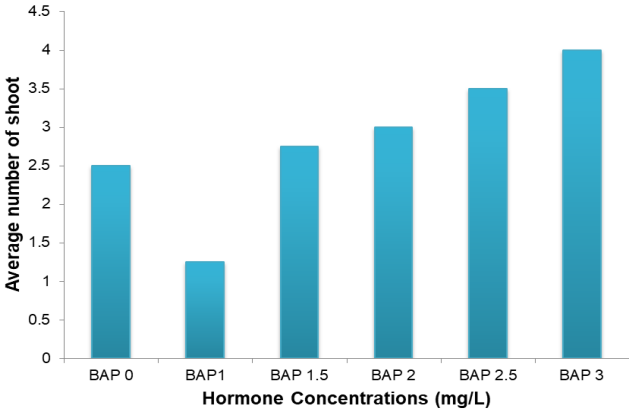


Figure 1: Effect of different concentrations of BAP (0, 1, 1.5, 2, 2.5, 3 mg/L) on the average number of shoot after one month of culture period in shoot formation media

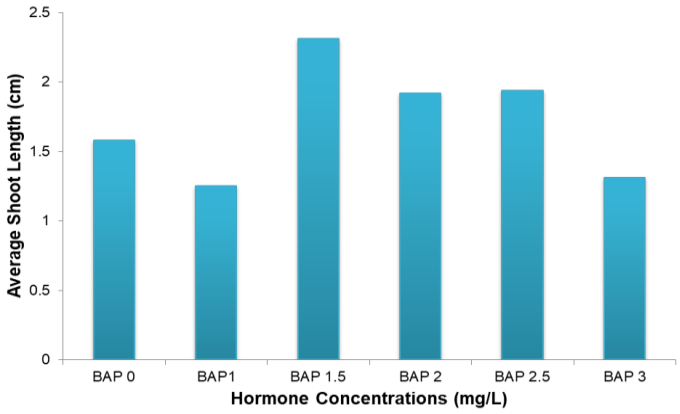


Figure 2: Effect of different concentrations of BAP (0, 1, 1.5, 2, 2.5, 3 mg/L) on the average shoot length after one month of culture period in shoot formation media

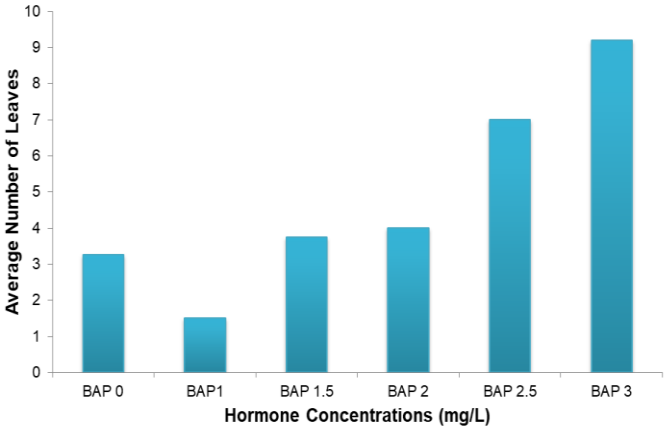
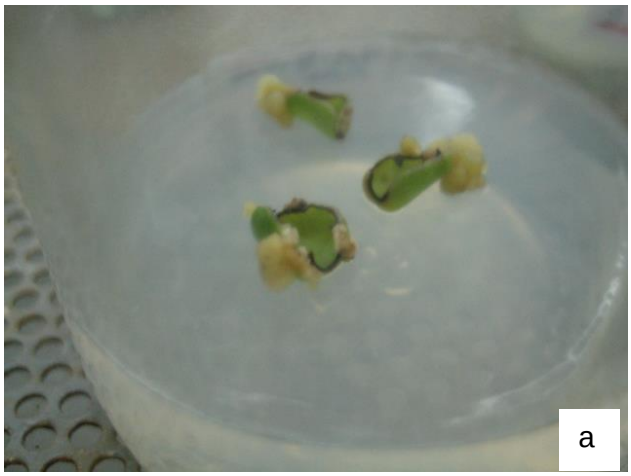
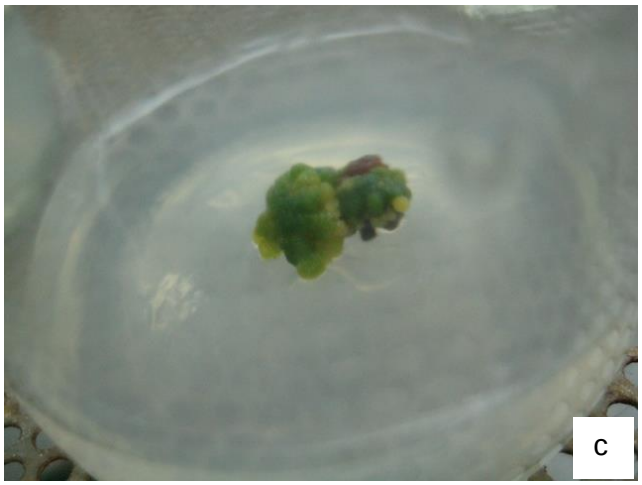
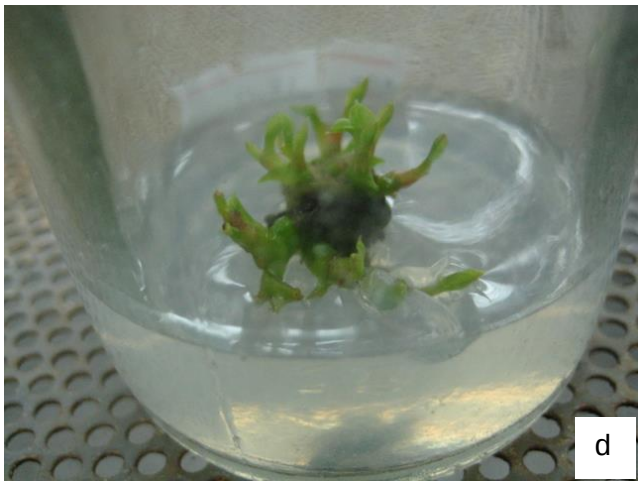


Figure 3: Effect of different concentrations of BAP (0, 1, 1.5, 2, 2.5, 3 mg/L) on the average number of leaf after one month of culture period in shoot formation media







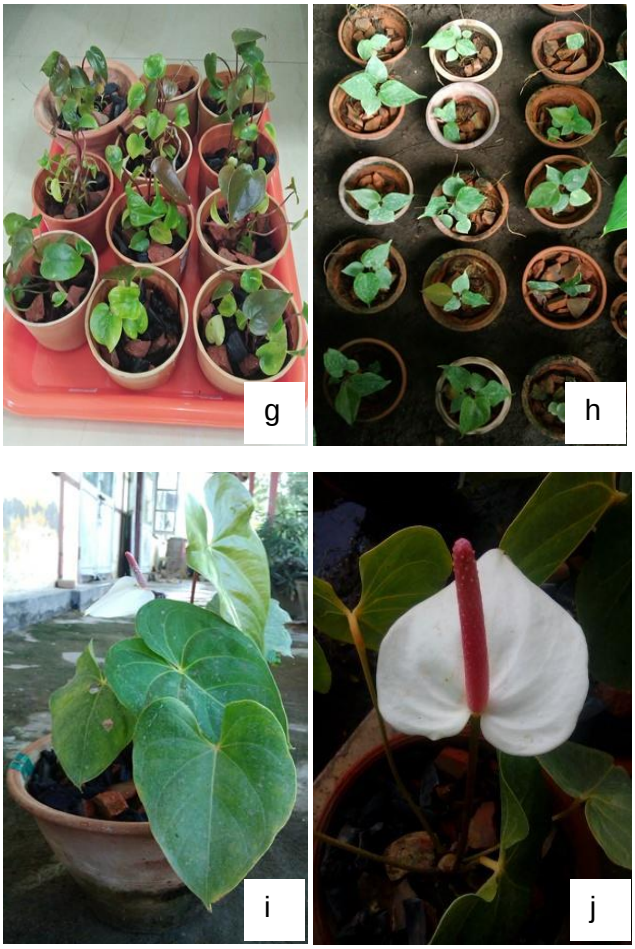


Figure 4: Micropropagation of *Anthurium andraeanum* Lin. through the use of leaf lamina and petiole segments as explants materials; (a) Callus induction from the excised leaf lamina explant, (b) Callus development from the petiole segment, (c) Proembryogenic callus formation, (d) Indirect organogenesis of shoot development from the proembryogenic callus, (e) Multiple shoot formation with a little root development, (f) Rooting from the developing well-grown shoot, (g) Primary hardening stage, (h) Secondary hardening stage, (i) Adult young *Anthurium* plantlet bearing large elegant white flower, (j) A beautiful unique white *Anthurium* flower

CONCLUSIONS

The callus formation was found in full strength MS media supplemented with the range of 0.4 to 0.8 mg/L concentrations of 2,4-D but the best result was achieved in 2,4-D 0.6mg/L in both type of *Anthurium* explants such as leaf lamina and petiole segments. Shoot development was occurred in full MS media alone and also full strength MS media supplemented with different concentrations of BAP (1, 1.5, 2, 2.5 and 3 mg/L). The highest number of both shoot and leaf development were observed in MS media supplemented with BAP 3mg/L. But for the elongation of shoot, BAP 1.5mg/L gave the best result among other remaining treatments. The rooting process occurred spontaneously from the well-developed shoots in the shooting media and there was no requirement of any phytohormone. The good production of roots facilitates the adaptation of plantlets to *ex-vitro* conditions under controlled environment. The healthy plantlets with strong roots were primary acclimatized in the plant growth chamber and the successive secondary hardening process carried out in the net house. After about one year in the net house, the tissue culture derived *Anthurium* plantlet grown up into adult plant and bloomed very beautiful, gorgeous white flowers.

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