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Micropropagation of *Anthurium spp.*

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1. Introduction

Micropropagation as an alternative method to conventional propagation, the culture of somatic cells, tissues and organs of plants under controlled conditions is a suitable way to produce a large number of progeny plants which are genetically identical to the stock plant in a short time. The important property of the plant cells is totipotency which is a capacity to produce the whole plant from different plant parts. Micropropagation has some features to be chosen in commercial production such as multiplicative capacity in a relatively short time, healthy and disease-free production capacity and ability to generate population during a year [1-5].

The genetic pattern of the plant is key element to select the propagation method. Using micropropagation techniques in plant biotechnology applications are costlier than conventional propagation methods. Propagation by using *in vitro* techniques instead of conventional methods offer some advantages like utilizing small pieces of plants called as explants to maintain the whole plant and increase their number. The main point is to evolve new strategies to lower the time and cost consumed per plant [2,3]. In tissue culture applications selection of initiating material is important in the beginning of the culture. Therefore it is easy to provide virus-free clones in a short time. Production of plants during all year long independent of seasonal changes, long storage periods make micropropagation preferable to propagate plants in short time. There are also some disadvantages of micropropagation. Adaptation of cultured plants to the environmental conditions need transitional period to allow the plants to produce organic matter by photosynthesis [2,4].

The main methods of *in vitro* propagation can be classified in two groups:

1. Propagation from axillary or terminal buds
2. Propagation by the formation of adventitious shoots or adventitious somatic embryos

The meristem and shoot tip cultures are used to establish virus-free plant culture. Many important horticulture crops were propagated by meristem culture for rapid growth and

virus elimination. Adventitious shoots or adventitious somatic embryos are established directly or indirectly. Cultures are directly started with the excised explants from the mother plant tissues for organogenesis or embryogenesis. If shoots or embryos regenerate on previously formed callus or in cell culture, they are called as indirect organogenesis or embryogenesis [3,6,7].

When propagation occurs via an indirect callus phase, the genetic identity of the progenies decreases. This is an important problem in commercial propagation to affect the uniformity of progenies. Callus formation also increases the somaclonal variation. Increasing of somaclonal variation incidence is a crucial result of long term period of callus growth. Origin of the callus also causes somaclonal variation.

Propagation from axillary or terminal buds is the most ensurable method to have the highest genetic stability during *in vitro* propagation of plants.

George et al. [2008] described five stages of micropropagation which are mother plant selection and preparation [Stage 0], *in vitro* culture establishment [Stage 1], shoot multiplication [Stage 2], rooting of microshoots [Stage 3] and acclimatization [Stage 4]. These stages are necessary for a successful micropropagation.

Establishing aseptic culture conditions can be classified as Stage 0 which contains pre-surface sterilization applications of explants to reduce contamination of stock plants. The success of Stage 2 depends on different factors such as plant species, cultivar or genotype, plant growth regulators, the ingredients of the medium and physical culture conditions. Stage 3 is responsible of rooting of microshoots. It depends on the factors given in Stage 2. Transplantation of rooted shoots to the environment is the main step of Stage 4. This is also the important part of micropropagation. Acclimatization needs to be well controlled to avoid loss of propagated plants [4,5].

2. Propagation of *Anthurium*

The commercial production of ornamental pot plants has a great potential in international markets. In the global market, *Anthurium* cultivars with valued flowers are the second beside the Orchids among tropical cut flowers. *Anthurium* species and hybrids in Araceae family have an importance in monocotyledonous ornamental plants and they are commercially produced as cut flowers and potted plants in tropical and subtropical countries [2, 8-12].

The propagation rate of *Anthurium* by seeds is very low and it is not recommended. The cultivation has also been limited because of the inherent heterozygosity. The time between pollination and seed maturity and the development time take three years in a breeding program. To grow plants from seed may not provide a practical method of making new planting areas, in such circumstances vegetative propagation [stem cutting] seems the only way of multiplying a unique individual. Propagation method selection for a plant depends on its genetic potential and its intended use. Stem cutting methods are also not practical to propagate in large scale. Today *Anthurium* can be multiplied in large number by

micropropagation. Application of biotechnology on *in vitro* propagation of *Anthurium* is important to increase the productivity of *Anthurium* [3, 6, 13].

2.1. Tissue culture of *Anthurium*

In vitro propagation methods have several advantages over conventional propagation like flexible adjustment of factors affecting regeneration such as explant type, nutrient and plant growth regulator levels and conditions of the environment, production of clones in desired rate, continued production during seasonal changes using tissue culture methods also increase the multiplication rate of plants [14].

Explant type

The success of tissue culture is related to the correct choice of explants. Shoot or shoot tips and node cultures are the most commonly used culture types in micropropagation of plants. Explants from shoot tips and nodal stem segments are suitable for enhanced axillary branching. *Anthurium* micropropagation from axillary buds, shoot tip, lamina explants, node, petiole, and microcuttings have been successfully utilized [15-18]. Among these plant parts, leaves are the most used explant source in *in vitro* culture of *Anthurium*.

The genotype of *Anthurium* plays an important role in *in vitro* propagation. The studies showed that different genotypes had different responses to the same culture conditions. For this reason, it is necessary to establish a suitable procedure for each varieties of *Anthurium* that can be adapted to commercial production [3,4,19,20].

Selection of explant type to induce callogenesis and organogenesis is important for plants. In direct and indirect organogenesis studies, using young leaf explants are important for the success of culture. Martin et al.[2003] observed higher number of shoots in the brown young lamina explants than young green lamina. Viégas et al.[2007] also indicated the importance of using new brown leaves for callus induction. Bejoy et al.[2008] reported that the explants excised from pale green leaves showed better callus development than pale brown leaves. Atak and Çelik [2009] also used young brown and green leaves of *Anthurium andreanum* to evaluate the effectiveness of callus formations. They achieved to decrease the callus formation time by using brown leaf explants and induced the callus formation percentage 50% more than performed by green leaves.

Establishing aseptic culture

The second important step in micropropagation is to obtain aseptic culture of plant material. Aseptic culture systems are effective to eradicate the bacterial, fungal and insect contaminants. The sterilization protocols used for different *Anthurium* explant sources were given in Table 1. NaOCl is the main disinfection material used in establishing aseptic culture conditions of *Anthurium*. NaOCl has been used for the concentrations differ from 1%-5% [Table 1]. The incubation times of the explants in sodium hypochloride showed differences due to its concentrations. There is also need to used extra disinfectant solutions to eradicate the fungal and bacterial contaminants. Benomyl [commercial name

is Benlate], Cetrimate, gentamicin and streptomycin sulphate are effectively used for this aim [11,13,15,18,20,22].

A. species	Explant Source	Sterilization method	Reference
<i>A.andreanum</i>	Leaf	0.6% Benlate [30 min]+70% ethanol [30 sec]+1.5% NaOCl containing two drops of Tween 20 [20 min]	[15]
	Leaf	0.1% HgCl ₂	[19]
	Leaf	70% ethanol [1 min]+gentamicin [30 min]+20% [v/v] commercial bleach [5% NaOCl,12 min]	[20]
<i>A.andreanum</i> L.	Apical shoot buds	Teepol+ antifungal solution Cetrimate [5min.] +NaOCl [5 min]+ 0.1% [w/v] HgCl ₂ [5min.]	[11]
	Spadices	Washing under running tap water [30-60 min]+1% pesticide solution of 50% benomyl and 20% streptomycin sulphate [30 min] +5 times distilled water [5min each rinse.]+ 1% NaOCl [10 min] + 2% NaOCl [5min] +80% alcohol [30s.] +5-6 times distilled water [5min each rinse.]	[22]
	Leaf and spadix Segments	Washing under running tap water [30 min]+0.5% [v/v]Trix [Commercial detergent]+70% ethanol [1 min.] + 1.5% NaOCl containing 0.01% Tween 20 [8 min]	[23]
<i>A.andreanum</i> cv Rubrun	Seeds from plant spadix	1%NaOCl [20 min]	[7]
	Separate fruits from spadix	3% [v/v]NaOCl [15 min]+3 times distilled water [5min.]	[18]
	Isolate seeds	1% [v/v]NaOCl [20 min]+2 times distilled water [10min.]	
<i>A.andreanum</i> Hort	Lamina segments	5% [v/v] Extran [5 min. with detergent]+0.1% [w/v] mercuric chloride [10-12 min]	[1]
	Leaf	15% [v/v] commercial bleach [20 min]+0.1%HgCl ₂ [7 min]	[21]

Table 1. Sterilization methods used in *Anthurium* tissue culture

Culture medium

Culture medium influences the propagation efficiency in plant tissue culture applications. Organic compounds, vitamins and plant growth regulators are used to stimulate healthy growth. The rate of tissue growth and morphogenetic responses highly affected by the features of nutrients included.

There are several basal media such as Chu [N₆] [24], Gamborg's B5 [25], Murashige and Skoog [MS] [26], Murashige and Tucker [MT] [27] and Nitsch and Nitsch [NN] [28]. These media are successfully used for establishing tissue cultures of different explants of various plants [22].

In plant tissue culture studies, different combinations of every medium based on different concentrations of macro and micronutrients have been used to develop efficient protocols. The rapid and efficient tissue culture protocols are important for micropropagation of *Anthurium* as much as in other plants.

The success of plant tissue culture depends on the composition of the medium used. Different combinations of macronutrients as nitrogen, potassium, calcium, phosphorus, magnesium and sulphur and micronutrients [trace elements] as iron, nickel, chlorine, manganese, zinc, boron, copper and molybdenum change the nature of the medium.

Each plant species has its own medium composition or it should be improved for better results. The modifications can be made up in macro and micronutrients, sugar content, plant growth regulators, vitamins and other nitrogen supplements.

MS media with some modifications have been frequently applied in tissue culture of *Anthurium*. The differences caused by using different concentrations of plant growth regulators in combination with MS organics used to obtain desired tissues [Table 2].

Nitrogen is an essential macronutrient in plant life. It is an important component of proteins and nucleic acids. Nitrate [NO₃⁻] is the main source of nitrogen. NO₃⁻ is reduced to ammonium [NH₄⁺] after uptake. Plants have ability to use the reduced form of nitrogen for their metabolism. Nitrate uptake happens effectively in an acidic pH. But after nitrate uptake, the medium are becoming less acid. When ammonium uptake, it makes the medium more acidic. The pH of the plant culture media is important because in a buffered media, existence of both ions affects efficient nitrogen uptake. The form and the amount of nitrogen in media have significant effects on cell growth and differentiation. pH controlling in the media is not the only reason of using both ions, excessive ammonium ions are toxic to the plants. Media containing high levels of NH₄⁺ also inhibits chlorophyll synthesis [4].

It has been known that the root growth is induced by NO₃⁻ and reduced by NH₄⁺. But morphogenesis is being controlled by total amount of nitrogen in the medium and it needs both of NO₃⁻ and NH₄⁺. Because of using optimum NH₄⁺: NO₃⁻ has a key role in morphogenesis, therefore the balance between NO₃⁻ and NH₄⁺ differs for different plants and different kinds of cultures. This situation implies that this ratio should be specifically adjusted for each plant species and for different purposes. Changing the NO₃⁻ to NH₄⁺ ratio by small alterations affects differentiation and growth.

<i>Anthurium species</i>	Explant source	Medium components	Aim	Reference
<i>A. andreanum</i>	Leaf	MS+2.2-4.4µM BA+0.9µM 2,4-D	Adventitious shoots	[33]
	Root	Modified MS+2.2µM BA	Multiple shoots	[34]
	Leaf	Modified Nitsch [200mg/l NH ₄ NO ₃] +1mg/l BA+0.1mg/l 2,4-D	Callus initiation	[15]
		Nitsch [720mg/l NH ₄ NO ₃] +0.5mg/l BA	Shoot development	
		Nitsch [720mg/l NH ₄ NO ₃] +1.0mg/l IBA+0.04% AC	Roots	
	Leaf	½MS+0.6mg/l 2,4-D+1mg/l BAP	Callus induction	[20]
		½MS+250mg/l NH ₄ NO ₃ +0.1mg/l 2,4-D+1mg/l BA	Shoot regeneration	
		½MS+1mg/l IBA+0.04% AC	Roots	
	Leaf, spadix	¼MS+1mg/l BAP	Multiple shoots	[23]
		¼MS+1mg/l IBA	Roots	
	Seed	MS+2mg/l BA+0.5mg/l NAA	Callus proliferation	[18]
	Petiol	½MS+0.1mg/l 2,4-D+0.5 mg/l BA	Callus	[36]
		½MS+0.1mg/l 2,4-D+1.0 mg/l BA	Shoot	
		½MS+0.5mg/l 2,4-D	Root	
<i>Anthurium ssp.</i>	Leaf	½MS+1mg/l BA+0.08mg/l 2,4-D	Callus induction	[19]
		½MS+1mg/l BA	Callus multiplication	
		½MS[206 mg/l NH ₄ NO ₃] +1mg/l BA	Shoot regeneration	
		¼MS+1g/l AC	Roots	
<i>A. andreanum</i> André cv.	Leaf, petiole	Modified Pietrik medium+0.36µM 2,4-D+4.4µM BA	Callus	[32]
<i>Anthurium andreanum</i> cv Rubun	Microcutting from germinated seed	MS+4.4µM BA+0.05µM NAA	Multiple shoots	[7]
<i>A. andreanum</i> Lind.	Apical shoot bud	MS+0.1mg/l NAA+0.25mg/l BAP	Multiple apical shoots	[11]
		MS+0.5mg/l BAP+60mg/l adenine sulphate	Multiple shoots	

<i>Anthurium</i> species	Explant source	Medium components	Aim	Reference
		MS+0.5mg/l IAA+2g/l AC	Roots	
	Half anther culture	NWT+0.25mg/l 2,4-D +0.02mg/l NAA+1.5mg/l TDZ + 0.75 mg/l BAP	Callus Shoot regeneration	[22]
		NWT+ 0. 2mg/l NAA+1.0 mg/lKIN	Roots	
<i>A.andreanum</i> Lindl.cv.	Nodal segments	MS+4.44mM BAP+2.89mM GA ₃	Shoot induction	[16]
<i>A.andreanum</i> Hort	Lamina	½MS+1.11µM+BA+1.14µM IAA+0.46µM Kin	Shoot induction	[1]
		½MS+0.44µM BA	Multiple shoots	
		½MS+0.54µM+NAA+0.93µM Kin	Roots	
	Leaf	¼MS+0.88µM BA+0.9µM 2,4-D+0.46µM Kin	Callus	[35]
		¼MS+0.88µM BA+0.54µM NAA+0.46µM Kin	Multiple shoots	
		½MS+0.54µM NAA	Roots	
		½MS+0.5mg/l 2,4-D+1mg/l BAP	Adventitious shoots	[21]
	Leaf, petiole	½MS+0.90µM 2,4-D+8.88µM BA	Callus induction	[17]
		½MS+0.90µM 2,4-D+4.44µM BA	Callus proliferation	
		MS+5.71mM NAA	Roots	
<i>A.scherzerianum</i>	Leaf	½MS+0.08mg/l 2,4-D+1mg/l BAP+1mg/l 2-iP	Callus	[10]
<i>A.scherzerianum</i> Schott	Leaf	MS+0.5mg/l BAP Modified MS+2.5 mM NH ₄ NO ₃ +18µM 2,4-D+6% sucrose	Shoots Embryo induction	[6]

BAP, 6-benzylaminopurine; BA, N⁶-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid, IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2-iP, N⁶-[2-isopentenyl]adenine; Kin, kinetin; NAA, α-naphthalene acetic acid; TDZ, thidiazuron; AC, activated charcoal; AS, adenin sulphate; MS, Murashige Skoog [1962] medium; WM: Winarto-Teixeria medium, NWM: New Winarto-Teixeria medium GA₃, giberellic acid.

Table 2. *In vitro* culture medium components for *Anthurium* cultivars (Modified from [22]).

MS media used frequently for tissue culture of *Anthurium* and the ratio of NO₃⁻ to NH₄⁺ is 66:34 at this medium. For this reason generally modified MS medium used at *Anthurium* organogenesis. The modifications of ammonium nitrate concentration have been studied at *Anthurium* media by researchers. Hamidah et al. [1997] used half-strength MS

macroelements with 2.5 mM ammonium nitrate for *in vitro* stock cultures. While Puchooa [2005] used 200 mg/L reduced ammonium nitrate concentration for callus culture, they increased the amount to 720 mg/L for regeneration. Dufour and Guérin [2005] used different compositions of NO_3 and NH_4 to evaluate the developmental results. According to their results, the ratio of 0.37 showed better plant growth and development. Atak and Çelik [2009] preferred to use half-strength MS salts with NH_4NO_3 lowered to 250 mg/L for shoot regeneration. Winarto et al. [2011] were improved a protocol for callus induction and plant regeneration and NWT-3 media contains 750 mg/l NH_4NO_3 .

In culture conditions, using synthetic chemicals with similar physiological activities as plant hormones have capabilities to induce plant growth as desired. Auxin and cytokinins are the most important hormones regulating growth and morphogenesis in plant tissue culture. Their combinative usage promote growth of calli, cell suspensions, root and shoot development and have capability to regulate the morphogenesis [4,29]. There are synthetic auxin and cytokinins beside naturals. Different combinations and concentrations of plant growth regulators such as 2,4-dichlorophenoxyacetic acid [2,4-D], naphthalene acetic acid [NAA], benzylaminopurine [BAP] and kinetin [Kin] were used to indicate callus formation from different kinds of explants of *Anthurium* cultivars. In preliminary studies, induction and regeneration of callus followed by shoot and root regeneration are the main steps of tissue culture of whole plants. As an important commercial plant, to develop a rapid and more effective tissue culture protocol to shorten the time is the main objective of *Anthurium* tissue culture [7,10, 22,23].

As given in Table 2, combination of 2,4-D and BA in culture media to induce callus initiation from leaf explants in different varieties of *Anthurium* is frequently used. Also, adding of BAP and 2-iP to the callus medium has been evaluated by different researchers. The concentrations of 2,4-D used in the callus medium is ranging from 0.08 mg/l to 1 mg/l 2,4-D. The BA concentrations are changing between 0.1 mg/l and 1 mg/l.

Micropropagated plants require a developed root system to resist the external environmental conditions. Rooting of the shoots take place *in vitro*. Therefore, determination of the appropriate auxin type and levels in the media required to promote rooting [4].

Activated charcoal [AC] is added to medium for promoting root growth [11, 13, 15, 19, 20]. AC is composed of carbon and it is often used in plant tissue culture to absorb gases and dissolved solids. It is not a growth regulator but it has an ability to modify medium composition [4].

There are several advantageous uses of charcoal on the type of culture. These are adsorption of secreted compounds from cultured tissues, decrements in the phenolic oxidations, pH changes of the medium to optimize for morphogenesis, prevention of unwanted callus growth, simulation of soil conditions because of the ability to promote root formation, capability to use in production of secondary plant products in culture conditions [4, 30].

The most important effect of using AC to the medium is the rigorous decrease in the concentrations of plant growth regulators and other organic supplements. AC shows greater adsorptive capacity to phenolics commonly produced by wounded tissues, plant hormones like IAA, NAA, IBA, BA, kinetin, zeatin and other hormones [30,31]. The adsorptive property of AC changes with purity, pH and density [3]. The *Anthurium* seedlings propagated by Atak and Çelik [2009] were rooted in medium containing AC and given in Figure 1.



Figure 1. *In vitro* propagation of *Anthurium* cultivars [Arizona]. The shoots with root were growth inplant tissue culture medium with AC [20].

3. The importance of subculturing in micropropagation

In plant propagation applications, subculturing has an importance to prolong the life of plants and expand the number of cultured seedlings. At *in vitro* propagation of *Anthurium andreanum* cultivars, the number of shoots per explants was increased subsequent subcultures. Atak and Çelik [2009] observed that shoot multiplication for two *Anthurium andreanum* cultivars Arizona and Sumi was increased in the next multiplication stage. At every subculture, shoot numbers regenerated form nodal explants gradually increased [Table 3]. Bejoy et al.[2008] reported that multiplication was enhanced in the next multiplication stage. They succeeded to increase the rate of shoot production in the second multiplication stage.

Subculture	Arizona		Subculture	Sumi	
	Number of Explants	Number of Shoots per Explant [Mean±SE]		Number of Explants	Number of Shoots per Explant [Mean±SE]
Va₁	50	15.64±1.69 ^a	Vs₁	50	12.24±1.18 ^a
Va₂	50	22.70±1.46 ^b	Vs₂	50	15.98±1.36 ^b
Va₃	50	26.76±1.30 ^c	Vs₃	50	21.82±1.87 ^c
Va₄	50	33.70±1.09 ^d	Vs₄	50	26.96±1.46 ^d

Data presented as means with different letters within a column indicating significant differences at $P < 0.05$ according to Duncan's Multiple range test. Each mean represented 5 replications.

Abbreviations: **Va₁** = initial shoot [Shoot regeneration from callus cultures of Arizona variety] **Va₂**, **Va₃**, **Va₄** = subcultures of Arizona variety, **Vs₁** = initial shoot [Shoot regeneration from callus cultures of Sumi variety], **Vs₂**, **Vs₃**, **Vs₄** = subcultures of Sumi variety.

Table 3. Shoot multiplication of *Anthurium andreanum* cultivars [20].

4. Acclimatization

In micropropagation studies, the last and the critical step is acclimatization of the rooted seedlings to the environment. In this stage, plant losses have been due to different reasons [37,38]. Directly rooted shoots in soil show higher survival rate in the field than rooted under *in vitro* conditions. Therefore, there are several methods to high the survival rate of *in vitro* rooted shoots.

Cultured plants must adapt to low humidity, high light intensities and large temperature fluctuations with *ex vitro* acclimatization techniques. However these methods are expensive, time consuming and labor-intensive, *in vitro* acclimatization techniques have been improved. Using growth chambers which have relative humidity, controlled ventilation and possibility to change the components of the media make it possible to reduce the steps need for the process [37,39].

The success of acclimatization of *in vitro* cultured plants depends on the nutrients reserved in the leaves during development [40]. The important point in acclimatization is to keep the rooted plants in incubator in order to keep the humidity high.

Different acclimatization protocols for *in vitro* regenerated *A. andreanum* plantlets have been reported. Soilrite-perlite with the rate of 10:1, vermicompost and sand mixture [1:3], vermiculite and perlite [1:1], soil and organic humus [1:1] are the most used acclimatization mediums with the high survival ratios ranging from 60% to 98% [1,10,13].

During *in vitro* development stage, the cultural conditions such as humidity, air turbulence, CO₂ concentration, sugar content in medium effect acclimatization ability of plants to *ex vivo* conditions. Therefore, for each *Anthurium* varieties, efficient acclimatization protocols have to be improved to prolong the success of micropropagation.

5. Discussion and conclusion

In micropropagation studies, the success of the protocols depends on the variety of *Anthurium*, explant type, the components of the media used for shoot and root regenerations. Different combinations of plant growth regulators and additives used in relation to increase the regeneration potential of the explants should be evaluated for each cultivars to determine the efficient tissue culture protocol. In this chapter, we compared the explant types and tissue culture components for *Anthurium* species which is an important ornamental pot plant.

Stages of the leaves show different response to propagation by indirect organogenesis. Explants prepared from brown leaves have higher callus formation rates in a shorter time than green leaf explants. Therefore, selection and using the right leaf explants at the appropriate leaf stage is the first step of establishing a successful tissue culture. Using different combinations of plant growth regulators and nitrogen additives should be evaluated to control the organogenesis for *Anthurium* varieties. $\text{NO}_3^- : \text{NH}_4^+$ balance in the growth medium has to be adjusted for each *Anthurium* varieties to obtain desired differentiation and growth. Developing an ideal acclimatization condition is important to increase the survival rate of micropropagated and rooted seedlings to adapt to *ex vivo* conditions.

In conclusion, the primary point to be remembered is the effects of genotypical differences on culture efficiencies. Different genotypes of varieties show different organogenesis responses in explant cultures. Therefore for each *Anthurium* varieties suitable micropropagation methods should be determined.

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