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Indirect Shoot Regeneration in *Anthurium andreanum* 'Clisto' from Leaf Explant

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Abstract

Anthurium is commercially produced as cut flowers and potted plants and known for its colorful spathes. It is traditionally propagated by stem cutting and suckers, so micropropagation is an alternative production method. In this experiment we carried out shoot regeneration in *Anthurium andreanum* 'Clisto'. The leaf explant was used and after sterilization, they cultured on two different media (half-strength MS and Nitsch's media) which supplemented with different compositions of 2,4-D (0, 0.25, 0.5 and 0.75 mg/L) and BA (0, 0.5, 1 and 1.5 mg/L). After three months callus size and percentage of explant that produced callus were measured and callus transferred to MS media with different concentrations of BA (0, 0.25, 0.5 and 0.75 mg/L). After 6 months, number of shoot, shoot length and leaf number was recorded. The highest callus proliferation was obtained in Nitsch' media supplemented with 0.25 mg/L 2,4-D and 0.25 or 0.5 mg/L BA. The callus was creamy color and compact. The highest shoot regeneration was observed in media with 0.75 mg/L BA. We cloud achieved 31 shoot per callus segment in the best treatment. Rooting was easily performed in peat: perlite (1:2) pots and they well acclimated and transferred to greenhouse and 99% plants were survived.

Keywords: *Anthurium,* BA, Shoot multiplication, Tissue culture, 2,4-D.

INTRODUCTION

Anthuriums are known as an exotic ornamental crop. The commercial flower is a combination of a colorful modified leaf, termed spathe, subtending bisexual flowers carried in a spiral fashion on a spadix (Kuehnle *et al.*, 2001). *Anthurium* species and hybrids belongs to 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 and also in the global market. *Anthurium* cultivars with valued flowers are the second beside the Orchids among tropical cut flowers (Dufour and Guerin, 2008; Gantait and Sinniah, 2011).

Anthurium is conventionally propagated by seeds and, this method is associated with some problems due to the inherent heterozygosity. *Anthurium* traditionally is propagated via stem cutting and suckers and these are they are tedious and not practical when carried out on a large scale (Puchooa, 2005).

In recent years, propagation of a wide range of ornamental plants by tissue culture has become accepted commercial practice and the rapid strides were achieved in the field of micropropagation of ornamental plants (Read and Preece, 2009). Ornamental industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties (Rout *et al.*, 2006). Tissue culture offers an alternative tool for rapid multiplication of selected clones in a short period (Bejoy *et al.,* 2008).

The tissue culture of *Anthurium* was first reported by Pierik *et al*., (1974) and they achieved regeneration of *Anthurium andreanum* through adventitious shoots formation from callus.

Many researchers studied on optimization of *Anthurium* tissue culture. Micropropagation of *Anthurium* is performed via adventitious shoot regeneration from callus (Kuehnle and Sugii, 1991; Beyramizade *et al*., 2008; Khorrami Raad *et al*., 2012) and direct shoot regeneration (Martin *et al.*, 2003; Islam *et al.*, 2010). 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 (Atak and Çelik, 2012). Kuehnle and Sugii (1991) explained callus proliferation in seven cultivars of Hawaiian anthuriums with leaf and petiole explants. They concluded that callus proliferation was best with modified Pierik medium containing 0.36 µM 2,4-D and 4.4 µM BA for leaf explant and Pierik modified Pierik, and Finnie and van Staden media for petiole explant and also for adventitious plantlets, callus was subcultured on a Kunisaki medium containing 2.2 or 22 µM BA. Beyramizade *et al.* (2008) investigated micropropagation of *Anthurium andreanum* 'Tera'. They achieved best result for callus induction on half strength MS medium containing 0.08 mg/L 2,4-D and 1 mg/L BA, whereas medium without phytohormones induced the highest number of shoots from the callus. Khorrami Raad *et al.*, (2012) reported that callus production in *Anthurium andreanum* L. different cultivars was best in medium containing 0.5 mg/L NAA + 3 mg/L BA in dark conditions. Then, the best proliferation of shoots per callus (22.83 shoots per cm^2 of callus) was observed on medium supplemented with 0.01 mg/L NAA + 1 mg/L BA after 8 weeks in a 16/8 h light and dark cycle under a photoperiod of 50 $\mu mol/m^2/s$. Afterward, in root induction media, the largest number of root (11.50 roots per plantlets) was obtained on medium supplemented with 1 mg/L IBA + 0.2 mg/L KIN.

The aim of this study was to investigate the effects of culture media and different plant growth regulators (BA and 2,4-D) and their concentrations on callus induction, and adventitious shoot formation of *Anthurium*, approaching an efficient *Anthurium* micropropagation system.

MATERIALS AND METHODS

The 3-4 year old *Anthurium andreanum* 'Clisto' was commercially selected from Ashianeh Sabz greenhouse used as donor plant. The research was conducted in Plant Physiology Tissue Culture Laboratory, College of Science, University of Tehran. Young and fresh leaves were taken and used as plant material. Leaf segments were pre-washed in 0.1% commercial dish washing and luke

warm water solution for 5 min and rinsed with running tap water then divided to smaller segment and were surface sterilized with solution of 10% (v:v) sodium hypochlorite (NaOCl) with two drops of Tween-20 for 20 min and finally rinsed 3 times in sterile distilled water. The aseptic explant were cut into 1 cm × 1 cm segment and placed on Petri dishes containing callus induction media. The media was half-strength Murashige and Skoog medium (Murashige and Skoog, 1962; MS) or Nitsch's medium (Nitsch and Nitsch, 1969) with 30 g/L sucrose and solidified with 7 g/L agar (Agar-agar 1614, Merck, Darmstadt, Germany) and supplemented with different composition of 6-Benzyladenine (BA) with concentrations 0, 0.5, 1 and 1.5 mg/L and 2,4-dichlorophenoxyacetic acid (2,4-D) with concentrations 0, 0.25, 0.5 and 0.75 mg/L. The pH of all medium was adjusted to 5.8 prior to autoclaving. The cultures were incubated at 25 ± 1°C and darkness. They were subcultutred per 20 days. After 3 months, callus size (observational) and percentage of explant that produced callus were measured. Produced callus were divided to same size segment and transferred to shoot media regeneration. This media was MS that supplemented with BA (0, 0.25, 0.5 and 0.75 mg/L) and 6 g/L agar. Six callus segments were plated in a 300 ml glass jar with 33 ml media and maintained in growth room with 25 ± 2°C temperature and 16/8 photoperiod with florescent lamp. After 6 months, number of shoot, shoot length and leaf number was recorded. Regenerated shoots were removed from calli and rinsed with sterilized water and planted in plastic pot filled with sterile peat: perlite (1:2) and covered with a transparent plastic container in order to moister preservation and maintained in growth room under 25 ± 1°C temperature and photoperiod 16/8 for two months. Plantlets acclimated and finally transferred to greenhouse.

This study was conducted in a factorial experiment based on completely randomized design. Each experiment included 3 replications. Data were analyzed using the SAS software (version 9.1). Percent data were arcsine transformed before performing ANOVA. The mean values were compared using Duncan's Multiple Range Tests ($P<0.05$).

RESULTS AND DISCUSSION

Callus induction was performed from cutedge of the explants which was most reactive zones. The 25-30 days after culture of explant on media creamy color and compact callus was induced. Smilar to our results, Kuehnle and Sugii (1991) reported that *Anthurium* cultivars were produced callus along cut edges of both leaf and petiole explants for all cultivars tested and it was pale yellow and firm. But they cited that callus produced after 2 to 3 months on a modified Pierik medium. Also we observed that callus induction was more from cut edge and midrib. Khorrami Raad *et al*. (2012) explained that lamina explants exhibited more potential for callus formation when they contained midrib.

Plant growth regulators (PGRs) composition showed significant effect on callus size and percentage of explant that produced callus (Table 1). Exogenous application of auxin and cytokinin induces callus in various plant species. Generally speaking, an intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin-to-cytokinin or cytokinin-to-auxin

Table 1. Analysis of variance for the effect of culture media and PGR concentrations on callus induction trait in *Anthurium andreanum* 'Clisto'.

S.O.V	df	Mean square	
		Callus size	Callus percent
Media culture (M)	1	4.55 **	14.89 ns
PGR composition (P)	15	3.86 **	62.24 **
M×P	15	0.76 ns	5.59 ns
error	64	0.49	4.55
CV (%)		29	28

ns, nonsignificant; **, significant at P<0.01.

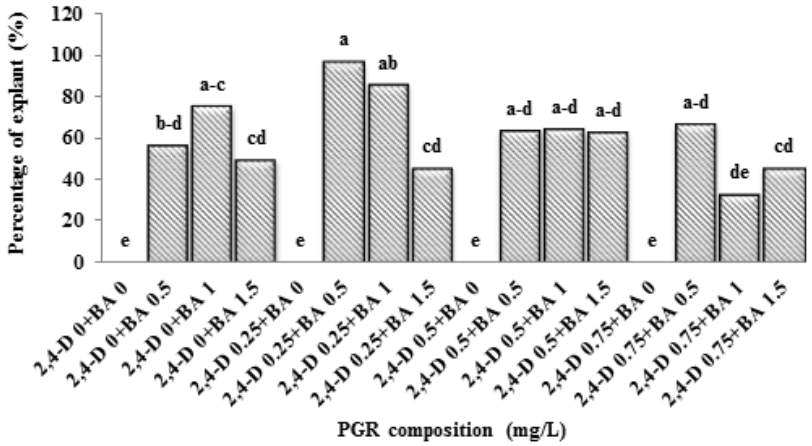

Fig. 1. Effect of PGR composition on percentage of explant that produced callus per treatment in callus induction stage of *Anthurium andreanum* 'Clisto'. Means with different letters were significantly different at the 5% level as determined by Duncan's multiple range test (DMRT).

induces root and shoot regeneration, respectively (Skoog and Miller, 1957). The highest percentage of explant that produced callus was obtained with 0.25 mg/L 2,4-D + 0.5 mg/L BA, however it was not statistically different from 0.25 mg/L 2,4-D + 1 mg/L BA. The lowest percentage of explant that produced callus was observed in media without BA (Fig. 1). These results show that BA has an important role in callus induction of this plant.

The results for callus size determined same as callus production percent and the media culture without BA showed lower callus size (Fig. 2). Our result was in agreement with Bejoy et al. (2008). They concluded that PGR balance in culture regulated explant response and they found that BAP along with 2,4-D were better in respect to rate of responsiveness and extent of callus development. They observed best dedifferentiation in 1 mg/L BAP and 0.5 mg/L 2,4-D in 6 week. Beyramizade *et al.* (2008) reported that best result for callus induction was obtained on half strength MS medium containing 0.08 mg/L 2,4-D and 1 mg/L BA.

Culture media significantly influenced callus size (Table 1) and Nitsch's medium produced higher callus size in comparison to ½ MS (Fig. 3). In the study of Puchooa and Sookun (2003) tissue culture response of three variety of *Anthurium andraeanum* (Nitta, Osaki and Anouchka) on two different media (modified MS and modified Nitsch) for callus induction were evaluated. Nitsch medium with reduced ammonium nitrate concentration (200 mg/L), proved to be the best for callus

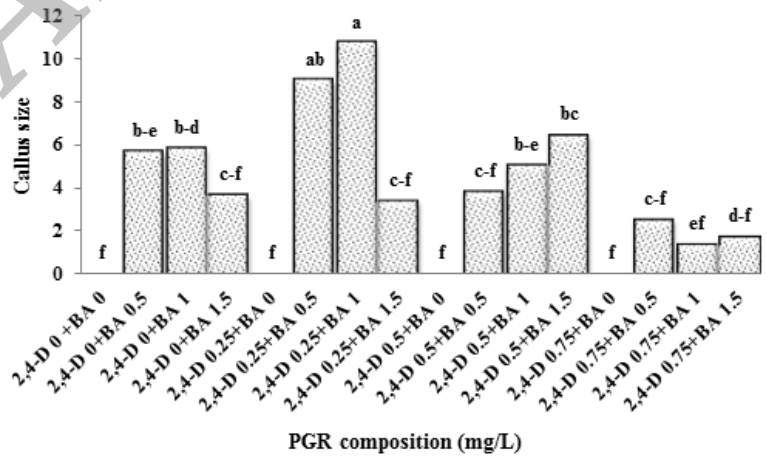

Fig. 2. Effect of PGR composition on callus size per treatment in callus induction stage of *Anthurium andreanum* 'Clisto'. Means with different letters were significantly different at the 5% level as determined by Duncan's multiple range test (DMRT).

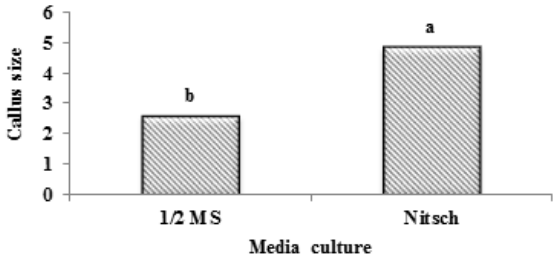

Fig. 3. Effect of media culture type on callus size per treatment in callus induction stage of *Anthurium andreanum* 'Clisto'. Means with different letters were significantly different at the 5% level as determined by Duncan's multiple range test (DMRT).

Table 2. Analysis variance for effect of BA concentration on shoot regeneration trait in *Anthurium andreanum* 'Clisto'.

S.O.V	df	Mean square		
		Shoot length	Number of shoot	Number of leaf
BA	3	8.93 *	11.36 *	4.88 *
error	20	2.51	3.70	1.33
CV (%)		25.3	27.3	26.78

ns, nonsignificant; **, significant at P<0.01.

induction. The media was supplemented with BA at 1 mg/L and 0.1 mg/L 2,4-D.

Callus was transferred to shoot formation media and light condition changed to green color and started to shoot formation and after six months data collected and shoots were removed and planted in pots. Jahan *et al.* (2009) explained that developed calli by leaf and spadix explants which maintained in dark condition for three months, no multiple shoots were regenerated and so, in order to induction of multiple shoots, callus was cultured on MS medium supplemented with different concentrations of BAP and KIN by shifting them from dark to light condition.

In shoot regeneration, BA concentration treatment showed significant effect on number of shoot, shoot length and number of leaf (Table 2). The tallest shoot length determined with medium without BA and 0.25 mg/L BA and with 0.5 and 0.75 mg/L shoot length was shorter (Fig. 4).

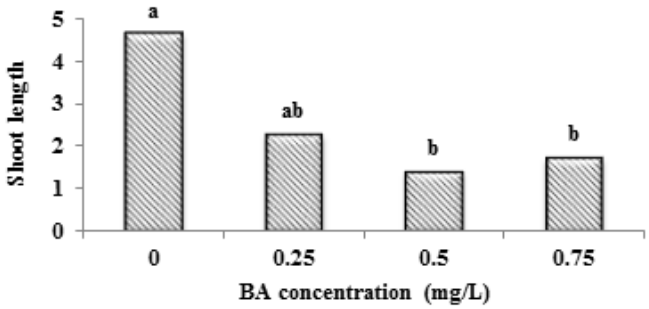

Fig. 4. Effect of BA concentration on shoot length in shoot regeneration stage of *Anthurium andreanum* 'Clisto'. Means with different letters were significantly different at the 5% level as determined by Duncan's multiple range test (DMRT).

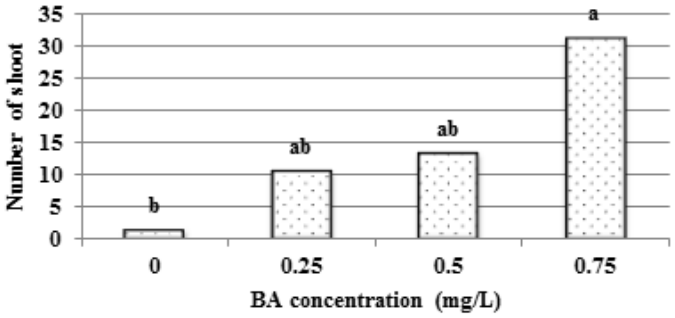

Fig. 5. Effect of BA concentration on number of shoot in shoot regeneration stage of *Anthurium andreanum* 'Clisto'. Means with different letters were significantly different at the 5% level as determined by Duncan's multiple range test (DMRT).

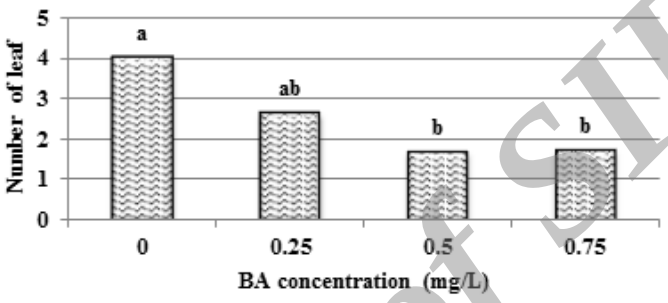

Fig. 6. Effect of BA concentration on number of leaf in shoot regeneration stage of *Anthurium andreanum* 'Clisto'. Means with different letters were significantly different at the 5% level as determined by Duncan's multiple range test (DMRT).

The highest number of shoot was achieved with medium with 0.75 mg/L BA (31 shoot per callus) and it was not significantly different from 0.25 and 0.5 mg/L BA. The lowest number of shoot was observed with medium without BA (Fig. 5). Budiarto (2008) reported that presence of BA affected on the number of newly developed shoots. They found that the highest shoot formation was in Nitsch and Nitsch media supplemented with 2 and 3 mg/L BA in several potted *Anthurium* accessions. Viegas *et al*. (2007) observed shoot formation of *Anthurium andraeanum* 'Flamingo' during 70 day culture and it is increase positively when MS media supplemented with 0.0, 0.5 and 1.0 mg/L BA. They concluded that a medium containing this cytokinin resulted in constant regeneration and growth of shoot until 10th week of culture.

Viegas *et al*. (2007) reported that each shoot produced 3 to 4 leaves throughout the 70 days of observation and these leaves were morphologically normal, dark green and cordiform. It was similar to our results. The results presented that the highest leaf number observed with medium without BA and it was statistically not different from 0.25 mg/L BA. The lowest number of leaf was determined with 0.5 and 0.75 mg/L BA (Fig. 6).

Whereas this plant easily is rooted, we directly transferred multiplied shoots to pots and after acclimation period transferred them to greenhouse. We could obtained an effective acclimation and 99% plantlet rooted and survived. 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 (Atak and Celik, 2012).

Fig. 7. A: Callus induction from cut edge of leaf explant. B: Callus proliferation with subculturing. C: Transfering callus to light condition for shoot regeneration. D: Shoot regeneration in different BA concentration 0, 0.25, 0.5 and 0.75 mg/L from left to right. E: Shoot proliferation. F: Removing shoot from callus. G: Planting shoot in pot and rooting. H: Plantlet acclimation in greenhouse.

CONCLUSION

In this experiment, we successfully regenerated shoot from leave explants of *Anthurium andreanum* 'Clisto'. Callus induction was the best in 0.25 mg/L 2,4-D + 0.5 mg/L BA and Nitsch's medium. In shoot regeneration phase also results was excellent and 31 shoot per callus segment were obtained. The highest shoot multiplication was achieved in MS media supplemented with 0.75 mg/L BA. Regenerated plantlet showed admirable adaptation and subsequent well growth. We could optimize shoot regeneration protocol for *Anthurium andreanum* 'Clisto'.

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باززایی غیرمستقیم شاخساره آنتوریوم رقم 'Clisto' از ریز نمونه برگ

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چکیده

آنتوریوم به عنوان یک گل بریده و گلدانی پرورش داده می‌شود و بخاطر اسپات‌های رنگی اش معروف است. این گیاه بطور سنتی با قلمه ساقه و تقسیم پاجوش تکثیر می‌شود و ریزازدیادی می‌تواند یک روش جایگزین باشد. در این آزمایش، ما باززایی شاخه در *Anthurium andreanum* رقم 'Clisto' را مورد بررسی قرار دادیم. ریزنمونه برگ مورد استفاده قرار گرفت که بعد از ضدعفونی شدن روی دو نوع محیط کشت مختلف (MS ۱/۲ و محیط کشت Nitsch) کشت شدند این محیط کشت با ترکیب‌های مختلفی از تنظیم کننده‌های رشد توفوردی با غلظت‌های ۰، ۰/۲۵، ۰/۵ و ۰/۷۵ میلی گرم بر لیتر و BA با غلظت‌های ۰، ۰/۵، ۱ و ۱/۵ میلی گرم بر لیتر تکمیل می‌شدند. سه ماه بعد اندازه کالوس و درصد ریزنمونه‌های کالوس زا اندازه گیری شدند و به محیط کشت MS با غلظت‌های مختلف BA (۰، ۰/۲۵، ۰/۵ و ۰/۷۵ میلی‌گرم بر لیتر) برای شاخه‌زایی منتقل شدند. بعد از ۶ ماه، تعداد شاخه، طول شاخه و تعداد برگ ایجاد شده ثبت شدند. بیشترین تولید کالوس با محیط کشت Nitsch همراه با ۰/۲۵ میلی‌گرم بر لیتر توفوردی و ۰/۲۵ یا ۰/۵ میلی‌گرم بر لیتر BA به دست آمد. بالاترین باززایی شاخه نیز در محیط کشت با ۰/۷۵ میلی‌گرم بر لیتر BA حاصل شد. ما ۳۱ شاخه در هر قطعه کالوس کشت شده در بهترین تیمار مورد آزمایش به دست آوردیم. ریشه‌زایی شاخه‌های تولید شده به آسانی در گلدانی با بستری از پیت: پرلیت (۲:۱) انجام شد و آن‌ها سازگار شده و به گلخانه انتقال داده شدند و ۹۹٪ گیاهان انتقال یافته زنده ماندند.

کلید واژگان: آنتوریوم، ازدیاد گیاه، کشت بافت، BA، توفوردی.