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Effect of Cultivar, Type and Age of Explants, Light Conditions and Plant Growth Regulators on Callus Formation of Anthurium

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Abstract: This paper investigate the effect of cultivar, type and age of explants, light conditions and plant growth regulators (NAA and BA) on callus formation of *Anthurium andreanum* Linden cultivars Casino and Antadra, an ornamental species. Results showed that the explants source and age, growth conditions, type of cultivar and different concentrations of growth regulators significantly influenced on callus production. Segments of lamina and petiole (micro-cuttings or explants) were cultured in MS basal medium with different concentrations of NAA (0.01, 0.1, 0.5, 1 and 2 mg/L) and BA (0.5, 1, 2 and 3 mg/L) to produce callus. After 65 days, high frequency of callus (0.74 g) was obtained from lamina segments, when they were cultured in MS medium containing 0.5 mg/L NAA + 3 mg/L BA in dark conditions. Callus development was observed along the cut margins and midrib regions of the lamina explants. Younger explants grown in dark conditions exhibited better responses to callusing than that of older explants and grown in light conditions. Callus production was better in Antadra than that of Casino. For explants types, permanent callus was produced on the lamina explants, not on the petiole explants.

Abbreviations: BAP-6-Benzylaminopurine · NAA-α-Naphthaleneacetic acid · 2,4-D-2,4-Dichlorophenoxyacetic acid · 2iP-6-(γ-γ-Dimethylallylamino) Purine · KIN-Kinetin · BA-N^6-Benzyladenine

Key words: Araceae · *Anthurium andreanum* Linden · Ornamental plants · Plant growth regulators · Propagation · Callus induction

INTRODUCTION

Anthurium is economically important genus in the family Araceae and *Anthurium andreanum* is one of the ten most cultivated ornamental plants in the world [1]. Many researchers have applied tissue culture methods for multiplication of Anthuriums using various media and explants. Researchers found that there was great variation in the requirements of different genotypes. Micropropagation of *Anthurium* has been achieved with various tissues including lamina, petiole, seed, shoot tips, lateral bud, spadix and spathe [2-3]. Plant regeneration of *Anthurium andreanum* has been achieved by callus, derived from various explants, especially lamina [2, 4-5]. Callus is an important source for indirect plant organogenesis and embryogenesis. Over one thousand plant species have been regenerated *in vitro* via organogenesis and embryogenesis [6]. These two methods are among the most striking processes in plant micropropagation [6]. Callus induction is hard and time consuming in many monocotyledons such as Anthuriums. Recently, an embryogenic-like callus of *Anthurium andreanum*, cultured on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), N^6-benzyladenine (BA) and kinetin (KIN) was described [7-8]. Various physical and biological factors, including media and cultivars, play role during *in vitro* propagation of *Anthurium* [6, 9]. Geier [10] showed that plant genotype and plant age influence plant regeneration of *Anthurium andreanum*. The establishment and improvement of micropropagation by *in vitro* culture through callus production is desirable. Thus, the aim of

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this paper was to obtain the best cultivar, type and age of explants, light conditions and plant growth regulators related to the callus formation of *Anthurium andreanum* Linden cv. Antadra and Casino.

MATERIALS AND METHODS

Two commercial cultivars of *Anthurium andreanum* Linden (Casino, with orange spathe and Antadra, with pink spathe) were used in this study. These two cultivars were prepared from a greenhouse in Nashtaroud and Abbasabad cities, Mazandaran province in the northern part of Iran. Plants were watered with a s olution containing Krystallon as spray on leaves (0.5 g/L) and drench (1 g/L). After 15-20 days, new leaves were formed. Laminas and petioles of new leaves were used as explants for callus induction. The explants (leaves) were applied in two age steps including; young explants (7 days after opening) and adult explants. Laminas and petioles were divided into sections of approximately 2 cm^2 (containing mid vein) and 1.5 cm size, respectively. Explants were washed under running tap water for 30 min with some drops of dishwashing. Then, explants were dipped on 70% (v/v) ethanol for 30-40 sec. Surface sterilization was done with 1% (w/v) NaOCl and 2-3 drops of Tween-20 for 10 min followed by three rinses with sterile distilled water for 2, 5 and 10 min. Margins of the surface disinfected lamina and petiole w ere cut. Explants were cultured in Petri dishes containing basal MS [11] media supplemented with plant growth regulators; NAA (0.01, 0.1, 0.5, 1 and 2 mg/L) and BA (0.5, 1, 2 and 3 mg/L) (Table 1). Sucrose (3%) was used as carbon source and media were solidified with Agar–agar (0.8%). Macro- and micro-elements, vitamins, plant growth regulators and sucrose were prepared from Sigma co., England and Agar from Duchefa, the Netherland. The acidity of culture media were adjusted to pH 5.7±0.1 prior to autoclaving at 121°C and 102 kpa for 20 min. Four lamina and petiole per Petri dishes were inoculated and ten replicates taken. Lamina and petiole were cultured on solidified MS media supplemented with NAA (0.01, 0.1, 0.5, 1 and 2 mg/L) and BA (0.5, 1, 2 and 3 mg/L) to induce callus (Table 1). For evaluation of the role of lightness and darkness on callus induction and growth, 50 percent of cultures were covered by aluminum foils and 50 percent of cultures were kept in light (without aluminum foils). Subculture was done at 3-week intervals. The effects of culture media, cultivars and their interaction on callus induction were evaluated by days to the beginning of callus induction and weight of callus. Lamina and petiole explants were used to test the role of light and dark on callus induction. Explants were placed on MS medium supplemented with 0.5 mg/L NAA + 3.0 mg/L BA (the best medium for callus weight and induction) (Table 1). Cultures were placed in a culture chamber with 16 h photoperiod. Semi of cultures was covered by aluminum foils. Two traits, days to the beginning of callus induction and weight of callus, were evaluated for the

Table 1: Mean comparison of the effects of different concentrations of BA and NAA added in culture media for callus weight and days to callus induction of *Anthurium andreanum* Linden*

Culture media and varieties**	BA (mg/L)	NAA (mg/L)	Callus weight (g)	Days to callus induction
M1	0.5	0.01	0.31d	-
M2	0.5	0.1	0.32d	-
M3	1	0.5	0.40cd	-
M4	2	0.5	0.63b	-
M5	3	0.5	0.74a	-
M6	2	2	0.43c	-
M7	2	1	0.36cd	-
M8	2	0.1	0.32d	-
M1V1	0.5	0.01	-	52.36a
M2V1	0.5	0.1	-	51.25a
M3V1	1	0.5	-	45.32bcde
M4V1	2	0.5	-	38.80fgh
M5V1	3	0.5	-	36.66gh
M6V1	2	2	-	42.44cdefgh
M7V1	2	1	-	39.75efgh
M8V1	2	0.1	-	48.20abcd
M1V2	0.5	0.01	-	50.12ab
M2V2	0.5	0.1	-	47.42abc
M3V2	1	0.5	-	40.45cdefg
M4V2	2	0.5	-	37.62gh
M5V2	3	0.5	-	35.00h
M6V2	2	2	-	39.29defgh
M7V2	2	1	-	46.00bcdef
M8V2	2	0.1	-	46.85bcdef

*: In each column, means with the similar letters are not significantly different at 5% level of probability using Tukey's test **: M; Culture media, V; Varieties (V1; Casino, V2; Antadra)

effect of light on callus induction after 14 week of culturing. Lamina explants were used to test the role of age on callus induction. Young (7-days-old) and adult explants were placed on MS medium supplemented with 0.5 mg/L NAA + 3.0 mg/L BA (the best medium for callus weight and induction) (Table 1). Two traits, days to the beginning of callus induction and weight of callus, were evaluated for the effect of explants age on callus induction. The cultures were incubated in growth chamber whose environmental conditions were adjusted to 25±2°C and 75-80% relative humidity, under a photosynthetic photon density flux 50 µmol/m^2/s with a photoperiod of 16 h per day. Data were recorded at 4-14 weeks after culturing. The experimental design was factorial with randomized completely blocks design (R.C.B.D), which was done with unequal repetition. All experiments were carried out in three to six replicates. For statistics analysis complementary approach were tested: an ANOVA was performed and means were compared using the Tukey's test ($p<0.05$) using SAS software package, version 9.1. Data processing of the results was carried out by an EXCEL.

RESULTS

Lamina and petiole were applied for callus induction. Between the two explants, no callus formation occurred from most petiole segments, because of the presence of phenol compounds, except for petiole explants of Casino grown on the medium supplemented with 0.1 mg/L NAA + 0.5 mg/L BA. Callus induction and formation (0.34 g) in this medium was observed after 52 days, but the callus became brown. Callus induction was observed on MS basal medium containing different concentrations of BA (0.5-3 mg/L) in combination with NAA (0.01-2 mg/L) in lamina segments. Callus development was observed along the cut margins and midrib regions of the leaf lamina (Fig. 1). Selection was based on days require to callus induction and callus weight. Callus formation was higher in mid vein area than the other regions of leaves (Fig. 1b). There was a wide range of variation in callus induction and formation depending on the concentrations and combinations of hormones, culture medium and type of cultivar (Figs. 1, 2 and 3).

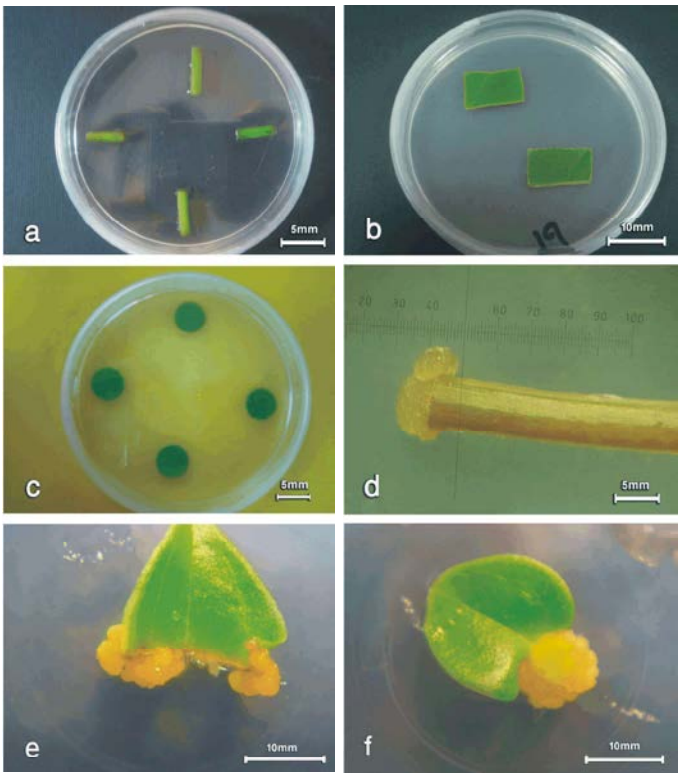

Fig. 1: (a-f) Process of lamina and petiole culture of *Anthurium andreanum* Linden and callus induction through *in vitro* culture. (a-c) Culture of petiole and lamina segments on MS basal medium with hormones. (d) Callus induction of petiole grown on MS basal medium containing 0.5 mg/L NAA + 3 mg/L BA. (e-f) Callus induction of lamina grown on MS basal medium containing 0.5 mg/L NAA + 3 mg/L BA

Table 2: Mean comparison of the effects of light and age conditions on callus weight and days to callus induction of *Anthurium andreanum* Linden*

Treatments	Callus weight (g)	Days to callus induction
Light	0.71b	46.00a
Dark	0.84a	34.00b
Young explants	0.77a	37.33b
Adult explants	0.65b	46.66a

*: In each column, means with the similar letters are not significantly different at 5% level of probability using Tukey's test

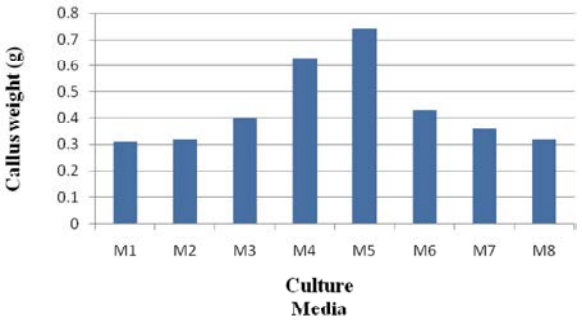

Fig. 2: Mean comparison of the effect of different media on callus weight of *Anthurium andreanum* Linden (M; MS culture media with different concentrations of BA and NAA)

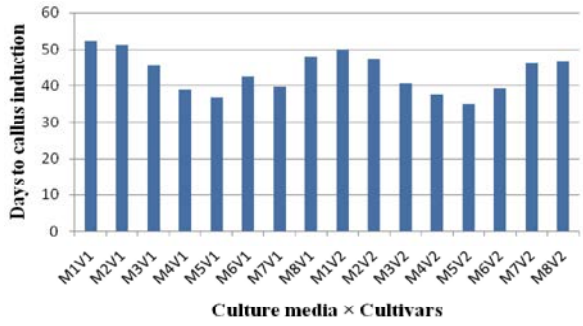

Fig. 3: Mean comparison of the interaction effect of different media and cultivars on days to callus induction of *Anthurium andreanum* Linden (M; MS culture media with different concentrations of BA and NAA, V1; cv. Casino, V2; cv. Antadra)

Our study on the effect of culture medium on callus weight revealed BA and NAA had important effect on callus weight (Table 1). Statistical analysis of data showed that culture medium had significant effect on callus weight and days to callus induction ($P \leq 0.01$). No the effect of cultivar kind was significant on callus weight and days to callus induction. The interaction effect of culture medium and cultivar was significant on callus induction ($P \leq 0.05$), but not on callus weight. The highest callus weight (0.74 g) was observed on MS medium containing 3 mg/L BA + 0.5 mg/L NAA (Table 1, Fig. 2). Callus weight (0.63 g) on MS medium containing 2 mg/L BA + 0.5 mg/L NAA was also good (Table 1, Fig. 2). The lowest callus weight (0.31 g and 0.32 g) was observed on MS media containing 0.5 mg/L BA + 0.01 mg/L NAA and 0.5 mg/L BA + 0.1 mg/L and NAA, respectively (Table 1, Fig. 2). There was significant difference between callus weights grown on MS media supplemented with 1 mg/L BA + 0.5 mg/L NAA (0.40 g), 2 mg/L BA + 0.5 mg/L NAA (0.63 g) and 3 mg/L BA + 0.5 mg/L NAA (0.74 g) (Table 1). NAA concentration was similar in these tree media (0.5 mg/L) but BA concentration was different (1, 2 and 3 mg/L). The best medium for least days to callus induction (35.00 and 36.66 days) in cv. Antadra and Casino was MS medium supplemented with 3 mg/L BA + 0.5 mg/L NAA (Table 1, Fig. 3). Mean comparison of the interaction effect of culture medium and cultivar on days to callus induction showed that the least days to callus induction (35.00 days) was observed in culture medium of Antadra containing 3 mg/L BA + 0.5 mg/L NAA (Table 1, Fig. 3). Maximum days to callus induction (52.36 days) was observed in culture medium of Casino containing 0.5 mg/L BA + 0.01 mg/L NAA (Table 1, Fig. 3). Culture medium supplemented with 3 mg/L BA + 0.5 mg/L NAA is suitable for decreasing the days to callus induction in both cultivars, but its effect on Antadra is more. Callus induction was faster in explants grown in dark conditions (34.00 days) than tha of light conditions (46.00 days) (Table 2). Callus weight was higher in explants grown in dark conditions (0.84 g) than that of light conditions (0.71 g) (Table 2). Callus induction was faster in young explants (37.33 days after culturing) than that of adult one (46.66 days after culturing) (Table 2). Also, callus weight of young leaves (0.77 g) was higher than that of adult leaves (0.65 g) (Table 2).

DISCUSSION

In current study, two types of explants; lamina and petiole segments, were used for callus formation. The success of *in vitro* culture is related to the correct choice of explants material [12]. In current study, petiole explants was not proper for callus induction. Contrary to our result, Kuehnle and Sugii [7] and Guo *et al*. [13] showed the better callusing on petiole than that of lamina.

Also, Devinder-Prakash *et al*. [14] obtained callus from *Anthurium andreanum* petioles after culture on MS medium with 0.5 or 1.0 mg/L 2,4-D. Our result confirmed many studies regarding to be better callusing on lamina than petiole [15-16]. Several researchers were reported the induction of callus on leaf explants of Anthuriums, especially lamina [1-3, 17-18]. Jahan *et al*. [1] showed that between the two explants of *Anthurium andreanum* L., leaf and spadix, leaf segments appeared to be best for callus induction. Te-chato *et al*. [6] used leaves, nodes and internodes of three genotypes of *Anthurium* and found internodes gave the highest callus formation. Our study showed that lamina explants exhibited more potential for callus formation when they contained midrib or vein. This result was consistent with other results reported by some workers [18-19]. The age of explants is an important factor relating to the callus formation. In our study, younger explants showed better responses to callus formation than that of older explants. Contrary to our finding, Bejoy *et al*. [18] showed that relatively older explants of leaves in *Anthurium andreanum* Hort. cv. Agnihothri, exhibited better responses to callus induction. Studies of Reddy *et al*. [20] on micropropagation of *Anthurium digitatum* revealed that the young leaf showed excellent callusing capacity.

In our work, the best medium for callus formation was MS medium containing 3 mg/L BA + 0.5 mg/L NAA. Hormonal regulation of auxin and cytokinin balance is a key factor in the control of cell division in tissue culture [21]. The importance of auxins (2iP and 2,4-D) and cytokinins (BA, BAP, KIN and zeatin) for callus induction in Anthuriums was demonstrated by some researchers [4, 7, 22-24]. Puchooa and Sookun [25] revealed that exogenously applied BA (1.0 mg/L) and 2,4-D (0.1 mg/L) was necessary for callus induction from leaf explants of *Anthurium andreanum* cv. Nitta, Ozaki and Anouchka. Vargas *et al*. [5] obtained callus from micro-cuttings, when placed on MS medium supplemented with 4.4 µM BAP and 0.05 µM NAA. Reddy *et al*. [20] obtained callus from leaf explants, when placed on half strength MS media with 2.0 mg/L BAP and 0.5 mg/L 2,4-D. Viégas *et al*. [26] concluded that callus formation was induced from leaf explants on half MS medium containing 0.08 mg/L 2,4-D and 1 mg/L BAP, with or without 1 mg/L 2iP. Jahan *et al*. [1] showed high frequently of callus on N6 medium containing 2.5 mg/L BAP and 0.2 mg/L 2,4-D. Studies of Bejoy *et al*. [18] on foliar regeneration of *Anthurium andreanum* showed that individual treatment of cytokinins induced callus to a maximum of 35.3% when explants were incubated in 1.0 mg/L BAP. KIN was less effective for the callus formation. BAP along with 2,4-D were found better in the extent of callus development. These researchers demonstrated that the best callus formation (53%) was recorded in 1 mg/L BAP and 0.5 mg/L 2,4-D. Totally, most studies related to callus induction of Anthuriums have been performed on leaf (especially lamina) using 1 mg/L BA and 0.1 mg/L 2,4-D [22, 24, 27-28]. Study of Kaviani *et al*. [29] on micropropagation of *Matthiola incana* (an ornamental plant) showed that MS media containing 0.5 mg/L NAA (100%) and 0.5 mg/L Kn + 0.5 mg/L NAA (100%) were most effective for callus induction on leaf micro-cuttings.

Present study showed that callus induction and callus weight were better in explants grown in dark conditions than those of light conditions. There are some findings in agreement with the present study [1, 30]. Study of Bejoy *et al*. [18] on callus formation of *Anthurium andreanum* Hort. cv. Agnihothri demonstrated that the callus could be established at various frequencies in most of the growth regulators regime within 5 weeks in then the dark. The explants kept in the light did not develop callus. Studies of Reddy *et al*. [20] on micropropagation of *Anthurium digitatum* revealed that incubation of leaf explants in dark conditions for 25 days caused better callusing. Callus induction from leaf explants depends strongly on genotype [3, 12]. Our study showed that callus formation of Antadra was higher than Casino. Nhut *et al*. [17] investigated the effects of 10 different *Anthurium* genotypes on callus induction derived from leaf explants and found some differences between genotypes. Atak and Çelik [3] showed that while callus induction rate for Arizona variety was 80%, this rate was 70% for Sumi variety. Te-chato *et al*. [31] reported that genotype affects callus formation in Anthuriums. The difference might be due to intra-metabolism of plant which affected cell division and differentiation [6]. These researchers showed that cv. Valantino gave the highest callus formation (83.73%) significantly different from cv. Sonat (78.73%) and cv. Plew Thien Phuket (45.66%).

In conclusion, current study emphasizes that younger leaf (lamina) of *Anthurium andreanum* cv. Antadra, grown on MS medium containing suitable concentrations of BA and NAA and kept in dark conditions were optimal for callus formation. Callus induction in *Anthurium andreanum*, like many other monocotyledons, is problematic and time consuming. Maximum of callusing obtains when all chemical, physical and environmental conditions for growing of explants in tissue culture are optimal. Callus plays an important role

in organogenesis, embryogenesis (especially, somatic embryogenesis), production of secondary metabolites, protoplast fusion and genetic manipulations.

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