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Callus induction and organogenesis capacity from lamina and petiole explants of *Anthurium andreanum* Linden (Casino and Antadra)

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Abstract

This paper describes a rapid and efficient protocol to propagate *Anthurium andreanum* Linden cultivars Casino and Antadra in presence of IBA, NAA, 2,4-D, KIN and BA through callus induction and organogenesis. Segments of lamina and petiole (micro-cuttings or explants) were cultured in MS basal medium with different concentrations of NAA (0.0, 0.01, 0.1, 0.5, 1 and 2 mg/L) and BA (0.0, 0.5, 1, 2 and 3 mg/L) to produce callus. After 65 days, the most callus production was observed in medium containing 0.5 mg/L NAA + 3 mg/L BA in dark conditions. Production of callus in younger explants grown in dark was better than that the older explants grown in light conditions. The development of shoots and plantlets was initiated later from calluses. NAA (0.0, 0.005, 0.01 and 0.02 mg/L), 2,4-D (0.00, 0.05 and 0.1 mg/L), KIN (0.0 and 1.0 mg/L) and BA (0.0 and 1.0 mg/L) were used for shoot proliferation. 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 µmol/m^2/s. Callus production and shoot proliferation were better in Antadra cv. than those of Casino. IBA (0.0, 0.5, 1.0 and 2.0 mg/L), NAA (0.0, 0.05, 0.1 and 0.25 mg/L) and KIN (0.0 and 0.2 mg/L) were applied for rooting of proliferated shoots. 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. Rooting was significantly higher in Casino cv. Regenerated plants were transferred to peat: perlite: sand (1:1:1) after hardening and they showed 96% of survival.

Keywords: Araceae, *Anthurium andreanum* Linden, ornamental plant, plant growth regulators, propagation.
Abbreviations: IBA-Indole-3-butyric acid, NAA-Naphthalene acetic acid, 2,4-D- 2,4-Dichlorophenoxyacetic acid, KIN-Kinetin, BA-6-Benzylaminopurine.

Introduction

Anthuriums has been cultivated for many decades as cut flowers in the tropical and subtropical countries and is economically important genera in the family Araceae, consisting of more than 1000 species. Among the several commercially important Anthurium species, *Anthurium andreanum* is one of the ten most cultivated ornamental plants for cut flower in the world (Jahan et al., 2009). *Anthurium* is propagated by seed, traditional vegetative (offshoots and nodal cuttings) and tissue culture. Most of the commercially grown Anthuriums are hybrids with high genetic variability, thus cultivators multiply them through vegetative and seed cultures (Kuehnle and Sugii, 1991). The seed propagation is not proper because of cross-pollination, poor germination rate, variation in color, quality and yield of flowers, and low viability (2-3 days). The progenies are heterozygous, and also propagation by seed leads to genetic segregation (Martin et al., 2003, Jahan et al., 2009). In addition, seeds cannot be conserved, and must be collected immediately after fruit maturation (Viégas et al., 2007). Vegetative propagation of newly developed plants is laborious and time consuming (Kuehnle and Sugii, 1991; Martin et al., 2003; Reddy et al., 2011). Many researchers have applied *in vitro* culture methods for multiplication of Anthuriums sing various media and explants. Micropropagation is being suggested as an alternative to increase the production of Anthuriums these days (Jahan et al., 2009). Under the ideal environment, Anthuriums produces long lasting flowers all year round (Mairo et al., 2010). The in vitro culture techniques are suitable for micropropagation and the establishment of disease free stock materials. Nowadays, most pot-type Anthuriums available in the market are produced by tissue culture methods (Mairo et al., 2010). Micropropagation of Anthurium has been achieved with various tissues including lamina, petiole, seed, shoot tips, lateral bud, spadix and spathe (Martin et al., 2003, Atak and Çelik, 2009). Plant regeneration of Anthurium andreanum has been achieved through adventitious shoot formation from callus (Pierik et

al., 1974; Kuehnle and Sugii, 1991; Vargas et al., 2004), direct shoot regeneration from lamina explants (Martin et al., 2003), axillary buds (Kunisaki, 1980), and root explants (Chen et al., 1997). Various physical and biological factors play important roles during in vitro propagation of Anthurium (Silva et al., 2005). Geier (1986) showed that plant genotype and plant age influence plant regeneration of Anthurium andreanum. Multiplying Anthuriums by callus induction implies a long explant-plant cycle (12 months) (Geier, 1986). This paper describes a detailed protocol for in vitro regeneration of Anthurium andreanum cv. Antadra and Casino from micro-cuttings (lamina and petiole) and callus tissue through organogenesis in order to reduce the time required for the various production stages.

Results and Discussion

Callus formation

Lamina and petiole explants were applied for the induction of callus. No callus formation occurred from petiole explants with the exception of 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/explant) in this medium was observed after 52 days, but the callus became brown after transfer to the regeneration medium supplemented with 0.02 mg/L NAA + 1 mg/L BA. Devinder-Prakash et al. (2001) obtained callus from *Anthurium andreanum* petioles after culture on MS medium containing 0.5 or 1.0 mg/L 2,4-D. In our work, 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 leaf segments. The data were calculated based on days until callus induction and callus fresh weight. The success of *in vitro* culture is related to the correct choice of explants material (George et al., 2008). In current study, petiole explants was not proper for callus formation. Several researchers were reported the induction of callus on leaf explants of Anthuriums especially lamina (Martin et al., 2003; Nhut et al., 2006; Bejoy et al., 2008; Jahan et al., 2009). Te-chato et al. (2006) used leaf, node and internode of three genotypes of *Anthurium* and found internode gave the highest callus formation. Lamina explants exhibited more potential for callus formation when they contained midrib (visual observation), which was agreement with results reported by Kumar et al. (1992) and Bejoy et al. (2008). Our studies on the effect of plant growth regulators on callus fresh weight revealed that BA induced higher callus weight than NAA. Statistical analysis (ANOVA) of the data showed that the effect of culture medium on callus fresh weight and days until callus induction was significant ($P \leq 0.01$). No the effect of cultivar kind was significant on callus fresh weight and days until callus induction. The interaction effect of culture medium and cultivar was significant on callus induction ($P \leq 0.05$), but not on callus fresh weight (Table 1). The highest callus fresh weight (0.74 and 0.63 g/explant) was observed on MS medium containing 3 mg/L BA + 0.5 mg/L NAA, and 2 mg/L BA+ 0.01 mg/L NAA, respectively (Figs. 1 and 2). The lowest callus fresh weight (0.31 g and 0.32 g/explant) was observed on MS medium containing 0.5 mg/L BA + 0.01 mg/L NAA and 0.5 mg/L BA + 0.1 mg/L NAA (Fig. 1). There was significant difference between callus fresh weights grown on MS media supplemented with 1 mg/L BA + 0.5 mg/L NAA (0.4 g/explant), 2 mg/L BA + 0.01 mg/L NAA (0.63 g/explant), and 3 mg/L BA + 0.5 mg/L NAA (0.74 g/explant). NAA concentration was similar in these tree media (0.5 mg/L) but BA concentration was different (1, 2 and 3 mg/L). The better medium for days until callus induction (35 days) in both cultivars was MS medium supplemented with 3 mg/L BA + 0.5 mg/L NAA (Fig. 3). The importance of auxins (NAA, 2iP and 2,4- D) and cytokinins (BA, BAP, KIN and zeatin) for callus induction in Anthuriums was demonstrated by some researchers (Joseph et al., 2003; Vargas et al., 2004; Puchooa, 2005; Reddy et al., 2011). Viégas et al. (2007) and Jahan et al. (2009) showed high frequently of callus on medium containing BAP and 2,4-D. Totally, most studies related to callus formation in Anthuriums have been performed on leaf explants using 1 mg/L BA or BAP and 0.1 mg/L 2,4-D (Zhao et al., 2004; Puchooa, 2005). Mean comparison of the interaction effect of culture medium and cultivar on days until callus induction showed that the least days (35 days) observed in culture medium of Antadra containing 3 mg/L BA + 0.5 mg/L NAA (Fig. 3). The highest number of days until callus induction (52 days) was observed in culture medium of Casino containing 0.5 mg/L BA + 0.01 mg/L NAA (Fig. 3). The least days until callus induction (55 days) were observed in culture media of Casino cultivar than that of Antadra (65 days) (Fig. 3). Culture medium supplemented with 3 mg/L BA + 0.5 mg/L NAA is suitable for decreasing the days until callus induction in both cultivars, but its effect on Antadra is more. Callus induction from leaf explants depends strongly on genotype (George et al., 2008). Our study showed that callus formation of Antadra was higher than Casino. Nhut et al. (2006) investigated the effects of 10 different *Anthurium* genotypes on callus induction derived from leaf explants and found some differences between genotypes. Atak and Çelik (2009) showed that while callus induction rate for Arizona variety was 80%, this rate was 70% for Sumi variety. Te-chato et al. (2002, 2006) demonstrated that the kind of genotype affects on callus formation in Anthurium. Callus induction was faster in explants grown in dark conditions (34 days) than that of light conditions (46 days). Also, callus fresh weight was higher in explants grown in dark conditions (0.84 g/explant) than that of light conditions (0.71 g/explant). There are some findings in agreement with the present study (Bejoy et al., 2008; Jahan et al., 2009). Studies of Reddy et al. (2011) on micropropagation of *Anthurium digitatum* revealed that incubation of leaf explants in dark conditions for 25 days caused better callusing. Callus induction was faster in young explants (37 days after culturing) than that of adult one (46 days after culturing). Also, callus fresh weight produced on media containing young leaves (0.77 g/explant) was higher than that of adult leaves (0.65 g/explant). Contrary to our finding, Bejoy et al. (2008) showed that relatively older explants of leaf in *Anthurium andreanum* Hort. cv. Agnihothri, exhibited better responses to callus induction. Studies of Reddy et al. (2011) on micropropagation of *Anthurium digitatum* revealed that the young leaf showed excellent callusing capacity.

Shoot multiplication and elongation

Statistical analysis of results showed that culture medium and cultivar type had significant effects on shoot number and days until shoot regeneration ($P \leq 0.01$). No interaction effect of culture medium and cultivar was significant on shoot number, but interaction effect of culture medium and cultivar was significant on days until shoot regeneration ($P \leq 0.01$) (Table 1).

Table 1. Analysis of variance (ANOVA) for the effect of cultivar, culture medium and interaction effect of cultivar and culture medium on the callus weight and days to callus induction, shoot number and days to shoot induction and root number and root length of *Anthurium andreanum* Linden.

Source of variations	df	M.S.		df	M.S.		df	M.S.	
		Callus weight	Days to callus induction		Shoot No.	Days to shoot induction		Root No.	Root length
Cultivar	1	0.0075^{ns}	7.52^{ns}	1	25.35^{**}	1310.29^{**}	1	58.77^{**}	13.08^{ns}
Medium	7	0.152^{**}	207.23^{**}	8	53.94^{**}	256.87^{**}	5	67.11^{**}	48.77^{**}
Cultivar × Medium	7	0.00098^{ns}	14.42^{*}	8	1.22^{ns}	29.33^{**}	5	3.11^{ns}	0.21^{ns}
Error	32	0.0032	5.58	36	1.09	5.25	24	1.44	0.33

**: Significant at α = 1%, *: Significant at α = 5%, n.s=Not significant

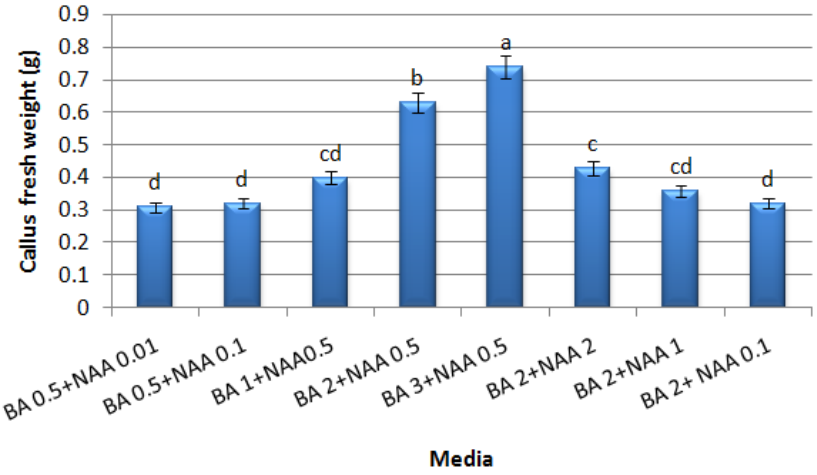

Fig 1. Mean comparison of the effect of different media on callus weight of *Anthurium andreanum* Linden.

shoot number, but interaction effect of culture medium and cultivar was significant on days until shoot regeneration (P≤0.01) (Table 1). The combination of BA and NAA improved the shoot proliferation in *Anthurium andreanum*. The combination of 1 mg/L BA +0.01 mg/L NAA with 22.83 shoots per explants was found to be the most suitable growth regulator regime (Fig. 4). Montes et al. (1999), Trujillo et al. (2000), Bejoy et al. (2008) and Jahan et al. (2009) obtained the highest proliferation of Anthuriums on medium enriched with BAP. BAP was not used in the present study. Some other researchers obtained the highest proliferation of Anthuriums in the presence of BA alone or in combination with others cytokinins or auxins (Yang et al., 2002; Lee-Espinosa et al., 2003; Martin et al., 2003; Lara et al., 2004; Duong et al., 2007; Maira et al., 2009). Our findings were in parallel with those studies. A low concentration of auxin along with a high concentration of cytokinin was most promising for the induction and multiplication of shoots in Anthuriums. In some reports, 2,4-D in low concentrations has been used in combination with cytokinins to induce multiple shoots in Anthurium (Orlikowska et al., 1995; Atak and Çelik (2009). In current study, the least number of shoot per explant (13.33) was observed on MS medium containing 1 mg/L KIN + 0.1 mg/L 2,4-D (Fig. 4). Regarding to constant of BA concentration in all shoot proliferation medium (1 mg/L), NAA had the important role in shoot proliferation. The better shoot proliferation in terms of shoot number was obtained using BA and NAA as compared with KIN and NAA. In media containing KIN, only 1 to 3 shoots per explant was produced that they lost their viability after several days. In contrast with our results, Jahan et al. (2009) showed the positive effect of KIN on increasing the number of shoots from callus of *Anthurium*. It seems that the main cause of this difference might be due to the type of cultivars. Mean comparison of the effect of cultivar type on shoot number showed that Antadra with 16.81 shoots per explants was better than Casino with 15.44 shoots per explant (Fig. 5). The best medium for days until shoot regeneration (49 days) in both cultivars was MS medium supplemented with 1 mg/L BA + 0.01 mg/L NAA (Figs. 6 and 7). Mean comparison of the interaction effect of culture medium and cultivar on days until shoot induction showed that minimum days (45 days) was observed in culture medium of Casino containing 1 mg/L BA + 0.01 mg/L NAA (Fig. 6). Maximum days until shoot induction (77 days) was observed in culture medium of Antadra containing 1 mg/L KIN + 0.1 mg/L 2,4-D (Fig. 6). Several researchers reported that the type of cultivar affects shoot induction in *Anthurium* (Te-chato et al., 2002 and 2006). This difference might be due to intra-metabolism of cultivars which affects cell division and differentiation (Te-chato et al., 2006).

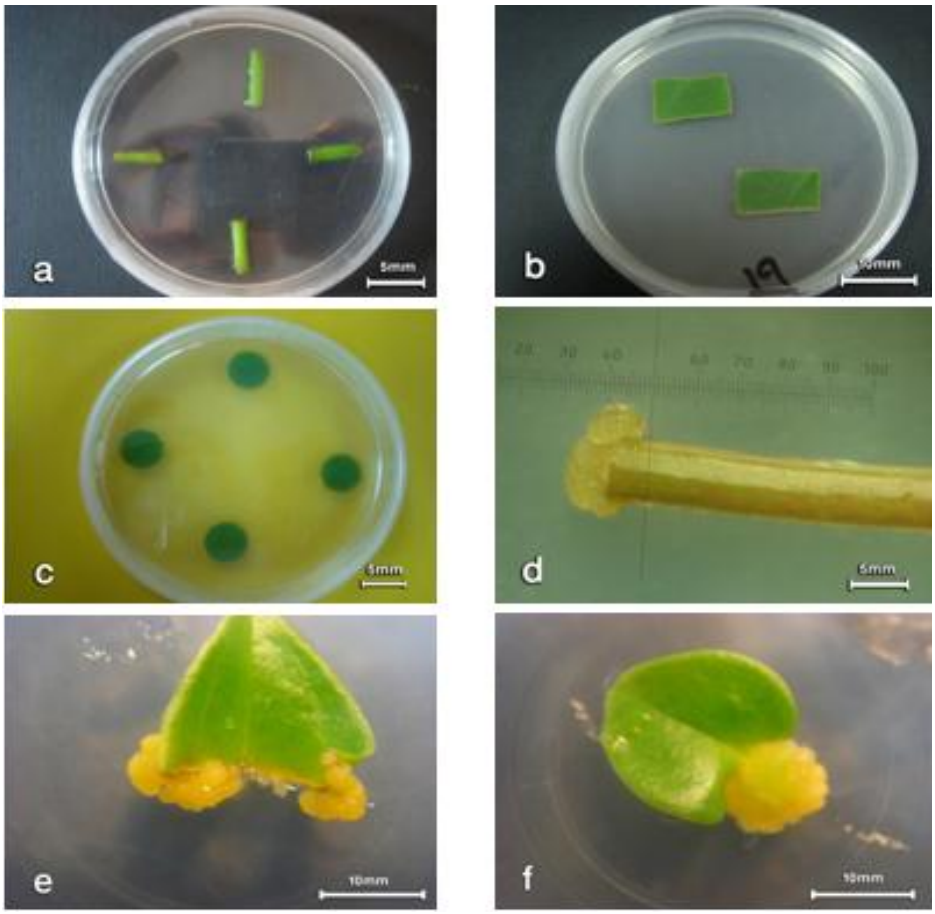

Fig 2. Process of lamina and petiole culture of *Anthurium andreanum* Linden and callus induction through *in vitro* culture (a-f). Culture of petioles and laminas segments on MS basal medium with hormones (a-c). Callus induction of petiole grown on MS basal medium containing 0.5 mg/L NAA + 3 mg/L BA (d). Callus induction of lamina grown on MS basal medium containing 0.5 mg/L NAA + 3 mg/L BA (e-f).

Adventitious rooting of multiplied shoots and plant acclimatization

Investigation of rooting was done by assessment of the number and length of roots. Our study on the effect of culture medium on rooting showed the importance of KIN and IBA. Statistical analysis of results showed that culture medium and cultivar type had significant effects on root number and root length ($P \leq 0.01$). Interaction between culture medium and cultivar type was no significant on root number and root length (Table 1). The largest number of roots per shoot (11.50) was obtained on MS medium containing 0.2 mg/L KIN + 1 mg/L IBA (Figs. 4 and 8). According to results of ANOVA and regarding to constant of KIN concentration in all rooting medium (0.2 mg/L), NAA and IBA had the important role in rooting. Media containing KIN and IBA was better than KIN and NAA. Auxin type and concentration significantly influenced rooting percentage and root length. Rooting is usually induced by auxins, and IBA is more effective for rooting compared with other auxins as reported for Anthuriums (Malhotra et al., 1998; Puchooa and Sookun, 2003; Jahan et al., 2009). Similar to our finding, in most of these studies, suitable concentration of IBA is 1 mg/L. The least number of roots per shoot (2.10) was observed on MS medium containing 0.2 mg/L KIN + 0.05 mg/L NAA (Fig. 4). In contrast with our result, Martin et al. (2003) cultured shoots of *Anthurium andreanum* in medium supplemented with 0.54 µ M NAA and 0.93 µ M KIN for *in vitro* rooting. *In vitro* rooting percentages were 100%. Also, Bejoy et al. (2008) achieved 98% root from shoots in medium supplemented with 0.5 mg/L NAA. Other auxins, like IBA and IAA had less effective for rooting from *in vitro* shoots. The best medium for increasing the root length per explant (10.90 cm) was MS medium supplemented with 0.2 mg/L KIN + 2 mg/L IBA (Fig. 9). The least increasing in root length per explant (2.50 cm) was observed in culture medium supplemented with 0.2 mg/L KIN + 0.05 mg/L NAA (Fig. 9). Mean comparison of the effect of cultivar type on root number showed that Casino with 7.66 roots per shoot was better than Antadra with 3.76 roots per shoot (Fig. 5). Mean comparison of the effect of cultivar type on root length

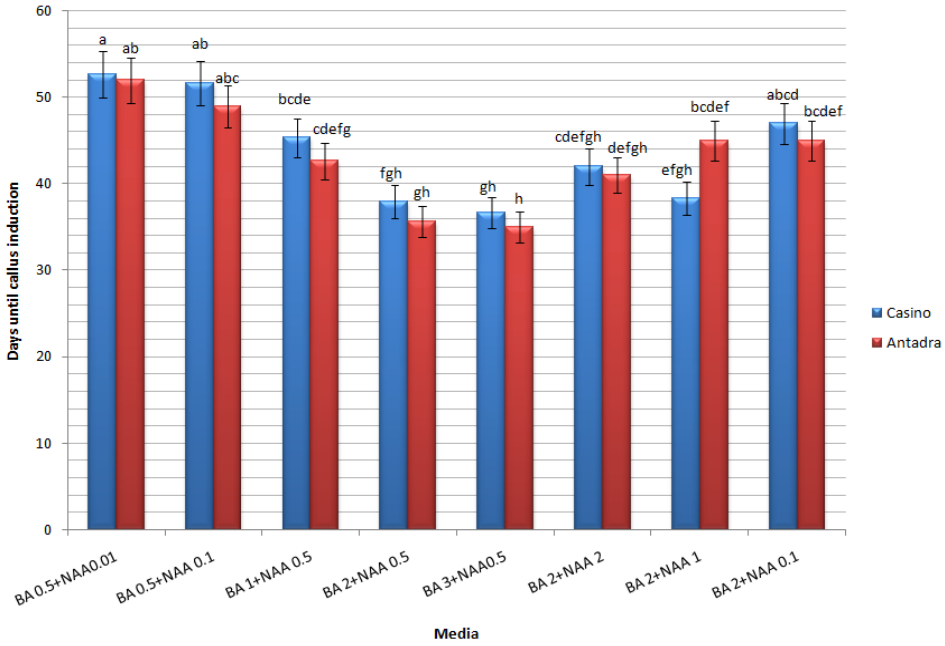

Fig 3. Mean comparison of the interaction effect of different media and cultivars on days to callus induction of *Anthurium andreanum* Linden.

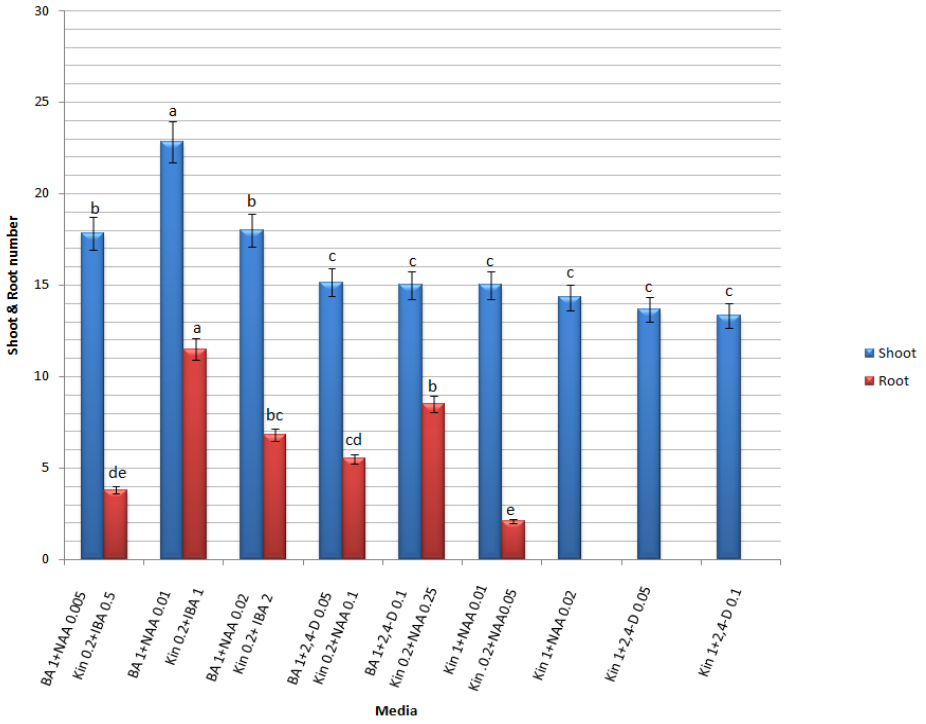

Fig 4. Mean comparison of the effect of different media on shoot number of *Anthurium andreanum* Linden.

showed that Casino with 7.30 cm per shoot was better than Antadra with 6.10 cm per shoot (Fig. 10). Generally, most studies related to rooting of Anthuriums shoots regenerated from callus have been performed using IBA and NAA (Zhang et al., 2001; Joseph et al., 2003). The results of acclimatization showed that the 96% of plantlets were survived to grow under greenhouse conditions and were morphologically similar to mother plants (Fig. 11). A mixture of light soil with good drainage is suitable for acclimatization of these plants. The plantlets at 4-5 cm long stage were transferred in greenhouse.

Materials and methods

Plant materials and sterilization

Two commercial cultivars of *Anthurium andreanum* Linden (Casino and Antadra) with orange and pink spadixes were prepared from a greenhouse in Nashtaroud and Abbasabad cities, Mazandaran province in the northern part of Iran. Plants were watered with a solution containing Crystallon as spray on leaves (0.5 g/L) and drench (1 g/L). After 15-20 days, new leaves were formed. Lamina and petiole were divided

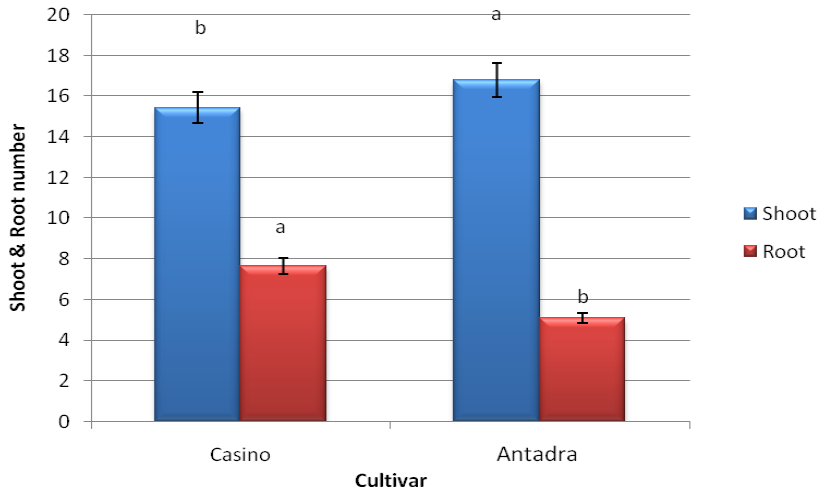

Fig 5. Mean comparison of the effect of different cultivars on shoot number of *Anthurium andreanum* Linden.

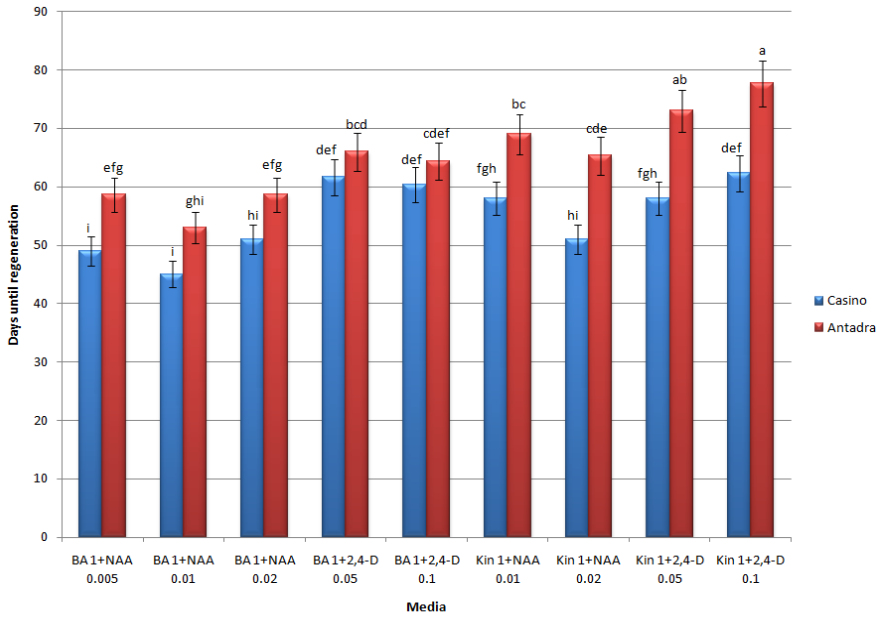

Fig 6. Mean comparison of the interaction effect of different media and cultivars on days to shoot induction of *Anthurium andreanum* Linden.

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 were cut.

Culture medium conditions

Explants were cultured in Petri dishes containing basal MS (Murashige and Skoog,1962) medium supplemented with plant growth regulators (NAA, IBA, 2,4-D, BA and KIN). Sucrose (3%) was used as carbon source and media were solidified with Agar–agar (0.8%). Micro- and micro-elements, vitamins, plant growth regulators and sucrose were prepared from Sigma co., England, and Agar from Duchefa, the Netherland. The pH was 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.

Callus induction

Lamina and petiole were cultured on solidified MS media supplemented with NAA (0.0, 0.01, 0.1, 0.5, 1 and 2 mg/L) and BA (0.0, 0.5, 1, 2 and 3 mg/L) to induce callus. Subculture was done at 3-week intervals. The effects of

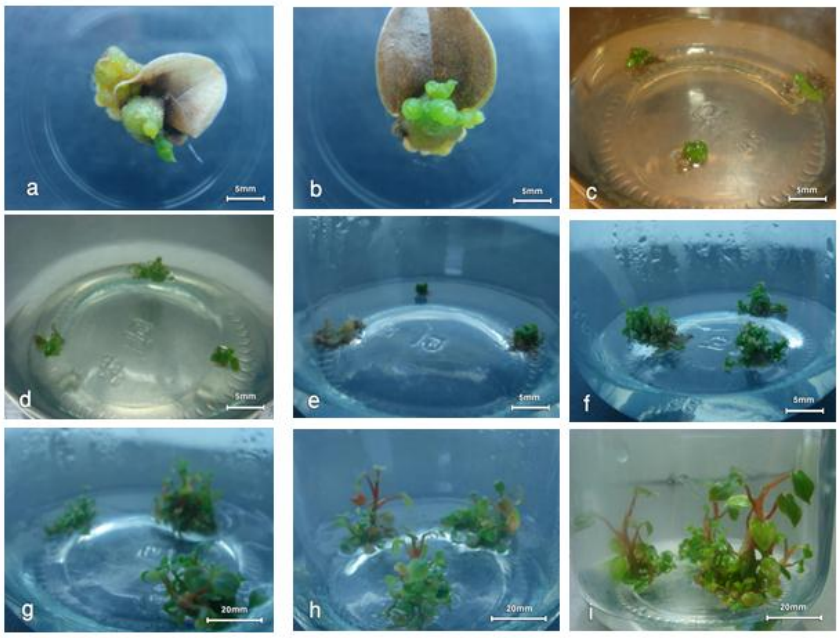

Fig 7. *In vitro* propagation of *Anthurium andreanum* Linden through shoot proliferation from callus produced from lamina explants (a-i). Callus induction (a). Initiation and development of shoot induction from callus in MS medium containing 0.01 mg/L NAA+ 1 mg/L BA (b-e). Growth and elongation of multiple shoots in MS medium containing 0.01 mg/L NAA + 1 mg/L BA (f-i).

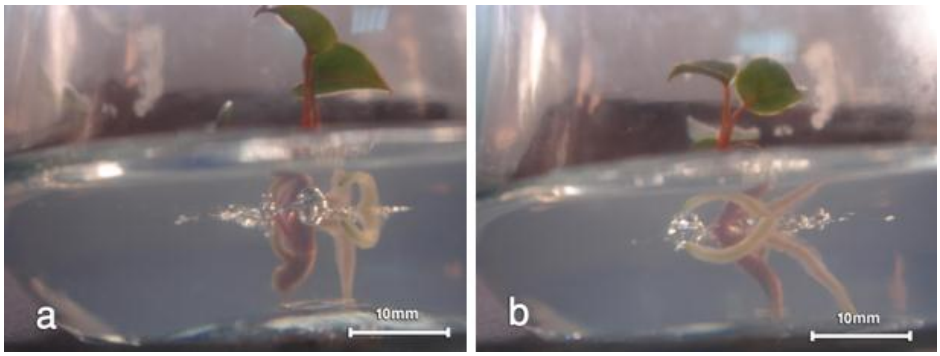

Fig 8. Induction and growth of roots from regenerated shoots in MS basal medium supplemented with 1 mg/L IBA + 0.2 mg/L KIN (a-b).

culture media, cultivars and their interaction on callus induction were evaluated by days to the beginning of callus induction and weight of callus.

Shoot induction and proliferation

Six to eight-week-old calli were transferred to proliferation media. Calli (0.2 g) were cultured on solidified MS media supplemented with NAA (0.0, 0.005, 0.01 and 0.02 mg/L), 2,4-D (0.00, 0.05 and 0.1 mg/L), KIN (0.0 and 1.0 mg/L) and BA (0.0 and 1.0 mg/L) to produce shoot. The effects of culture media, cultivars and their interaction on shoot proliferation were evaluated by days to the beginning of shoot induction and the number of regenerated plantlets.

Rooting

Shoots were rooted on the rooting induction media containing MS basal medium supplemented with IBA (0.0, 0.5, 1.0 and 2.0 mg/L), NAA (0.0, 0.05, 0.1 and 0.25 mg/L) and KIN (0.0 and 0.2 mg/L). The effects of culture media, cultivars and their interaction on rooting were evaluated by root length and number.

Effect of light and dark on callus induction

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/LNAA + 3.0 mg/L BA (the best medium for callus induction). Cultures were placed in a culture chamber with 16 h photoperiod. Semi of cultures was covered by aluminum foils. Two traits of days to the beginning of callus induction and weight of callus were evaluated for the effect of light on callus induction after 14 week of culturing.

Effect of explants age on callus induction

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 induction).

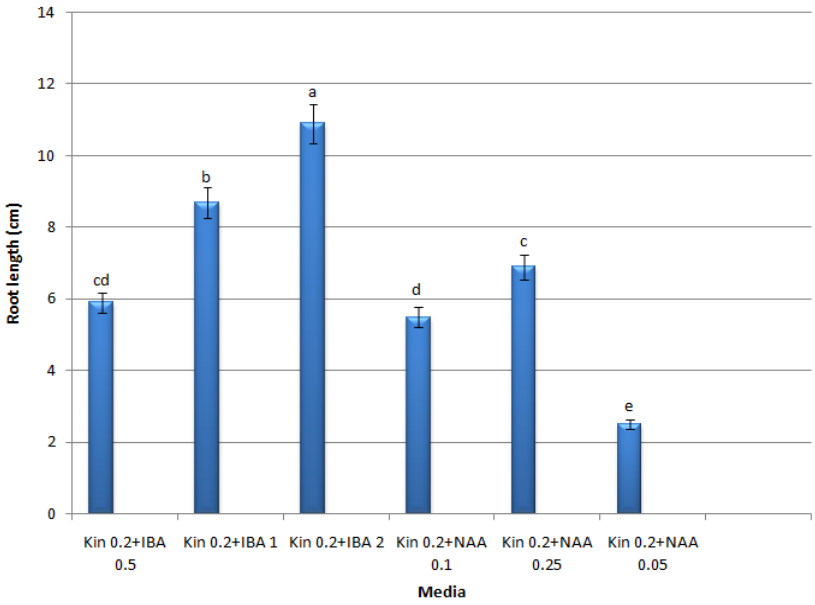

Fig 9. Mean comparison of the effect of different culture media on root length of *Anthurium andreanum* Linden.

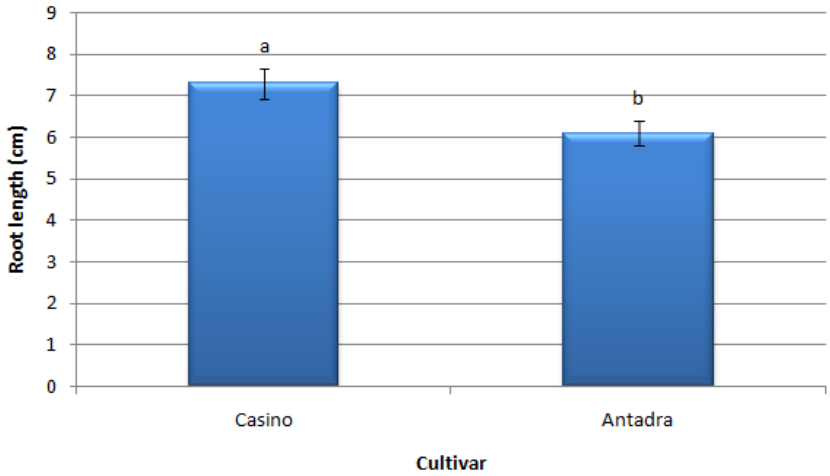

Fig 10. Mean comparison of the effect of different cultivars on root length of *Anthurium andreanum* Linden.

Fig 11. Process of plantlets hardening (a-c). Plantlet established in plastic cup filled with peat: perlite: sand (1:1:1) (a). Plantlet established in the greenhouse (b). Hardened plantlet in plastic pot (c).

Two traits of days to the beginning of callus induction and weight of callus were evaluated for the effect of explants age on callus induction.

Cultural conditions after treatments

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 µ mol/m^2/s with a photoperiod of 16 h per day. Data were recorded at 4-14 weeks after culturing.

Hardening

The well-developed seedlings were removed from culture vessels and thoroughly washed with tap water to remove adhering medium completely without causing damage to the roots. Then the plantlets were transferred to the plastic pots filled with a mixture of peat: perlite: sand (1:1:1) and placed into the greenhouse at 27 ± 1°C, light density of 4000 lux and 70% RH. Crystallon liquid fertilizer was added to irrigation water and plants were watered two times a day (morning and evening) as misting.

Experimental design and statistics

The experimental design was factorial with R.C.B.D design, 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.

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