Optimization of In-Vitro Propagation, Regeneration Through Somatic Embryogenesis and Establishment of Callus, Cell Suspension Culture of *Anthurium Andrenum* Plants

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ABSTRACT

INTRODUCTION

Anthurium industry plays a significant role in the global floriculture trade. To overcome the demerits of conventional vegetative propagation, micropropagation of Anthurium, through tissue culture is the most alternative techniques to increase the production. Anthurium andreanum, initiation was studied using mid, proximal and distal end of young leaves on full, half and ¾ strength MS media by reducing only the macro nutrients.

Initiation and development from embryogenic mass derived from leaf explants of *Anthurium andreanum* were conducted. Embryos showed a sequence of developmental patterns from globular to heart and finally cotyledon stages. Only BAP 1 mg/L and 2, 4-D 0.1 mg/L combination with ¾ strength MS was found to be the best for somatic embryogenesisusingmid and distal region of the leaf. Shoot multiplication was observed best on MS medium supplemented with BAP 1 mg/L and Kinetin 1mg/L. Optimal number of rooting was obtained on simple MS medium without any Plant Growth Regulator (PGR). The regenerated plants were shifted to a greenhouse for acclimatization, coconut husk and charcoal (1:1) presented the best result for acclimatization of *Anthurium*.

Friable callus of *A. andreanum* was established on ½ MS (Murashige & Skoog 1962)medium with BAP (1mg/L) and 2, 4-D (0.5 mg/L).

Anthurium is one of the most popular and largest genus with 500 species of Araceae family. Anthurium is known for its attractive long lasting inflorescence with beautiful colors and shapes. These characters make the Anthurium an ever demanding plant as it is being used continuously as indoor decors (Dufour and Guerin, 2003). Among tropical cut flowers, its trade value stands second after Orchids. One of the vegetative propagation methods for anthurium is through seed propagation which requires approximately three years for the plant to be completely developed in a breeding program (Hamidah et al., 1997). Moreover, slow germination rate and low seed viability are limiting factors for the large scale production of anthurium (Martin et al., 2003) plants obtained through seeds have usually poor uniformity and heterozygosity due to cross pollination (Bejoy et al., 2008). On the other hand, plant tissue culture technology has emerged as an alternative method for large scale production of true-to-type anthurium at faster rate than conventional methods (Chen et al., 1997).

Various explants like seedling, leaf, petiole, spathe, lateral bud, or shoot tips are used for anthurium micro-propagation (Atta-Alla et al., 1998; Joseph et al., 2003). The factors affecting regeneration of anthurium are age, genotype and choice of explant for the initiation.Although, regeneration from embryogenic calli is more reliable for rapid mass propagation while direct somatic embryogenesis results in high multiplication rate with high genetic integrity.

Some modifications of different hormonal and macronutrient concentrations in culture media such as reduced amount of macronutrients for somatic embryos induction may lead to ideal regeneration system for massive plant propagation of anthurium. This improvement could have economic importance by contributing towards enhanced production of anthurium plants along with limited consumption of nutrients, which leads to its commercial application on a large scale development of anthurium by tissue culture technology.

MATERIAL AND METHODS

Plant material and sterilization

Young green leaves of *Anthurium andraeanum* were collected from mature plants. Initially different explants were used such as spadix, petal, and leaves were sterilized with different sterilants such as NaOCl (bleach) (20%) and mercuric chloride (0.1%) for different time intervals (Graph 1a). Young leaf explants were sterilized with different concentrations of NaOCl (bleach) and mercuric chloride (Graph 1b). After 20 minutes of gentle agitation in sterilization, the leaves were rinsed thrice with sterile distilled water. Sterilized leaves were cut into, proximal, midrib and distal end of appropriate sizes ($1cm^3$) in the laminar flow cabinet under sterile conditions.

All different types of explants were inoculated on modified MS (Murashige and Skoog,1962) medium with macronutrients at full, 1/2 and 3/4 strength. The micronutrients usedwere full strength in each case with 100 mg/L myo-inositol and MS vitamins. Table sugar instead of sucrose was added to the media as a source of carbon at the concentration of 30g/L. Plant hormones like BAP, 2,4-D alone and in combinations were supplemented with MS ¾ +BAP (0, 0.5, 1, 1.5 and 2) mg/L with 2, 4-D (0, 0.1 and 0.2) mg/L for somatic embryogenesis. For multiplication MS + BAP (0, 0.5,1, and 1.5) mg/L + Kinetin (0, 0.5, 1) mg/L + Sugar 40 g/L were used. All cultures were placed under dark condition at 25 ± 2^0C. Somatic embryo formation was started in *Anthurium andraenum* after three weeks at ¾ MS in combination with 1 mg/L BAPand 0.1 mg/L 2, 4-D and then followed to embryoid differentiation. The embryos were white to pale yellow in color. After 2 months embryos turned green in color. The induced shoots were transferred to full MS supplemented with various concentrations of BAP (0-1.5mg/L) and KN (0-1mg/L) to enhance shoot multiplication. All cultures were placed at $25\pm2^{\circ}$C witha 16 h photoperiod.

Rooting

The newly formed shoots were excised and used for the induction of *in- vitro* rooting. MS media without any plant growth regulator with 30 g/L sugar was used for rooting. Twenty explants were selected for each treatment and all the best treatments were repeated twice.

Acclimatization

Plantlets with fully developed roots were removed from jars, washed in lukewarm water to ensure that all media/gel were removed and plants were transferred to pots containing different types of potting mix like charcoal (100%), coconut husk (100%), mixture of coconut husk + charcoal (50:50), soil (100%), marble peats (100%) and soil + manure (50:50) in the green house.

Callus culture

Leaf explants of *Anthurium andraeanum* cultures were transplanted onto the jars containing callus induction medium. This medium consisted of half-strength MS (Murashige & Shoog, 1962) basal salt and vitamins supplemented with 30 g/l sucrose, 6 g/l agar, with various concentrations of BAP (benzylaminopurine) and 2, 4-D (2, 4-dichlorophenoxyacetic acid). All cultures were placed in the dark at 27°C for one month for callus initiation (Cimen Atak & Ozge Celik 2009; Puchooa & Sookun 2003; Nhut et al., 2006; Te-chato et al., 2006).

RESULT AND DISCUSSION

In-vitro propagation

Various explants from different sources exhibited different responses on treatment with various sterilants (Graph-1). After screening of explants on the basis of low contamination rate, only leaf explants were processed for various concentrations of NaOCl (20 min.) and mercuric chloride (5-7s min.). 20% NaOCl for 20 min. showed maximum survival rate andconsidered as the best for sterilization (Graph- 1). Leaf was chosen as the best explant for further experiments for somatic embryo induction, as Leaf explants from micropropagated plants were found to be more responsive than other plant portions for somatic embryos induction in *Anthurium andraeanum* (Hamidah et al., 1997).

In a preliminary experiment, three portions of the leaf explants (proximal end, mid and distalregion) were selected for initiation on modified Murashige and Skoog (1962) medium of full,

½ and ¾ strength of macronutrients along with various concentrations of BAP and 2,4-D.Full and ½ strength MS media showed slow development of somatic embryos on all leaf portions whereas the mid and distal region of leaf grown on ¾ strength MS medium provedto be the best for somatic embryo induction after 8 weeks (Table- 1). Only M8 (BAP; 1 mg/Land 2, 4-D; 0.1 mg/L), M9 media (BAP 1 mg/L and 2, 4-D 0.2 mg/L), M10 (BAP; 1.5 mg/Land 2, 4-D; 0 mg/L) and M11 (BAP; 1.5 mg/L and 2, 4-D; 0.1 mg/L) showed its effect for all parts of leaf but maximum somatic embryo percentage was observed from mid and distal regions of leaf on M8 media (Table- **1a**). From this preliminary result, mid and distal regions of the leaf were chosen as the best part for further experiments to get somatic embryos (Table-**1a**). It is believed that proximal end of the leaf had more shoot regeneration potential as compared to the distal end which may be due to the difference in maturity between the regions (Welander, 1988; Martin et al., 2003). Typically, when embryogenic tissue is exposed to a medium lacking auxin (or to one containing only a low auxin concentration) numerous somatic embryos are formed,superficially. Together with embryos which may have been present in the tissue prior to the transfer, these sometimes develop into plantlets, but sometimes a second transfer ontoan auxin-free medium may be necessary. Somatic embryos formed in the presence of auxin give rise to clumps of embryogenic cells if they are again exposed to high auxin concentrations. Assuming that the level within cultured tissues is in equilibrium with that in the medium, there are several ways in which the concentration of exogenously supplied auxin may be reduced to promote embryo formation (George, 1996).

After 3 weeks, somatic embryo induction started in dark along cut edges of the explants. Only M8 media (BAP 1mg/L and 2, 4-D 0.1mg/L) induced the best somatic embryos, it was also observed at lower and higher concentrations of above mentioned plant growth hormones but with lower frequencies (Table -**1b**). The lowest concentration of 2, 4- D indicated that there is requirement for auxin free environment for somatic embryos induction. The continuous incubation in darkness was found to enhance the process of somatic embryogenesis. The calli were pale yellow in color but after 2 months when it was transferred to light it turned green in color with induced shoots Fig- **1 (a, b, c**). The differences in the regeneration capacity and mode of regeneration at higher concentrations and lower than optimum, may be explained by the variation in the endogenous levels of these growth hormones in the leaf tissues. The division and sub- culturing of somatic embryos were done after every 8 weeks. For the study of the different stages of the somatic embryo development, the embryoids were harvested at various time intervals, fixed, sectioned, stained and examined, microscopically.Transferring the somatic embryos from the dark to light and to full MS supplementedwith various concentrations of BAP and kinetin gave shoot induction and multiplication

(Table -**1c**). Effective regeneration and healthy shoot development were observed on R9 media (BAP; 1mg/L and KIN; 1mg/L) on MS full strength which showed maximumshoot formation with maximum number and length of the shoots (Table -**1c**; Fig- **1d**). Further increase in BAP concentration did not improve the rate of

shoot production whereas lower amount of BAP and light enhances shoot development and proliferation. These shoots were both adventitious and axillary in nature. The shoots were sub-cultured after 60 days intervals on the same media. Subsequent cultures exhibited faster multiplication of shoots at the rate of 50 shoots within 60 days of culture. Allowing the regenerated shoots to stand for two months on MS basal medium supplemented with BAP (1mg/L) and Kinetin (1mg/L) caused spontaneous rooting to occur. However, transferring the shoots to full strength MS medium without any plant growth regulators improved rooting by developing 7-9 roots on each shoot which was good for acclimatization Fig-**1(e)**.

After four weeks of development on the rooting medium, plantlets were transferred topots with different potting mixtures (Table- **1d**) which were kept in the green house for acclimatization. Under these conditions, coconut husk and charcoal (1:1) ratio gave the best growth for acclimatization, started new growth within 15 days and continuouslygrew quite well Fig-**1(f)**.

After 2-3 months of acclimatization, 95% of the plantlets survived in the green houses Fig-1 (m, n). Finally, the plants were shifted to the field.

Table 1a. Percentage of somatic embryo induction on different leaf portions of *A.andreanum.*

Code	PGRs		Somatic embryos (SE) % of leaf mid portion	SE % of leaf proximal end	SE % of leaf distal end
	Cytokinin	Auxin			
	BAP	2, 4- D			
M1	0	0	0±0	0	0
M2		0.1	29.8±0.62	0	34.1±0.9
M3		0.2	15.07±0.21	0	10.66±0.76
M4	0.5	0	10.33±0.34	0	10.73±0.66
M5		0.1	29.9±0.34	0	40.5±0.62
M6		0.2	20.0±0.20	0	24.53±0.50
M7	1	0	49.96±0.35	0	55.06±0.20
M8		**0.1**	**89.76±0.68**	**20.03±0.45**	**94.9±0.75**
M9		0.2	80.9±1.08	10.73±0.66	82.9±0.78
M10	1.5	0	19.96±0.65	8.67±0.58	14.96±0.65
M11		0.1	50.3±0.3	5.09±0.17	50.06±0.20
M12		0.2	29.33±0.58	0	40.6±0.65
M13	2	0	5.1±0.18	0	10.16±0.15
M14		0.1	25.39±0.45	0	30.3±0.32
M15		0.2	30.6±0.79	0	35.44±0.42

Means ± Standard Error

Table 1b. Somatic embryos induction at ¾ MS media with different plant growthregulators and their combinations.

Code	Plant Growth Regulators		% of explants produced SE	Response of Explant
	Cytokinin	Auxin		
	BAP	2, 4- D		
M1	**0**	**0**	0±0	-
M2		0.1	34.6±0.36	+
M3		0.2	15.10±0.26	+
M4	0.5	0	19.65±0.34	+
M5		0.1	44.80±0.53	+
M6		0.2	40.03±0.15	+
M7	1	0	60.93±0.81	++
M8		**0.1**	**89.76±0.58**	**++++**
M9		0.2	74.8±0.43	+++
M10	1.5	0	22.6±0.79	+
M11		0.1	54.5±0.78	++
M12		0.2	45.73±0.66	+
M13	2	0	10.12±0.12	-
M14		0.1	34.63±0.35	+
M15		0.2	40.26±0.92	+

Key: +, ++, +++, ++++ represents, 25%, 50%, 75% and 100% respectively

Means ± SE

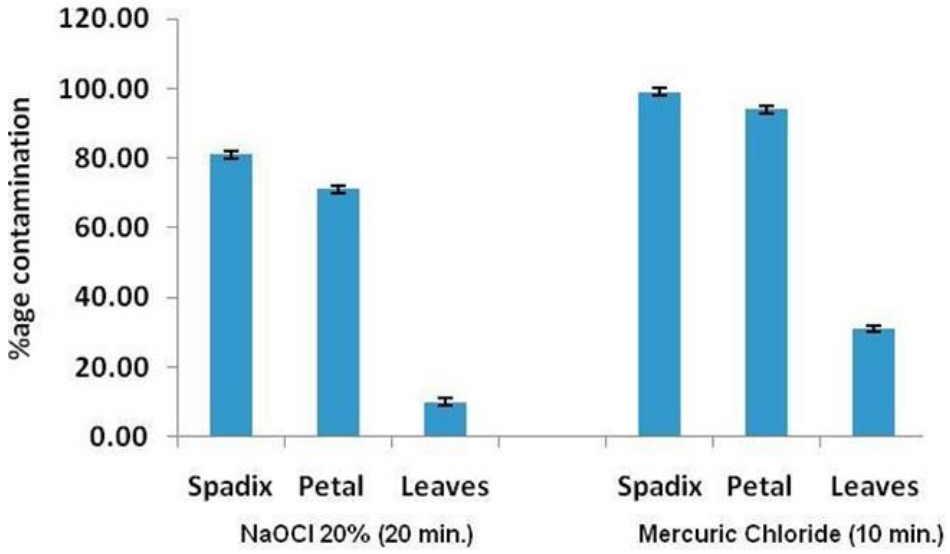
Table 1c. Effect of plant growth regulators on length and No. of shoots of *A. andreanum* regenerated from somatic embryos.

Code	Cytokinins		No. of regenerated shoots	Length of shoot
	BAP	KIN		
R1	0	0	0±0	0±0
R2		0.5	10±0.1	1.64±0.55
R3		1	14.8±0.32	1.44±0.05
R4	0.5	0	18.06±0.12	2.09±0.17
R5		0.5	37.43±0.51	3.38±0.15
R6		1	41.66±0.57	4.89±0.06
R7	1	0	46±0.1	4.98±0.01
R8		0.5	45±1	4.37±0.14
R9		**1**	**50.43±0.51**	**6.57±0.20**
R10	1.5	0	34.26±0.10	5.03±0.06
R11		0.5	32.1±0.1	6.04±0.05
R12		1	40.43±0.51	5.03±0.07

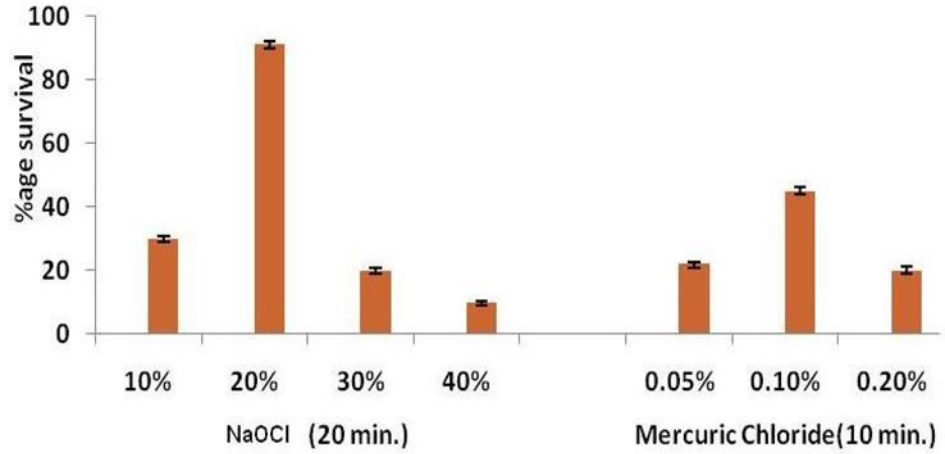
Means ± SE

Table 1d. Relative growth pattern of *A. andreanum* under different acclimatization supporting mediums.

support medium	% growth	Pattern	Remarks
Charcoal	70	Healthy Leaves and flowers	Very good
Soil	40	Small curling leaves, no flowering	Not good
Sand	45	Small curling leaves, no flowering	Not good
Farm Yard Manure	40	Small curling leaves, no flowering	Not good
Coconut Husk	60	Healthy leaves and flowers	Better
Coconut Husk + Charcoal	**95**	**Healthy, full size leaves and flowers**	**Best**
Soil + Farmyard Manure	45	Small curling leaves, no flowering	Not good
Soil + Sand	50	Small curling leaves and flowers	Not good



Graph 1a. Response of *A. andreanum* explants to different sterilants.



Graph 1b. Percent survival of leaf explants of *A. andreanum* for various sterilantconcentrations.

Figure 1a. Micropropagation studies of *Anthurium andreanum.*

Callus culture

Callus formation of Anthurium was observed on the leaf explants in ½ MS medium. Table **1e** shows different combinations of BAP, 2, 4-D and the percentage of explants that induced callus formation from the leaves. Explants showed callus formation in 6-7 weeks in medium with BAP (0, 0.5 and 1mg/l) and 2, 4-D (0, 0.1, 0.2, 0.5, 0.7 and 1mg/l). Out of 16 combinations (A1 to A16, Table-1e) best combination using PGRs was observed in A14 (80% callus with friable cum compact texture, Table-**1e**) for the callus induction and same medium devoid of agar was used for cell suspension culture preparation that was later used for biotransformation studies. Suspension thus prepared was good as callus easily dispersed when transferred to the liquid medium (Figure 1b). Rest of the media formulations resulted in greenish off white to off white color, while control did not yield any callus. Callus induction rate per explants had different responsesto the medium with different combinations of plant growth regulators Other treatments that showed higher callus induction rates were A9-10 (60%), but the calli were more on the compact side. i.e., not feasible for biotransformation studies.

The explants on ½ MS (Murashige & Skoog 1962) medium with BAP (1mg/L) and 2, 4- D (0.5 mg/L) observed highest callus formation.

Cell suspension culture

10-15 gms of the friable callus were found enough to form suspension in 25 days from inoculation time in the liquid medium (Figure-**1b**). Lesser amount of the callus such as 5 gms and less than that were not producing good cell suspension culture. Agitation of the suspension was at moderate speed i.e. 95 rpm.

Table 1e. BAP and 2, 4-D effect on the callus induction of *A. andreanum*.

MediaCodes	Growth Regulator		Callus Formation Frequency %	Description of Callus	
	(mg /L)			Color	Texture
	BAP	2,4-D			
A1	0	0	-	-	-
A2	0	0.1	30	Off white	Compact
A3	0	0.2	30	Off white	Compact
A4	0	0.5	40	Off white	Compact
A5	0	0.7	40	Off white	Compact
A6	0	1	30	Off white	Compact
A7	0.5	0	50	Light green	Compact
A8	0.5	0.2	50	Greenish off white	Compact
A9	0.5	0.5	60	Greenish 0ff white	Friable cum compact
A10	0.5	0.7	60	Greenish off white	Friable cum compact
A11	0.5	1	20	Greenish off white	Compact
A12	1	0	30	Greenish off white	Compact
A13	1	0.2	50	Greenish off white	Friable cum compact
A14	1	0.5	80	Greenish off white	Friable cum compact
A15	1	0.7	50	Greenish off white	Compact
A16	1	1	30	Greenish off white	Compact

(a) Callus (b) Cell suspension culture

Figure 1b. Callus induction and cell suspension culture of *Anthurium andreanum*

REFERENCES

1. Atak, Ç. and Ö. Çelik. Micropropagation of Anthurium spp. pp. 241-254. In: Dhal, N.K. and S.C. Sahu (eds.). Plant science. In: Tech Press, Rijeka, Croatia. (2012).
2. Adelbeid, R., & Kuehnle, N. S. Somatic embryogenesis and plant regeneration in Anthurium andraeanum. Plant Cell Reports, (1992). 11(9), 438-442.
3. Atak, C., & Celik, O. Micropropagation of Anthurium andraeanum from leaf explants. Pak J Bot, (2009). 41(3), 1155-1161.
4. Atta-Alla H, Mcalister BG, Van Staden J In vitro culture and establishment of Anthurium parvispathum. South African Journal of Botany (1998). 64, 296-298
5. Bhavana, G.P., Satyan, K.B. and Aswath,C. A regenerative protocol and SEM study for in vitro propagation of Anthurium crossed lines via indirect somatic embryogenesis. Biosci. Biotech. Res. Comm., (2018). 11(1): 31-40
6. Bejoy, M., Sumitha, V. R., & Anish, N. P. Foliar Regeneration in Anthurium andraeanum Hort. cv. Agnihothri. Biotechnology, (2008). 7(1), 134-138.
7. Brar, D. S., & Jain, S. M. Somaclonal variation: mechanism and applications in crop improvement. In Somaclonal variation and induced mutations in crop improvement Springer Netherlands. (1998). (pp. 15-37).
8. Chen, F. C., Kuehnle, A. R., & Sugii, N. Anthurium roots for micropropagation and Agrobacterium tumefaciens-mediated gene transfer. Plant Cell, Tissue and Organ Culture, (1997). 49(1), 71-74.
9. da Silva, J. A. T., Nagae, S., & Tanaka, M. Effect of physical factors on micropropagation of Anthurium andreanum (2005).
10. Desai, C., Inghalihalli, R., and Krishnamurthy, R. (2015). Micropropagation of Anthurium andraeanum-A important tool in floriculture. J. Pharmacogn. Phytochem., 4(3): 112-117. E-ISSN: 2278-4136
11. Dufour, L., & Guerin, V. Growth, developmental features and flower production of Anthurium andreanum Lind. in tropical conditions. Scientia Horticulturae, (2003). 98(1), 25- 35.
12. Gamborg, O. L., & Shyluk, J. P. The culture of plant cells with ammonium salts as the sole nitrogen source. Plant Physiology, (1970). 45(5), 598-600.
13. Gantait, S., & Mandal, N. Tissue Culture of Anthurium andreanum: A Significant Review and Future Prospective. International Journal of Botany, (2010). 6(3), 207-219.
14. Geier T Anthurium. In: Ammirato PV, Evans DA, Sharp WR, Bajaj YPS (Eds) Handbook of Plant Cell Culture, Ornamental Species (Vol 5), McGrawn-Hill, New York, (1990). pp 228-252.
15. Geier, T. Morphogenesis and plant regeneration from spadix fragments of Anthurium scherzerianum cultivated in vitro. In Plant tissue culture 1982: proceedings, 5th International Congress of Plant Tissue and Cell Culture held at Tokyo and Lake Yamanake, Japan, (eds. Akio Fujiwara). (1982). (pp. 137-142). Tokyo: Japanese Association for Plant Tissue Culture.

16. Hamidah, M., Karim, A. G. A., & Debergh, P. Somatic embryogenesis and plant regeneration in Anthurium scherzerianum. Plant Cell, Tissue and Organ Culture, (1997). 48(3), 189-193.
17. Hammerschlag, F. A., & Litz, R. E. Somaclonal variation. Biotechnology of perennial fruit crops, (1992). 35-55.
18. Hazra, S. K., Das, S., & Das, A. K. Sisal plant regeneration via organogenesis. plant cell, tissue and organ culture, (2002). 70(3), 235-240.
19. Jiménez, V. M. Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regulation, (2005). 47(2-3), 91-110.
20. Joseph, D., Martin, K. P., Madassery, J., & Philip, V. J. In vitro propagation of three commercial cut flower cultivars of Anthurium andraeanum Hort. Indian journal of experimental biology, (2003). 41(2), 154-159.
21. Kuehnle, A. R., Chen, F. C., & Sugii, N. Somatic embryogenesis and plant regeneration in Anthurium andraeanum hybrids. Plant Cell Reports, (1992). 11(9), 438-442.
22. Maalej, M., Chaari-Rkhiss, A., & Drira, N. Contribution to the improvement of olive tree somatic embryogenesis by mineral and organic analysis of zygotic embryos. Euphytica, (2006). 151(1), 31-37.
23. Martin, K. P., Joseph, D., Madasser, J., & Philip, V. J. Direct shoot regeneration from lamina explants of two commercial cut flower cultivars of Anthurium andraeanum Hort. In Vitro Cellular & Developmental Biology-Plant, (2003). 39(5), 500-504.
24. Matsumoto, T. K., & Kuehnle, A. R. Micropropagation of Anthurium. In High- Tech and Micropropagation VI (1997). (pp. 14-29). Springer Berlin Heidelberg.
25. Merkle, S. A., Wiecko, A. T., & Watson-Pauley, B. A. Somatic embryogenesis in American chestnut. Canadian Journal of Forest Research, (1991). 21(11), 1698-1701.
26. Prakasha, D.P., Ramya, G., and Srinivasalu, G.B. In Vitro Mass Multiplication of Anthurium andreanum (HORT) Cultivars. Int. J. Curr. Microbiol. App. Sci., (2017). 6(9): 2579- 2584
27. Puchooa, D., & Sookun, D. Induced Mutation and in vitro Culture of Anthurium andreanum.AMAS. Food and agricultural Research Council, Reduit, Mauritius (2003)..(1-12 paginas).
28. Saptari, R.T., Sinta, M.M., and Budiani, A. In Vitro Propagation of Anthurium adreanum cv. Nitta through Organogenesis. AGRIVITA J. Agric. Sci., (2017). 39(2): 192-200
29. Thokchom, R. and Maitra, S. Micropropagation of Anthurium andreanum cv. Jewel from leaf explants. J. Crop Weed, (2017). 13(1): 23-27. ISSN: 2349-9400.
30. Te-chato, S., Susanon, T., & Sontikun, Y. Cultivar, explant type and culture medium influencing embryogenesis and organogenegenesis in Anthurium spp. Songklanakarin J. Sci. Technol., (2006). 28(4): 717-722.