Volume 64, Issue 2, 2020
Journal of Scientific Research
Institute of Science,
Banaras Hindu University, Varanasi, India.

Callus Immobilization and Effect of Plant Growth Regulators on Callus Induction and Multiplication of *Annona Muricata*

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Abstract: ***Annona muricata*** **is the member of the family annonaceaae. Numerous studies have substantiated its antidiabetic, antimalarial, anticancer, antiparasitic, antiarthritic and hepatoprotective activities. In the present study, callus induction was done from leaf explants plant hormones, multiplied and immobilized for its conservation. Different plant growth regulators had various responses in callus induction and multiplication with the combination of 2, 4-d 2mg/l+BA 1mg/l showed best response of 97% in induction and of 95% in multiplication.**

Index Terms: Annona muricate, **callus immobilization, callus induction, callus multiplication, Plant Growth Regulators.**

I. Introduction

The medicinal plants are globally valuable source of herbal products and they are disappearing at a high speed. The conservation and sustainable utilization of this vital biological heritage is therefore imperative and paramount. *A. muricata* has been shown to possess significant DPPH scavenging activities. (Omolara *et al.,* 2016). Previous studies demonstrated significant cytotoxiceffect of *A. muricata* leaves against various cancer cell lines without affecting the normal cells (George*et al*., 2012, Mishra*et al*., 2013). Due to this tremendous antiproliferative effect, *A. muricata* was described as "the cancer killer" (Mishra*et al.,* 2013).

Based on the importance and its biomedical applications, a well-known Indian medicinal plant *Annona muricata* was selected for the present study since it can treat a myriad of conditions including hypertension, diabetes and cancer. *Annona muricata* is a tropical plant species belonging to the family Annonaceae, widely used in indigenous system of medicine in India and abroad. A total of 1,741 germplasm accessions of eleven identified species, one interspecific hybrid and various *Annona* spp. are documented (IPGRI, 2000), with a surprisingly low percentage of duplication across the 67 institutional collections in 34 countries. The *Annonas* are slow growing semi-deciduous trees (Samson, 1986) which drop their leaves during the cool season and remain bare and dormant for several months with the exception of *A. muricata*. The length of the juvenile period varies between accessions; however, the first important fruit production begins after eight years. The juvenile period is very variable and influenced by seedling root stock and type of scion. (Jordan and Botti, 1992).

Although several studies have appeared on Annona Tissue culture (Rasai*et al.*, 1995; Lemos and Blake, 1996 (a); Padilla, 1997; Encina *et al.*, 1999; Lemos, 1996(b)) a great deal of work remains to be done on the development of methods for invitro propagation of germplasm conservation of *Annona* species. In the present study, an attempt has been undertaken for tissue culture studies of the plant and *in vitro* conservation through callus immobilization.

II. Materials and Methods

A. Plant Material

The mother plant was collected from Siddha Vidya Abhyasalayam, Arayoor during March 2017, July 2017, September 2017, January 2018, February 2018 and June 2018. Arayoor is a small Village in Neyyattinkara Taluk in Thiruvananthapuram District. It comes under Chenkal Panchayath. It belongs to South Kerala Division. Arayoor is located 5 km from Parassala, 6 km from Neyyattinkara and 30 km from Thiruvananthapuram. Arayoor lies between Global Positioning System coordinates of 8.3615° N, 77.1299° E. The explant used was leaf segments. Healthy and fresh leaves of *Annona muricata* was collected from locally grown plants.

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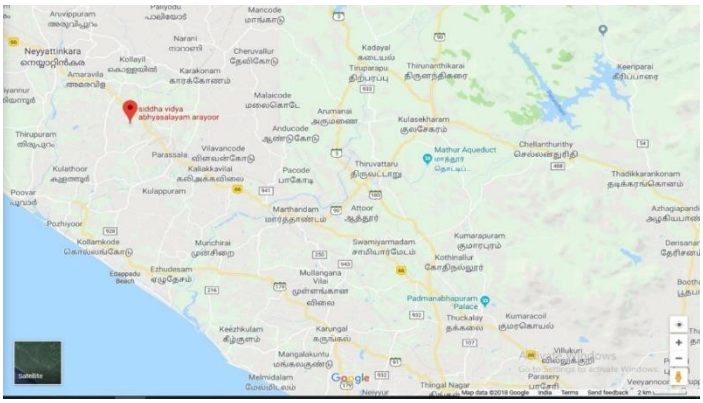
DOI: 10.37398/JSR.2020.640221

Fig 1. Plant sample used as source of explant for Tissue Culture and its locality



Map 1. Location Map of the Area of Collection of Plant Sample used for Tissue Culture. [Google map]

B. Glassware

Glasswares used during the from M/s. Borosil Glass Work Ltd.

C. Chemicals and other media ingredients

Chemicals required for media preparation were of analytical grade and were obtained from HiMedia Laboratories Pvt. Ltd.

D. Preparation of Explants

Leaves were detached and brought from the field to the Tissue Culture Laboratory. The leaves were cut into 1-2 cm^2 length cuttings and were used as explants to establish *in vitro* cultures. The procedure of surface sterilization followed is given here under.

Leaf explants were washed under running tapwater with 5.1%Teepol solution. Thereafter, they were immediately soaked in solution of 2%bavistin and K-cycline which served as fungicide and bactericide respectively. The explants were soaked for 1hrs. Then the explants were transferred to the Laminar Air Flow cabinet for Surface sterilization.

E. Surface sterilization

- Explants were washed with sterile water
- Explants were washed with 70% alcohol for 30 min
- Explants were washed with sterile distilled water for 2 or 3 min
- The explants were washed with 0.1% mercuric chloride + Tween 20 (1 or 2 drops) for 10 minutes.
- Then washed with sterile distilled water for four times.

First time -4 minutes
Second Time -4 minutes
Third Time-4 minutes
Fourth Time-12 minutes

After the surface sterilization was over, the explants were cultured on appropriate media.

F. Composition of tissue culture media

A modified Murashige and Skoog (1962) medium containing 3% sucrose solidified with 0.8% agar was used as the basal medium. The composition is given in the Table 1.

Table 1. Media Composition used in the present study.

Ingredients	Salt Concentration of stock solutions (g/l)	Amount taken for 1 liter medium (mg/1)	Final concentration of salt in a 1 liter culture	Strength of the final concentration
Macro-nutrients				
KNO_3	19		1900	
$NH4NO_3$	165	100	1650	10 x A
$CaCl_2.2H_2O$	4.4		440	
$MgSO_4.7H_2O$	3.7		170	
$KH_2.PO_4$	1.7		170	
Micro-nutrients				
$MnSO_4.4H_2O$	2.23		22.3	
$ZnSO_4.7H_2O$	0.86		8.6	
H_3BO_3	0.62	10	6.2	100 x B
$CuSO_4.5H_2O$	0.025		0.0025	
KI	0.083		0.83	
$Na_2MoO_4.2H_2O$	0.025		0.25	
$CoCl_2.6H_2O$	0.0025		0.025	
$FeSO_4.7H_2O$	2.78		27.8	100 x C
Na_2EDTA	3.73	10	37.3	
Vitamins				
Myo-inositol	10		100	
Thiamine	0.01		0.1	
Nitrotinine acid	0.05	10	0.5	100 x D
Pyridoxide HCl	0.05		0.5	
Glycine	0.2		2	

Poly Vinyl Pyruvate 2mg/l

G. Culture media preparation:

Vitamins, micronutrients and macronutrients are drawn from the stock solution were mixed in the required quantity. The growth substances are added as necessary. Carbon source was sucrose and was dissolved at the rate of 30g/l (3%).The final volume of Known quantity obtained by adding double distilled water. The pH was adjusted to 5.0 by an addition of Hcl or NaOH as required.Agar-Agar was added to the boiling media at the rate of 8g/l (0.8%) slowly and gradually with constant stirring to avoid formation of clumps. Then the medium was aliquoted into culture vessels. These vessels were plugged with polypropelene caps and were then autoclaved along with other instruments required for transfer process at 121℃ and at apressure of 15lbs for 20min

1) *Potassium iodide stock solution KI (1000X):*
0.0830 (83mg) of KI was dissolved in 100ml distilled water. (Usage =1ml/L).

2) *Solution of Na_2-EDTA (100X):*
37.2mg of Na_2 EDTA (Ethylenediamine tetra-acetic acid, disodium salt) was dissolved in 50 ml distilled H_2O.

Boil Na_2EDTAsolution and dissolve 27.8 mg of $FeSo_4.7H_2O$ gently by stirring and final volume was made up to 100 ml. Usage-1ml/L.

3) *Calcium chloride stock solution (100X):*
Calcium chloride (4.4g) was dissolved in 80 ml of sterile distilled water and final volume was made up to 100 ml. Usage-10 ml/l.

4) *Myoinositol stock solution (10X):*
1000mg of myoinositol was dissolved in 100 ml distilled water. Usage-1ml/L.

5) *Role of Plant Hormones:*
Some chemicals occurring naturally within plant tissue have a regulatory rather than a nutritional role in growth and development. Growth as well as differentiation of tissues invitro is controlled by various hormones.

2, 4-D: (2, 4 dichlorophenoxy acetic acid) is a synthetic auxin known primarily as a weedicide.it is used for callus induction.

NAA (Napthalene acetic acid): It is also a root inducing hormone and also promotes callus induction.

BAP (Benzylaminopurine): It is used to promote auxillary bud growth.

Kinetin (6-Furfurylaminopurine :): promote cell division

H. Growth Regulators

Plant Growth Regulators used in the preparation of culture medium include 6-benzyladenine (BAP), kinetin (kn) 2, 4-dichlorophenoxyacetic acid (2, 4-D), 1-Napthaleneacetic acid (NAA). These were prepared in desired concentra*tions (to be* maintained as stock solution) to induce plant regeneration from explants

1) *6-Benzyl Adenine (BAP)*
6-benzyl adenine (BAP) is acytokinin [a syntheticN--(Phenylmethyl)-1H-Purine-6-amine compound]. 10 mg of 6-benzyl adenine (BAP) was dissolved in 1 ml of 1N NaOH and the final volume was made up to 10 ml with distilled water,filter sterilized and stored at 4℃.

2) *Kinetin (Kn)*
Kinetin (Kn) is a cytokinin[a synthetic N-(2-furanyl methyl)-1H-Purine-6-amine compound].10mg of kinetin was dissolved in 1ml of 1 N NaOH, volume was adjusted to 10ml with distilled water, filter sterilized and stored at 4℃.

3) 2, 4-Dichlorophenoxyacetic acid (2, 4-D)
10 mg of 2, 4-D was dissolved in 1ml of 1N NaOH, the volume was adjusted to 8ml with distilled water. Then final volume was made up to 10 ml with distilled water, filter sterilized and stored at 4℃.

4) *Naphthaleneacetic acid (NAA)*
10 mg of NAA was dissolved in 1 ml of 1 N NaOH, volume was adjusted to 8ml with distilled water. Then final volume was made up to 10ml with distilled water, filter sterilized and stored at 4°C.

I. Transfer area preparation for aseptic culture

Aseptic culture works like final surface sterilization of explant, preparation and inoculation of explants and further subculturing of invitro cultures were carried out in a laminar air flow cabinet. Before the use of the laminar air flow cabinet, the working surface was sterilized by swabbing the surface of the laminar air flow cabinet with 70%ethyl alcohol. Then it is sterilized by switching on UV light 2500A° for about 15 min before use. Then the sterile airflow was switched on and left for atleast 10 min before use. During transfer of explants the instruments were dipped in absolute alcohol followed by dipping them in glass head sterilization for 15-20sec.

J. Incubation room

The culture was incubated in an air conditioned room with temperature 25 ± 2ºC under a micropropagation region of 16 hours' light and 8 hours' dark cycle.

K. Growth Room

Each growth room has mobile culture incubation racks fitted with 40 watts cool day white fluorescent tube lights for providing light for photosynthesis of tissues. Growthroom is maintained clean was of CL 1,00,000 and temperature of 25±27ºC for temperature crops 16 hours' photoperiod and 8 hours darkness are provided in each growth room. The photoperiod and temperature is maintained.

L. Culture maintenance in Laboratory

The invitro cultures of *Annona muricata* or all the experiments were maintained at 25±2°C and 3000 Lux Ilumination comprising a 16-hour photoperiod provided by cool fluorescent light and with a relative humidity of 50±20%.

M. Culture Initiation

After surface sterilization the explants were cut into specific size with a scalpel on pre sterilized petriplates and embedded on media with various concentrations of hormones and with the lower surface firmly touching the media. The cultures were maintained for 4 weeks and after proper callusing they were subcultured on different media. All the experiments were repeated 3 times and 12 replications. The cultures were observed regularly to watch the growth and recorded the observations. Inoculated tissue culture explants are shown in Fig 2.

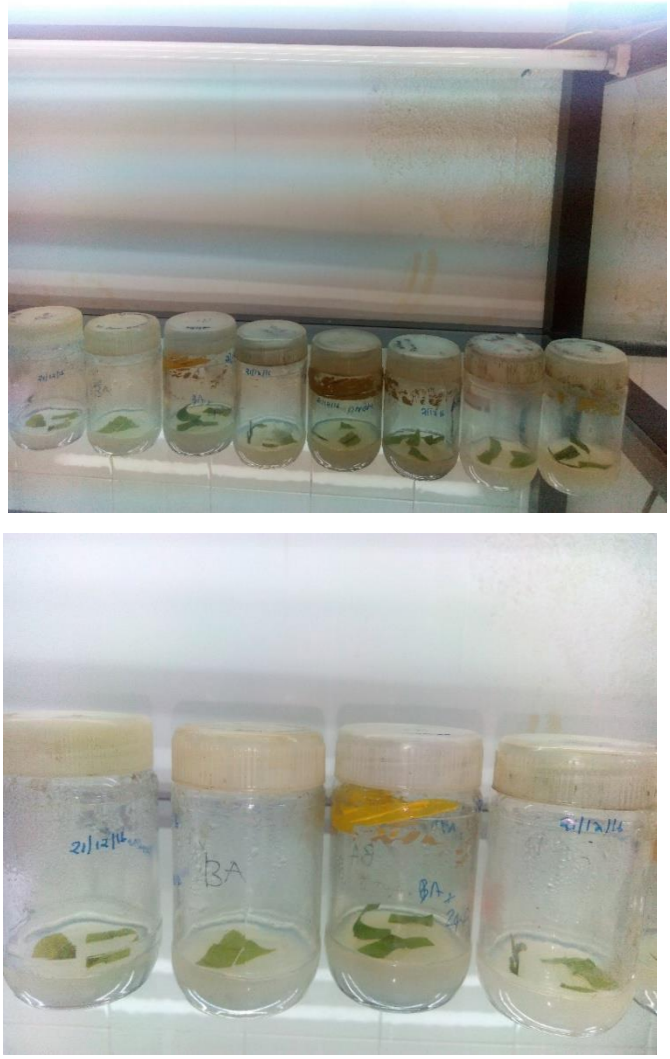


Fig 2. Innoculated Tissue Culture Explants

N. Callus induction

The MS basal medium fortified with various concentration of plant growth regulator tried for callus induction (Table 2).

Table 2: Plant Growth Regulators tried for the induction of callus

Treatment	Plant Growth Regulator (PGR) - (Mg/l)
T15	NAA 4 mg/l
T 22	2,4-D 1 mg/l+BA 1 mg/l
T 23	2,4-D 2 mg/l+ BA 1 mg/l

1) Callus Induction Frequencies (CIF)

Callus induction frequencies were calculated as the percent explants inducing callus by using following equation and were converted to mean CIF, asdescribed by Javed et al 2012.

Callus Induction Frequency (%) = number of calli producing explants/total number of explants in the culture ×100.

O. Callus multiplication

Actively growing callus can be initiated on culture media with an even physiological balance of growth hormones. After callus biomass increases two or four times (after 2-4 weeks of growth), callus can be divided and placed on fresh callus initiation medium for callus multiplication.

1) Surface sterilization

The callus was removed from the culture bottles and placed on sterile paper. Dried callus, hard callus and dead tissue were removed.

2) Callus subculture

The induced calli were sub cultured on MS medium fortified with various hormonal combinations (Table 3) after 14 days' interval and harvested after one month for further growth of the callus. The periodical observations were made during the experiment.

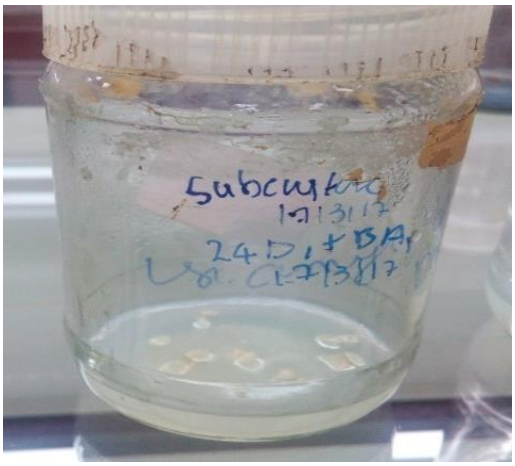


Fig 3. Callus subcultured for Multiplication

Table 3: The callus sub cultured for multiplication

Treatment	Plant growth (P G R) regulator (Mg/l)
M12	NAA4mg/l
M15	BAP2 mg/l
M16	BAP3 mg/l
M18	KINETIN2 mg/l
M27	2,4.D 1mg/l+BA1mg/l
M28	2,4.D 2mg/l+ BA1mg/l

P. In vitro conservation of Annona muricta by Callus Immobilization (Doblin et al., 2012)

In vitro conservation of *Annona muricta* was done by Callus Immobilization as described by Doblin*et al*., (2012).

1) Materials Required

Sodium alginate: 3.6 g of sodium alginate in 100 ml of distilled water.

Calcium Chloride: 4g of $CaCl_2$ in 100 ml of distilled water.

2) Procedure

Callus cells of *Annona muricata were* entrapped with 1.5% (wt/v) sodium alginate. The proliferated calli were cut into small pieces u and then cultured in a liquid medium with 5 mg/l 2, 4-D for a week with shaking at 90 rpm at 22°C for a photoperiod of 16 hours. Then the calli were placed on a sterilized filter paper and used forimmobilization. 1.5% (wt/v) sodium alginate was dissolved in ½ MS medium supplemented with 5 mg/l 2, 4-D. The calli were placed in sodium alginate solution sterilized at 121°C for 20 minutes. The sodium alginate solution was dropped into a swirling 50 mM $CaCl_2$ solution. Calcium alginate capsules were formed after 30 minutes and each capsules contained calli leave the beads of calcium

alginate entrapped callus in $CaCl_2$solution for about 1 hour. The beads were filtered using funnel and washed with distilled water. Then it was stored in respective hormonal medium at 4°C without photolight.

Fig 4. Callus immobilization

III. Result

A. Callus Induction

The plant *Annona muricata* was inoculated in based MS medium augmented with various concentrations of BA, 2,4-D and NAA, kinetin and BAP and combinations of BA, 2,4-Dfor callus induction (Table-5.62,Plate 5.88).Observations were taken based on the number of days taken for the induction of callus and nature of callus. The responses of calli are given in (Table 4, Fig 5).

Callus formation was observed from 4to 40 days of inoculation for callus induction. Callus formation on the explants was observed at wounding site of major veins and covered the whole explant. The best responses (97%) of callus induction was found on the medium containing 2, 4-D 2mg/l+ BA1mg/l (Table 4Fig 5, T.23). The calli formed were soft, friable or compact with white, grey-white brown and dark brown, pale green pale orange color. Although PVP was added, browning was observed but growth of callus was not affected due to browning (Fig 5. Table 4).

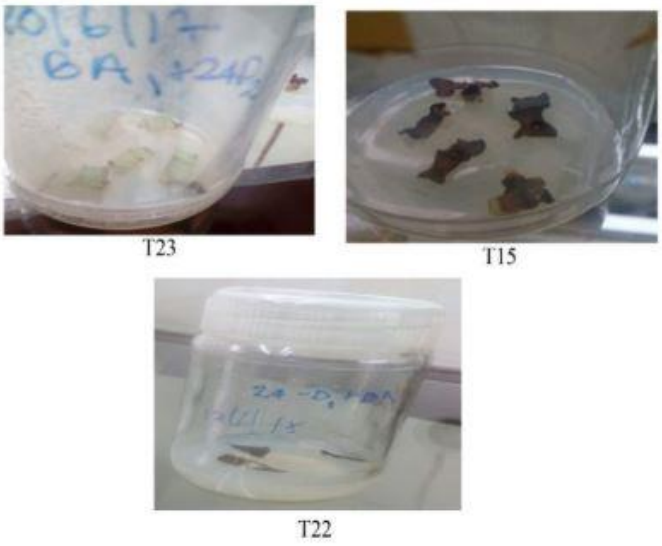


Fig 5. Effect of Plant Growth Regulators on Callus Induction of *Annona muricata* from leaf explants.

Table 4: Effect of Plant Growth Regulators on callus induction of *Annona muricata* from leaf explants.

Treatment	**Plant growth (P G R) regulator (Mg/l)**	Number of days taken for callus induction	Nature and colour of the callus produced	CIF %
T15	NAA4mg/l	23-25	Friable orange	20
T 22	2,4-D 1mg/l+BA1mg/l	26-29	Smooth white	24
T 23	2,4-D 2mg/l+ BA1mg/l	7-9	Friable yellow	97

B. Callus Multiplication

After 35 days of growth the induced calli which exhibited good response were sub cultured to basal medium augmented with various level of plant growth regulator. The growth responses of each calli in respective media are presented in (Fig 6, Table 5).

C. Callus immobilization

The calli produced by callus multiplication was immobilized by gel entrapment method. The well-developed calli of *Annona muricata* was encapsulated in sodium alginate beads and stored in MS basal medium with same growth regulators used for multiplication. The calli was retrieved from cultures in solid MS medium. The encapsulated calli (synthetic seeds) was stored in liquid MS medium with same hormone treatments used for multiplication. The immobilized callus was shown in Fig 7.

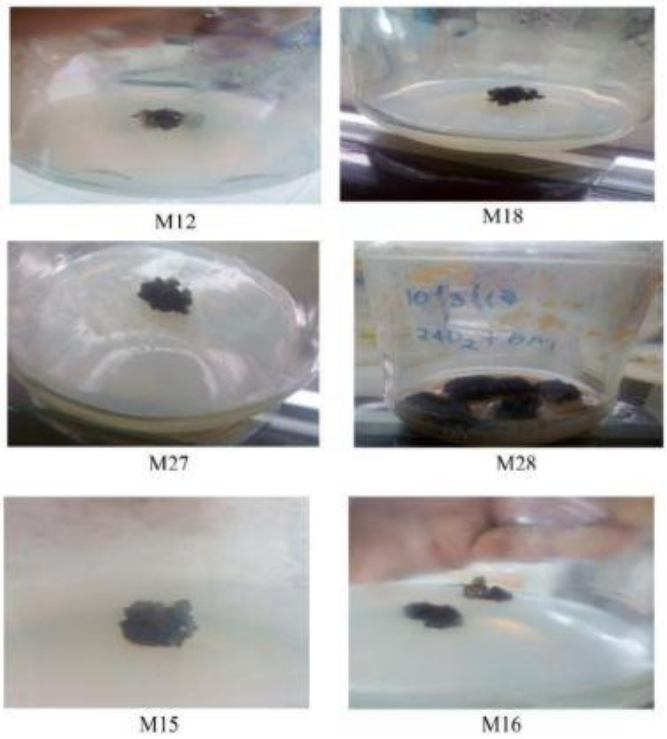


Fig 6. Effect of Plant Growth Regulators on Callus multiplication of *Annona muricata* from leaf explants.

Table 5: Effect of Plant Growth Regulators on Callus multiplication of *Annona muricata* from leaf explants

Treatment	Plant growth (P G R) regulator (mg/l)	Number of days taken for callus multiplication	Nature and colour of the callus	Percentage response
M12	NAA4mg/l	24-29	Friable pale green & brown	88
M15	BAP2 mg/l	17-20	Friable brown & pale green	88
M16	BAP3 mg/l	25-29	Friable brown & white	91
M18	KINETIN2 mg/l	22-25	Friable brown, white & pale green	82
M27	2,4.D 1mg/l+BA1mg/l	23-25	Friable pale green, white & brown	82
M28	2,4.D 2mg/l+ BA1mg/l	30-33	Friable brown	95

IV. Discussion

Conservation areas throughout America and central eastern Africa should be surveyed for the presence of Annona populations. However insitu conservations is not always possible or acceptable (Ndambuki, 1991). The seeds of Annona muricate tolerate dessication to 1.5% moisture content, and no viability loss occurred during 6 months of hermetic storage at -20°C (Hong*et al.*, 1996).

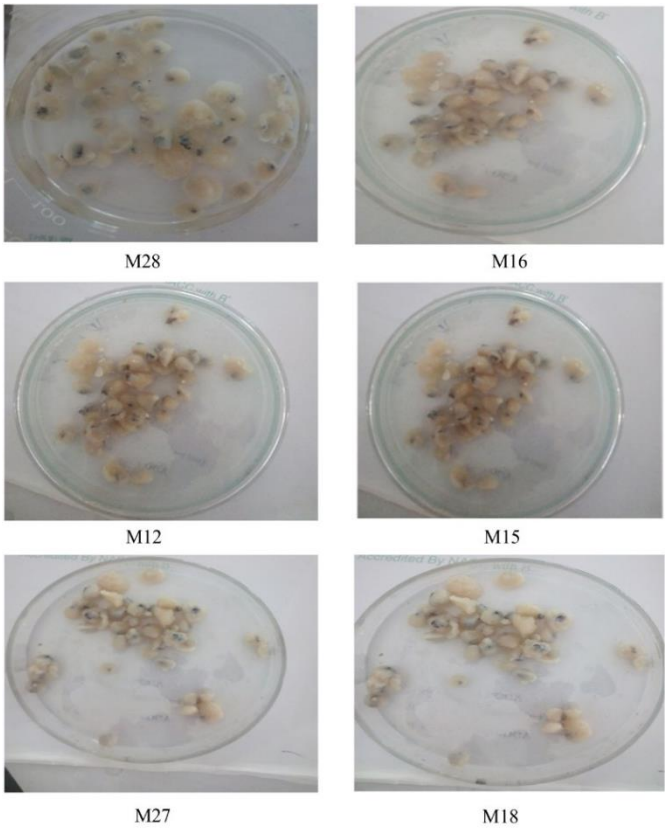


Fig 7. *In vitro* conservation of *Annona muricata* by Callus Immobilization

According to Gray and Purohit (1991), Gray*et al.,* (1995) and Litz and Gray (1995), the success in inducing dormancy and the accomplishment of long term storage, together with the achievement of encapsulation of somatic embryos have also opened up the possibility for their use in the synthetic seed technology. In the present study the calli produced by callus multiplication was immobilized by gel entrapment method. Callus immobilization of *Annona muricata* was not reported yet. This study thus validates conservational importance of *Annona muricata*.

The first haploid plants induced by another culture in fruit trees were reported by Nair et al (1983) with Annona muricate. The availability of haploids is very important for the fruit breeding, because of the long generation intervals, the highly heterozygous nature of most fruit species and the presence of parthenocarpy and self-incompatability. These researchers obtained callus differentiation, and formation of triploid roots and shoots from Annona muricate endosperm (Nair *et al.*, 1986). Their aim was to develop seedless fruits, but a complete plantlet was not obtained.

Van Beem*et al.,* 2014 reported the formation of compact callus of *Annona muricata* (light green color; diameter was 1.50x1.20cm) was showed at 86% in the medium of MS+0.2mg/l, BAP+0.2mg/l, NAA +0.8% and agar+2% sucrose. Lemos *et al.,* 1996 reported a complete micropropagation system using juvenile or mature explants of Annona muricate. Adventitious bud and shoot proliferation were achieved from hypocotyls of seedlings. In the present work 2, 4.D 2mg/l+ BA1mg/l showed maximum effect. There is no report on callus multiplication of *Annona muricata.*

CONCLUSION

Tissue culture-based propagation and conservation of *Annona muricata* can be a boon to farmers for faster propagation of this plant independent of seed germination and seed storage and viability problems, since *Annona muricate* has gained much commercial importance as an anticancer agent. Although there are numerous experimental protocols for Annona tissue multiplication, the final price of the plantlets is still too high for commercial use. .Given the potential of this technology, further research is needed to transform experimental protocols into commercial protocols.

REFERENCES

Doblin Sandai, Darah Ibrahim, Jain Kassim (2012) calcium alginate entrapped cells of Pencillium digitatum FETL DSL for the improvement of tannase production. Biotechnology an Indian Journal volume 6 issue 2 27-34.

Encina, C.L.; Padilla, I.M.G.; Cazorla, J.M.; Ruiz-Camacho, N. y Caro, E. (1999) Tissue culture in cherimoya. Acta Horticulturae 497: 289-301.

George VC, Kumar DN, Rajkumar V, Suresh P, Ashok R: (2012) Quantitative assessment of the relative antineoplastic potential of the n-butanolic leaf extract of annona muricata inn. In normal and immortalized human cell lines. Asian Pacific Journal of Cancer P, 13(2):699–704.

Googlemaphttps://www.google.com/maps/place/siddha+vidya+abhyasalayam+arayoor/@8.3614606,77.1277195,17z/data=!3m1!4b1!4m5!3m4!1s0x3b05ac75c0b80cfd:0x273322158d1b043c!8m2!3d8.3614606!4d77.1299082

Gray, D.J. and A. Purohit. (1991)Somaticembryogenesis and development of synthetic seed technology. Critical Reviews in Plant Sciences, 10: 33-61.

Gray, D.J., M.E. Compton, R.C. Harrelland D.J. Cantliffe(1995) Somatic embryogenesis and the technology of synthetic seed. In: Y.P.S. Bajaj (Eds.). Biotechnology in agriculture and forestry. Berlin, Springer-Verlag, pp. 126-151.

Hong T. D., Linington S. and Ellis R. H. (1996) Seed Storage Behaviour: A Compendium. IPGRI, Rome, Italy: 656 pp.

International Plant Genetic Resources Instit. (IPGRI), (2000) Rome (Italy) ISBN 10:92-9043-485-6 ISBN 13:978-92-9043-485-6.

Javed, B., Farhatullah, H., Shah, S.F. and Ali, I. (2012) In Vitro Analysis of Callus Induction in Interspecifically Hybridized Populations of brassica. Pakistan Journal of Botany, 44, 787-790.

Jordan M and Botti C (1992) Tropical and Subtropical Small Fruits. In: Hammerschlag F.A and Litz R.E. Biotechnology of Perennial Fruit Crops. *Biotechnology in Agriculture No. 8 series*. C.A.B. U.K.

Lemos, B. y J. Blake, 1996(a), Micropropagation of juvenile and mature Annona muricata L. Journal of Horticultural Science 72: 395-403.

Lemos, E.E.P. y Blake, J, 1996(b), Micropropagation of juvenile and adult Annona squamosa. Journal Plant Cell, Tissue and Organ Culture. Vol. 46 (1); 18-23A.

Litz, R.E. and D.J. Gray, (1995) Somatic embryogenesis for agricultural improvement. WorldJournal of Microbiology & Biotechnology, 11: 416-425.

Mishra S, Ahmad S, Kumar N, Sharma BK, (2013) Annona muricata (the cancer killer): a review. Global Journal of Pharmacological Research, 2(1):1613–1618.

Murashige T and Skoog F, (1962) A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. Physiologia Plantarum 15: 473-497.

Nair S, Gupta PK and Mascarenhas AF, (August 1983)Haploid plants from in vitro anther culture of Annona squamosa LinnArticle *in* Plant Cell Reports 2(4):198-200 · DOI: 10.1007/BF00270103 · Source: PubMed.

Nair S, Gupta PK, Shirgurkar MV and Mascarenhas AF, (1986) Studies of endosperm culture of Annona squamosa L. Plant Cell Reports. 5: 132-135.

Ndambuki B. M., (1991) "In Situ Conservation and Monitoring of Rangeland Genetic Resources." In: Crop Genetic Resources of Africa. Edited by F. Attere, Zedan H., Ng N. Q. and Perrino P. IBPGR/UNEP/IITA/CNR, Rome, Italy: 656 pp.

Omolara Jemimah Ojezele, Matthew Obaineh Ojezele and Abiola Muhammad Adeosun (2016) Comparative Phytochemistry and Antioxidant Activities of Water andEthanol Extract of Annona muricataLinn Leaf, Seed and Fruit.Advances in Biological Research 10 (4): 230-235.

Padilla, I.M.G. (1997) Micropropagation of cherimoya (Annona cherimola Mill.) cv. Fino de Jete.. 334 f. Thesis (PhD) - University of Málaga, Malaga, 1997.

Rasai, S., A. P. George y A. S. Kantharajah, (1995) Tissue culture of Annona spp. (Cherimoya, atemoya, sugar apple y soursop): A review. Scientia Horticulturae 62: 1-14.

Samson J.A (1986) "Tropical Fruits," 2nd Edition, Longman Inc., New York.

Van Beem-Usa Inwanna, Sainiya Samala, Charuvat Chanpradit Natthaya Choosingh, (2014) Effects of Culture Media and Explant Types on Callus and Shoot Induction of Annona muricataL.Malaya Journal of Biosciences, 1(3):155-159 ISSN 2348-6236 print /2348-3075 online.

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