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Effect Of 2,4-D On Callus Induction And Multiplication Of *Annona Muricata*

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Abstract: :- Annona muricata L., belongs to the family of Annonaceae and has been used for centuries by medicine men in South America to treat a number of ailments. The present investigation was carried out to determine the effect of 2,4-D on Callus Induction and Multiplication of Annona muricata. Callus cultures were initiated from the leaf of the medicinal plant Annona muricata, cultured on Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose and various concentrations (1, 2,3,4and 5 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4-D). Explants treated with various concentration of the same hormone formed calli with different morphologies. In the induction studies, 2,4-D 5 mg/lwas the most efficient formulation for callus formation. The callus induction response was for2,4-D 5mg/l treatment was 84%. In 2,4-D 5mg/l treatment callus was formed between 7-9 days .In 2,4-d3mg/l the callus induction frequency was 89% but the number of days taken for callus induction was 17-22.The least CIF response was in 2,4-D at 4mg/l The CIF response was 35%Calli were successfully Multiplied in 2,4-D 5mg/l treatment with percentage response of 81%. Addition of 2ppm polyvinylpyrrolidone (PVP) aided in overcoming the browning effects by absorbing the phenoliccompounds in the medium to some extent but not so efficiently growth of callus was not affected by browning .

Keywords: Annona muricata,callus induction,callus multiplication,2,4-D,tissue culture

INTRODUCTION

With a growing focus on the importance of medicinal plants and traditional healthsystems the international trade of medically significant plants has shown a phenomenal growth. Interest in natural materials by the dominant economic powers enhanced with emergence of new possibilities in biotechnology and drug synthesis Green plants synthesize and preserve a variety of biochemical products, many of which are extractable and used as chemical feed stocks or as Raw material for various scientific investigations. Many secondary metabolites of plant are commercially important and find use in a number of pharmaceutical compounds. However, a sustained supply of the source material oftenbecomes difficult due to the factors like environmental changes, cultural practices,diverse geographical distribution, labour cost and selection of the superior plant stock and over exploitation by pharmaceutical industry. Over–exploitation has even led to the detriment of natural habitats and indigenous populations.The scientific study of traditional medicines, derivation of drugs through bioprospecting and systematic conservation of the concerned medicinal plants are thus of great importance (Joy *et al*., 2001).

Annona muricata L., belongs to the family of Annonaceae and has been used for centuries by medicine men in South America to treat a number of ailments including hypertension, influenza,Rashes, neuralgia, arthritis, rheumatism, high blood pressure,

diarrhea, nausea, dyspepsia, ulcers, ringworm, scurvy, malaria, dysentery, palpitations, nervousness,insomnia, fever, boils and muscle spasms.(Rojas *et al.*, 2002; Rojas *et al.*, 2003;Sawantand Dongre, 2014).

Limited research has being carried out on tissue culture studies of *Annona muricata*nowadays. Therefore, cell and tissue culture techniques can be developed to overcome the problems such as slow-growing, rapid loss of seed viability, and low morphogenesis potentials. Thus, the plant tissue culture were carried out in this study in order to observe the effects of 2,4-D at various concentrations on the induction of callus from the leafexplants of *Annona muricata*, as well as to examine the appropriate concentrations for callus multiplication.

Experimental

Media Preparation :

Murashge and skoog basal media was prepared .Pipette out stock solution of macro and micro nutrients and vitamins 30 g / l sucrose + 100 mg / l inositol +2mg/l PVP were added.Plant growth regulator was added.Made up to 1000 ml by using distilled water. P H was adjusted to 5.7 .8 gm / l agar added . Boiled the medium for melting of agar. The medium was poured to presterilized culture vessels which were rinsed with double distilled water. Jam bottles and boiling tubes were used as culture vessels. The culture vessels containing medium were plugged tightly. They were then autoclaved at 121 ^{0}c at 15 lbs for 20 minutes.

Collection Of Plant Material

Annona muricata leaves were collected from Arayoor ,Thiruvanathapuram district,Kerala.Care was taken while collecting the plant material and the material which was showing systemic bacterial or fungal infections were discarded.

Surface Sterilization And Inoculation Of Explant :

To avoid the source of infection via plant material this was surface sterilized thoroughly before planting it into the nutrient medium. Plant material was first subjected to a jet flow of tap water.The material then was rinsed in an detergent bath and shaken well for 5 min. Again the plant material was washed to remove the detergent.Following this step the plant material was rinsed in 70 ethanol and rinsed in sterilized distilled water for three times until the smell of ethanol was lost.After that the explants were surface sterilized with 0.1 % $Hgcl_2$ for 10 minutes. After 10 minutes it is rinsed with double distilled sterile water for 4 times. The last two steps were done in a sterile area. The explants were then cut into small pieces (1 – 1 .5cm) with their mid ribs with sterile scalpels and inoculated into the medium with lower surface firmly touching the medium.

Callus Induction :

The MS basal medium fortified with various concentration of plant growth regulator tried for callus induction. (Table 1)

Table: 1.Plant growth regulator tried for the induction of callus

Treatment	Plant growth (P G R) regulator (Mg/l)
T1	2,4.D 1mg/l
T2	2,4.D 2mg/l
T3	2,4.D 3mg/l
T4	2,4.D 4mg/l
T5	2,4.D 5mg/l

Media: M S + Sucrose 30 gm / l + Inositol 100 mg / l + Agar 6.0g/l+ Polyvinyl pyruvate 2mg/l

Callus Induction Frequencies (Cif)

Callus induction frequencies were calculated as the percent explants inducing callus by using following equationand was converted to mean CIF, as described by Javed *et al*2012.

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

Incubation of Inoculated Explant :

The cultures were incubated with the light intensity of 3000 lux intensity at 25 + 2° c. The observations were recorded on the basis of the number of days taken for callus initiation and the difference of callus growth in each treatment.

Callus multiplication

Callus induced on the medium was allowed to grow initially for 30-35 days, in order to establish and maintain the callus the callus was transferred onto a freshly prepared medium containing same composition of2,4-d. After callus biomass increases two to four times (after 2–4 weeks of growth), callus can be divided and placed on fresh medium for callus multiplication. Multiplication procedures can be repeated several times (up to eight sequential transfers) before gross chromosome instability (orcontamination) occurs.

The ms basal medium fortified with various concentration of plant growth regulator tried for callus multiplication. (table 2)table: 2.plant growth regulator tried for the multiplication of callus

Treatment	Plant growth (P G R) regulator (Mg/l)
M1	2,4.D 1mg/l
M2	2,4.D 2mg/l
M3	2,4.D 3mg/l
M4	2,4.D 4mg/l
M5	2,4.D 5mg/l

Result And Discussion

Callus Induction

The plant *Annona muricata* was inoculated in basel MS medium augmented with various concentrations of 2,4-Dfor callus induction (Table-1).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 3Callus formation was observed from 4 to 30 days of inoculation for callus induction. Callus formation on the explants were formed at wounding site of major veins and covered the whole explant . The best responses (84%) callus induction was found on the medium containing 2,4-D 5mg/l (Table 3,Plate 1.5 T5). 2,4-D 3mg/l showed CIF response 89% but it took 17-22 days to respond.

The calli formed were different in morphology and texture for different concentration of the same hormone.Calli appeared Smooth ice white &glassy white,Smooth glassy white,Yellow friable&brown friable, Glassy whiteyellow friable.The least CIF response was in 2,4-D at 4mg/l The CIF response was 35%. In 2,4-D 2mg/l the days needed for the induction of callus was 25-27 days and CIF response was 43%. (Table 3,Plate 1) The induction responses of each calli in respective media are presented in Table3 .In a previous study by Van Beem *et al* 2014 2,4-D 2mg/l showed browning and very slow proliferation and the survival rate was only 20%.In the present work the same hormonal concentration showed 43% survival rate.Although PVP was added browning was observed but growth of callus was not affected due to browning (Plate 1Table3)

Table 3 Effect of plant growth regulators for callus induction.

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Treatment	Plant growth (P G R) regulator (Mg/l)	Number of days taken for callus induction	Nature and colour of the callus produced	CIF%
T1	2,4-D 1mg/l	27-30	Smooth ice white &glassy white	49
T2	2,4-D 2mg/l	25-27	Smooth glassy white	43
T3	2,4-D 3 mg/l	17-22	Yellow friable&brown friable	89
T4	2,4-D 4mg/l	28-30	Glassy white	35
T5	2,4-D 5mg/l	7-9	Yellow friable	84

Plate 1 Effect of plant growth regulators for callus induction.

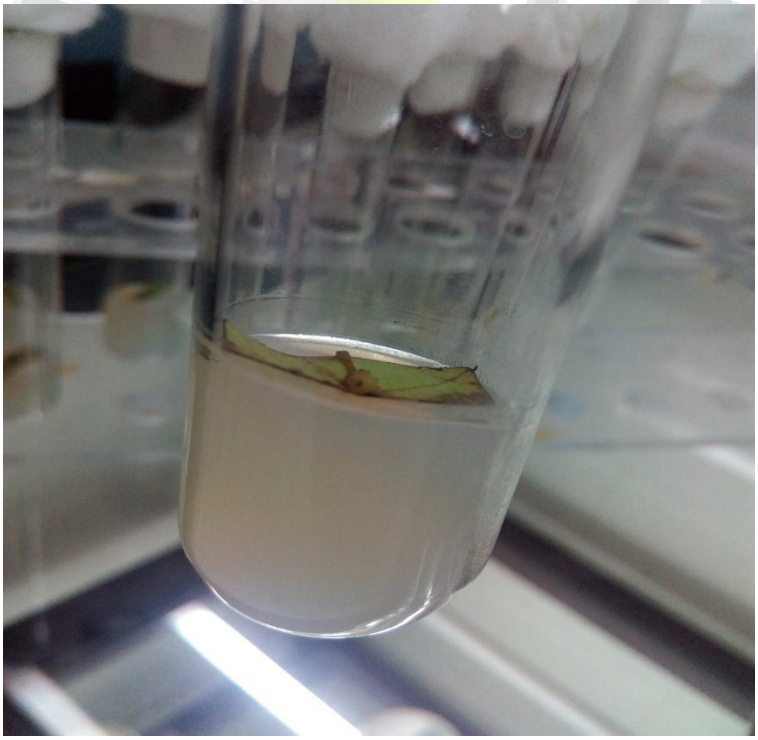
Plate1.1 T1

Plate1.2 T2

Plate1.3 T3

Plate 1.4 T4

Plate 1.5 T5



Callus Subculture and Multiplication

After 30 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 Table4.

The maximum callus was observed in basal MS medium fortified with 2,4.D 5mg/l (Table 4 Plate 2.5 M5). In 2,4.D 5 mg/l the callus was multiplied within23 -28 days and the percentage response was 81%The M3(MS+ 2,4.D 3 mg/l mg/l)also found to be good for callus multiplication. In 2,4.D 3 mg/l the callus was multiplied within24 -27 days and the percentage response was 76%Very least amount of callus was produced in M2 (MS+ 2,4.D 2mg/l). In 2,4.D 2mg/l the callus was multiplied in 29-32 days and the percentage response was 39%(Table 4 Plate 2).The calli formed were different in morphology and texture for different concentration of the same hormone.Calli appearedfriable black and pale green,Compact black and light orange,Friable black,brown,white&pale green.Browning was seenin all the treatments but growth was not much affected.There is no report on callus multiplication of Annona muricata.

Table 4 Effect of plant growth regulators for callus multiplication.

Treatment	Plant growth (P G R) regulator (Mg/l)	Number of days taken for callus multiplication	Nature and colour of the callus	Percentage response
M1	2,4-D 1 mg/l	25-29	Friable black and pale green	65
M2	2,4-D 2mg/l	29-32	Compact black and light orange	39
M3	2,4-D 3 mg/l	24-27	Friable black	76
M4	2,4-D 4 mg/l	23-28	Friable black,brown,white&pale green	69
M5	2,4-D 5 mg/l	23-28	Friable black	81

Plate 2 Effect of plant growth regulators for callus multiplication

Plate2.1 M1

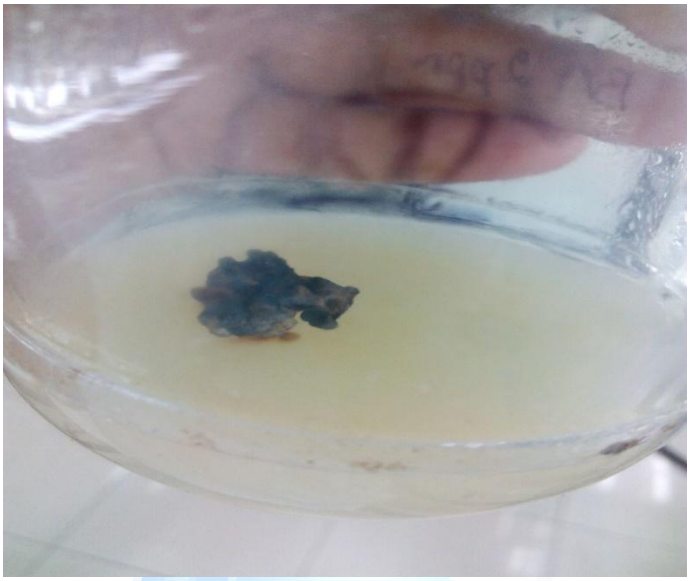
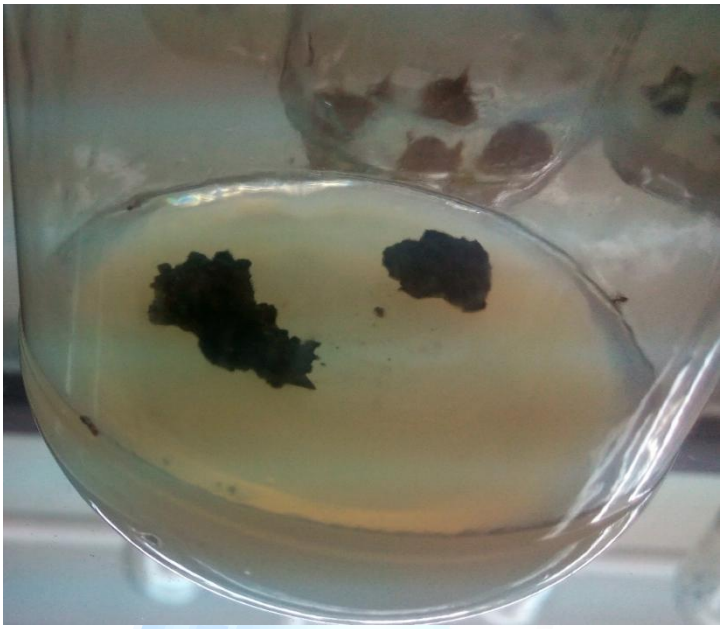


Plate2.2 M2

Plate 2.3 M3

Plate2.4 M4

Plate2.5 M5



Results obtained from this study confirms that considerable variability exists among calli for different concentrations of same hormone and optimisation of the concentration is highly essential for induction of morphogenesis

Statistical Data Analysis

The data obtained by treating A. Muricata leaf explants using different concentrations of growth regulator were analysed statistically for every treatment level having 3 replicates with 20 explants each.The results obtained are mean of the triplicate.

Conclusion

The in vitro protocol for tissue culture of *A. Muricata* will be useful in gaining more information for micropropagation technique of this rare tree member for conservation strategy. As far as we are aware, there are no published reports about micropropagation of *A. Muricata* through leaf explants and the aim of the present work was to determine the hormones formicropropagation of this plant from leaf explants. 2,4-D was successful in callus induction from leaf explants and its multiplication.We concentrated on leaf explants because it is the most available explant available in all seasons and procurement of this explant do not harm the plant

Future Perspectives

This study can be furthered by carrying out activities such as induction of somatic embryogenesis and mutation studies by exposure to ultraviolet rays which can develop into whole plant. Besides, organogenesis can help to introduce an intact plant and influence of elicitors can be studied. Optimization of medium formulation for callus maintenance can be improved bytesting with different concentrations of PVP and activated charcoal in order to overcome the browning effects. Extraction of secondary metabolites from the callus culture and their enhancement by elicitors can also be carried out to isolate the chemical compounds for the production of useful pharmaceutical products. Lastly plant transformation by *Agrobacterium*-mediated method, exposure to ultraviolet rays and treatment with any other desirable chemicals can be applied to introduce the pest-resistance plant as well as to produce more germination frequency of seeds or more chemical extracts.

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