WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.421
Volume 8, Issue 1, 741-753 Research Article ISSN 2278 – 4357

IN VITRO PROPAGATION OF CAYRATIA PEDATA VAR. GLABRA GAMBLE. WITH SOME GROWTH REGULATORS USING LEAF AND STEM

Dr. K. Kalaichelvi*

Associate Professor, PG and Research Department of Botany, Vellalar College for Women (Autonomous), Erode.

Article Received on
28 Oct. 2018,

Revised on 17 Nov. 2018,
Accepted on 07 Dec. 2018

DOI: 10.20959/wjpps20191-12742

***Corresponding Author**
Dr. K. Kalaichelvi
Associate Professor, PG and Research Department of Botany, Vellalar College for Women (Autonomous), Erode.

ABSTRACT

Cayratia pedata var. *glabra* is as old as civilization and through history it has been used as a popular folk medicine. Considering it's medicinal importance, after looking through its increasing demand, there is a global need to develop quality plant material. In the present investigation, an efficient *in vitro* propagation technology was developed for a rapid and large scale production of *Cayratia pedata* var. *glabra* by using stem and leaf from the mature healthy and approximately 5-6 year old parent plant. After proper sterilization all the explants were micropropagated on MS basal medium supplemented with 3% sucrose as carbon source and 0.8% agar-agar as solidifying agent along with different growth regulators. The maximum callusing frequency and more number of shoot formation was observed in lowerconcentration of BAP (0.5 mg/l) in combination with NAA (0.2 mg/l). The maximum frequency of root formation in leaf callus was 85% and 75% in stem callus and both were achieved on MS medium with NAA (1 mg/l) after two weeks. Therefore, the above protocol could be effectively used in rapid micropropagation of elite plant of *Cayratia Pedata* Var. *Glabra.*

KEYWORDS: *Cayratia Pedata* Var. *Glabra*, *in vitro* propagation, BAP, NAA.

INTRODUCTION

The most traditional medicines are developed from nature. They have not yet fulfilled the scientific requirements so as to be classified as modern medicines. Plants – derived natural products have been widely investigated for the discovery and development of new

pharmaceuticals. Natural products have been obtained through plant tissue culture techniques, such as callus and cell suspension culture. These methods of producing compounds with medicinal interest are of great value, since they allow controlled cultivation, providing continuous and homogeneous synthesis of raw material, regardless of environmental and seasonal factors. Plant tissue culture technology provides an attractive alternative for secondary metabolite production, offering the possibility of obtaining medicinal compounds and ensuring sustainable conservation and rational use of biodiversity.[1,2] Recently, plant tissue culture technology has been efficiently applied in secondary metabolites production.[3,4,5]

The potentiality of the plant cell can be enhanced for the production of useful secondary metabolites by applying various in vitro techniques. It is imperative that, viable strategies have to be taken to conserve the surviving population; at least, the critically important medicinal species from further loss. Since the species *Cayratia pedata* var. *glabra* is rare and endangered due to ecological anthropological pressure, it is need to be conserved. Hence in the present investigation, the endangered medicinal species namely *Cayratia pedata* var. *glabra* has been selected for in vitro micropropagation as a conservation strategy to produce large scale plantlets within a short time without any genetic variation. In the present investigation, a protocol has been developed for callus establishment and enhancement in production of sterols by addition of growth regulators.

Cayratia pedata[5], **(**Tamil: Pannikkodi, Kattupirandai, Sanskrit: Suvaha, Gobhapadi, Malayalam: Velutta sori valli, Tripadi) is an indigenous herb belonging to the family *Vitaceae*. It is a woody climber with cylindrical stem and grown mostly in semi evergreen to evergreen forest. Traditionally, the leaves of this plant were used in the treatment of ulcers and diarrhea. The decoction of the leaves was used to check uterine and other fluxes.[6] The plant has also found to possesses anti-inflammatory[7] and antinociceptive activities.[8]

MATERIALS AND METHODS

In vitro micropropagation of *C. pedata* var. *glabra*

The application of biotechnological principles for the establishment of micropropagation under *in vitro* conditions has been studied by following the methods quoted below.

Source of plant material

The *C. pedata* var. *glabra,* explants were collected from two to three years old plants from Thiashola, Manjoor, Nilgiris South Division, Western Ghats.

Explants selection and mode of sterilization

The explants namely leaf and stem harvested from *in vivo* plants were thoroughly washed with running water and kept under running water for 20 min. After that, the explants were washed thoroughly with teepol solution for 5-10 min and treated with 10% (w/v) of bavistin-methyl-3-benzimidizole carbomate solution and ampicillin and rifampicin for 15-20 min. Finally the explants were subsequently surface sterilized with 70% alcohol for 30-60 seconds and 0.1% (w/v) mercuric chloride solution for 3-7 min and washed for 3 - 4 times in sterile distilled water. The surface sterilized explants were trimmed gently with the help of sterile surgical blade (Lisyter No:10) and aseptically inoculated on to pre-cooled autoclaved medium.

Culture media employed and their composition

MS (Murashige and Skoog, 1962)[9] basal medium full strength (MSF) was employed in the present study (Table 1). The composition of the medium is given below.

Preparation of the medium

For the preparation of medium analytical reagents of "Hi-media" grade chemicals, "Borosil" glasswares and double distilled water were used. The nutrient medium basically consists of inorganic nutrients, carbon source, vitamins, iron source, amino acids and natural supplements (optional). The chemicals were weighed accurately in electronic weighing balance (Shimadzu AY 220). All the stock solutions were prepared and stored in well-stoppered sterilized bottles and preserved in a refrigerator at 4°C. Specific quantity of the stock solutions of the chemicals and growth regulators were pipetted onto a one liter beaker. Required sucrose and other organic supplements and complex additives (optional) were added. The final volume was made up with distilled water and the pH was adjusted to 5.8 - 5.9 with either0.1 N NaOH or 0.1 N HCl using a pH meter (Systronics).

To the above said media 0.8% (w/v) agar (extra pure gelling point 32 - 35°C, Hi-media-Bombay) or Phytagel (Hi-Media Bombay) was added, melted in water bath and the medium was dispensed into 150 ml bottles (30 ml of medium). The bottles after covering with a polyethylene cap were autoclaved at 1.06 kg pressure/sq cm for about 20 min at 121°C. The

autoclaved medium in the culture bottles were cooled and allowed to solidify and it was stored in dark for future use. The inoculations were done after four days to ensure that the bottles were free from contamination.

Growth regulators, growth adjuvant and their preparation

Two important groups of growth regulators such as auxins and cytokinins were used in the experiments. All the growth regulators are stored at 4°C until use.

Auxins and their preparation

Auxin namely α-naphthalene acetic acid (NAA) was used in these experiments. The stock solution was prepared by dissolving 10 mg of auxin individually in 1 ml of ethanol. Then the volume was made up to 100 ml with sterile distilled water. The required volume of auxins was added to the nutrient media, before autoclaving and was used in different concentrations.

Cytokinins and their preparation

The stock solution was prepared by dissolving 10 mg of 6-benzyl adenine (BAP) in 1ml of 0.1 N Hydrochloric acid (HCl) and the volume was made up to 100 ml by adding sterile distilled water. The required volume of cytokinin was added to the nutrient media, before autoclaving and was used in different concentrations.

Culture conditions

All the cultures were maintained in the culture room at a temperature of 25 ± 2°C and relative humidity of 65 – 70%. The cultures were kept under white light at intensity of 3000 Lux provided from white fluorescent lamps (Philips, India) with 14 hrs photoperiodic duration.

***In vitro* studies**

The present investigations on *in vitro* propagation of *C. pedata* var. *glabra* were carried out in the tissue culture laboratory of the Department of Botany, Vellalar College for Women, Erode, India. *In vitro* techniques like micropropagation, callus induction and regeneration were attempted.

Callus induction

The explants namely leaf and stem collected from Thiashola were used as primary explants. Explants measuring about 0.4 - 0.6 cm length were cultured on MS medium supplemented with growth regulators BAP and NAA. Twenty five explants were used for each culture. Each experiment was repeated twice. MS medium enriched with BAP and NAA in the

concentration range of 0.5 - 3.0 mg/l were tested for callus induction and morphogenesis.

Sub-culturing was carried out at the regular interval of 20-30 days. The *in vitro* developed microshoots were cut into segments (0.4 - 0.6 cm in length) each. These were sub-cultured on MS medium containing various growth regulators namely BAP and NAA in different combinations.

Percentage of callus induction of six week old cultures was calculated. Calli were subcultured regularly at an interval of three weeks. MS medium enriched with BAP in the concentration of 0.5 - 3.0 mg / l was tested for regeneration.

Callus frequency

The percentage of callusing was recorded at the end of fifth week. Frequency of callus induction was calculated as shown below and was represented as percentage:

$$\text{Frequency of response } (\%) = \frac{\text{Number of explants responded}}{\text{Total number of explants cultured}} \times 100$$

Rooting of *in vitro* multiplied shoots

The elongated shoots were transferred to MS medium supplemented with NAA at different concentrations for root induction.

RESULTS

Explants sterilization

The explants collected from the field (shola) were treated in different sterilants with various concentrations at different time for sterilization (Table 2). Among the various combinations tried, the Teepol treatment for 10 min followed by Bavistin 20 min, antibiotics namely Ampicillin and Rifampicin for 20 min, 70% alcohol for 30 seconds and 0.12% $HgCl_2$ for 3 min was found to be effective (Table 2) by having no dried explants and no contamination. The other treatment in Teepol for 10 min followed by Tween 20 for 10 min, Domestos for 10 min, Bavistin for 20 min, antibiotics for 20 min, 70% alcohol for 30 seconds and 0.12% of $HgCl_2$ for 3 min was however occupied next position to the previous treatment in terms of the degree of survivability of explants and contamination level (Table 2). But here the response was 90% and the dried buds were around 10%. Drying of explants is due to higher concentration as well as too many sterilants in the particular combinations. The explants responded along with surface contamination heavily to the 0.1% concentration, which proved

that the concentration and the exposure timing was not adequate to eliminate the surface contaminants of the explants.

Callusing and multiple shoot induction

Explants like leaf and stem were employed for the induction of callus. The explants were cultured in MS medium supplemented with various concentrations of BAP and NAA (Table 3). It has been noted that an increase in the concentration of BAP concomitantly reduced the frequency of callus formation. The morphological features of callus developed from leaf and stem explants were varied widely in the present study. The callus induced from the leaves was friable, soft and yellowish in colour and grow faster than the other explants (Plate 1). The maximum callusing frequency and more number of shoot formation was observed in lower concentration of BAP (0.5 mg/l) in combination with NAA (0.2 mg/l). The callus obtained from all the above combinations were sub-cultured on MS medium with some combinations of BAP and NAA.

The regenerated axillary shoots were excised and transferred to MS medium with different concentrations of NAA (Table 4). The maximum frequency of root formation in leaf callus was 85% and 75% in stem callus and both were achieved on MS medium with NAA (1 mg/l) after two weeks.

Table 1: Chemical composition of Murashige and Skoog (1962) medium.

S.No	Component	mg/l
Major salts		
1	NH_4NO_3	1650
2	KNO_3	1900
3	$CaCl_2.2H_2O$	440
4	$MgSO_4.7H_2O$	370
5	KH_2PO_4	170
Minor salts		
6	$MnSO_4.4H_2O$	16.8
7	$ZnSO_4.7H_2O$	8.6
8	H_3BO_3	6.2
Micro salts		
9	$Na_2MoO_4.2H_2O$	0.251 0.025
10	$COCl_2.6H_2O$	0.025
11	$CuSO_4.5H_2O$	0.830
12	KI	
Iron		
13	$FeSO_4.7H_2O$	27.86
14	Di sodium EDTA	37.26
Vitamins		

15 16 17	Nicotinic acid Thiamine HCl Pyridoxine HCl	0.5 1.0 0.5
Amino Acids		
18 19 20 21	Glycine Meso Inositol Sucrose Agar	2.0 100.0 30g 8g
	pH	5.8

Table 2: Effect of different sterilization treatments in initiation of callus (*in vivo* explants of *C. pedata* var. *glabra*).

S. No.	Mercuric Chloride		Teepol (min)	Tween 20 (min)	Domestos (min)	Bavistin (min)	Ampicillin & Rifampicin Antibiotics (min)	70% Alcohol (Sec)	No. of explants	No. of explants responded	No. of explants dried	No. of explants contaminated
	%	Time (min)										
1.	0.12	3 5 7	10 20 30	- - -	- - -	**20** 30 40	**20** 30 40	**30** 45 60	**20** 20 20	**20** 10 5	- 10 15	- - -
2.	0.12	3 5 7	10 20 30	10 20 30	- - -	20 30 40	20 30 40	30 45 60	20 20 20	15 7 5	5 13 15	- - -
3.	0.12	3 5 7	10 20 30	10 20 30	10 20 30	20 30 40	20 30 40	30 45 60	20 20 20	18 9 5	2 11 15	- - -
4.	0.1	3 5 7	10 20 30	- - -	- - -	20 30 40	20 30 40	30 45 60	20 20 20	6 4 3	2 9 14	12 7 3
5.	0.1	3 5 7	10 20 30	10 20 30	- - -	20 30 40	20 30 40	30 45 60	20 20 20	7 6 5	2 11 13	11 3 2
6.	0.1	3 5 7	10 20 30	10 20 30	10 20 30	20 30 40	20 30 40	30 45 60	20 20 20	9 5 4	2 9 12	9 6 4

Table 3: Effect of MS medium and Growth regulators on callus induction and shoot formation (in vivo explants of *C. pedata* var. *glabra*).

S. No.	MS medium + Growth regulators mg/l		Leaf		Stem	
	BAP	NAA	% of leaf producing callus	% of callus forming shoots	% of stem producing callus	% of callus forming shoots
1.	0.5	0.1	79.5	20.0	80.0	22.0
2.	1.0	0.1	90.0	15.0	85.0	15.0
3.	1.5	0.1	90.5	10.0	87.5	9.0
4.	2.0	0.1	72.5	2.0	65.5	2.0
5.	2.5	0.1	30.5	-	20.0	-
6.	3.0	0.1	25.5	-	18.5	-
7.	**0.5**	**0.2**	**95.0**	**90.0**	**80.0**	**75.0**
8.	1.0	0.2	85.5	60.0	70.0	55.0
9.	1.5	0.2	63.5	25.0	60.5	25.0
10.	2.0	0.2	60.5	5.0	58.5	3.0
11.	2.5	0.2	45.0	-	40.5	-
12.	3.0	0.2	40.0	-	35.0	-

Values are means of three replicates

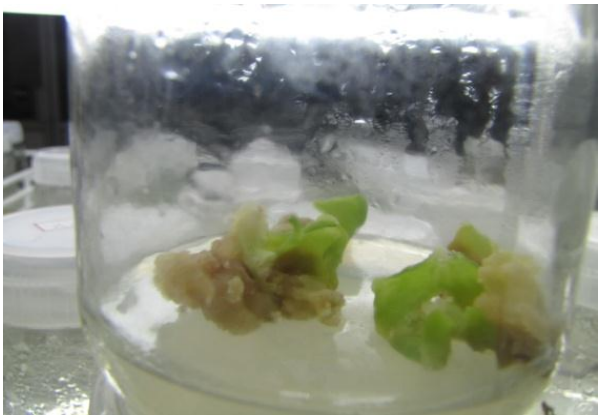
A column means followed by a common letter are not significant

Table 4: Effect of growth regulator on rooting of leaf and stem callus.

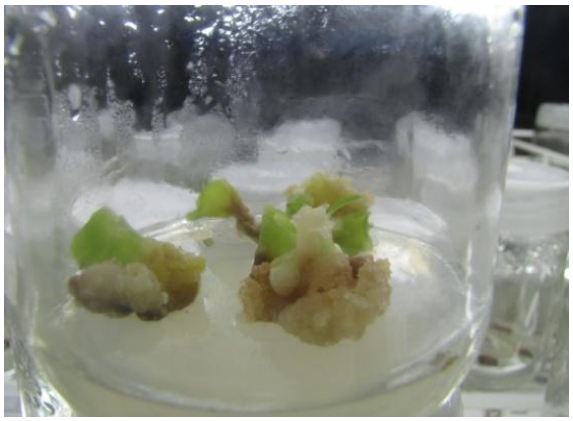
S. No.	MS medium + Growth regulator (NAA mg/l)	% of rooting	
		Leaf callus	Stem Callus
1.	1.0	85	75
2.	1.5	65	60
3.	2.0	50	50
4.	2.5	45	45
5.	3.0	30	30
6.	3.5	25	25

Values are means of three replicates

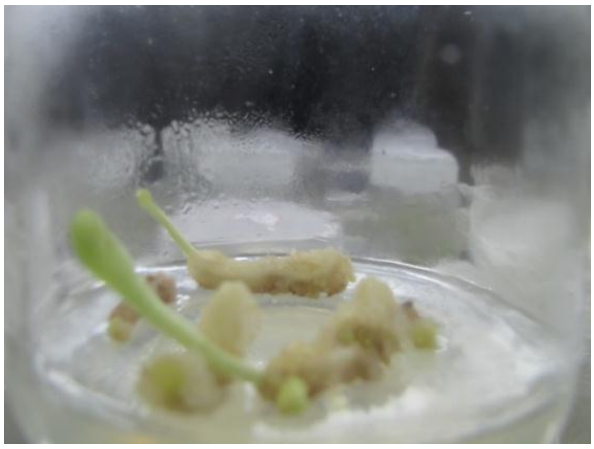
Means followed by common superscript letter are not significant at p<0.05 level



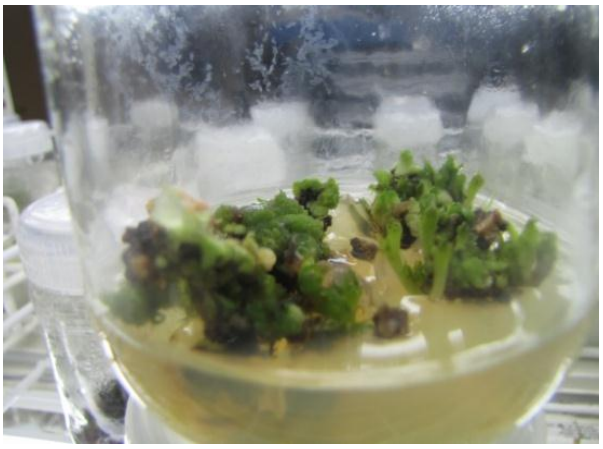
Initiation of callus from leaf explants.



Well developed callus from leaf explants.



Induction of shoots from the leaf derived callus.



Rhizogenesis from leaf derived callus.

Plate – 1: *In vitro* culture of *C. pedata* var. *glabra* through leaf and stem explants.

DISCUSSION

Generally, *in vitro* regeneration is an efficient means of ex situ conservation of plant diversity and it assists in suitable maintenance of the present day rapidly dwindling germplasm on long-term basis, especially for the medicinal plants. With this technology many threatened medicinal plants can be rapidly propagated and preserved from a minimum plant material and with little impact on wild populations. Many important Indian medicinal plants have been successfully regenerated *in vitro* [Dode *et al*., (*Ocimum basilicum*)[10], Sebastian *et al*., (*Phyllanthus emblica*) [11], Karuppusamy and Pullaiah, 2007 (*Bupleurum distichophyllum*)[12], Jamwal *et al*., (*Vitis vinifera*)[13], Khanam and Sharma, (*Aloe vera*)[14] Riberio *et al*., (*Hovenia dulcis*).[15] This technique facilitates the introduction of successfully produced in vitro plantlets into the suitable micro-sites in natural communities to enhance the population of valuable plant species which have been failed/less efficient in natural reproduction processes.

The success of *in vitro* culture is largely depended on three factors, such as explants choice, media composition and control of physical environment.[14,15] The importance of the choice of explants, which served as the inoculums for axenic culture, is well documented in the classical compilation of George and Sherrington.[16] The supplementation of growth hormones like auxins, cytokinins and gibberellic acids individually or in combinations in MS basal medium at different concentrations is having varied response with respect to callus formation and organogenesis in many plant species.[17]

The plant collected from shola was treated in different sterilants with various concentrations at different time for sterilization (Table 2). Among the various combinations tried, the Teepol treatment for 10 min followed by Bavistin 20 min, antibiotics namely Ampicillin and Rifampicin for 20 min, 70% alcohol for 30 seconds and 0.12% $HgCl_2$ for 3 min was found to be effective by having no dried explants and no contamination. This was in corroboration with the works of Thorpe and Patel[18], Ahmed *et al*.[19] and Khanam and Sharma. [14]

The presence of the plant growth regulators, particularly cytokinin in culture medium is the most important factors for shoot proliferation.[15] The two hormones cytokinin (BAP) and auxin (NAA) supplemented in the basal medium played greater role in the callus formation and subsequent shoot and root initiations (Table 3 and Table 4). Chalupa[20] stated out that the cytokinin and benzylaminopurine (BAP) are the hormones induced the morphogenesis

effectively in dicots. However, Khanam and Sharma[14] pointed out that the auxin – cytokinin ratio is species specific for the effective morphogenesis.

In the present study, the percentage of leaf discs for the species *C. pedata* var. *glabra* producing callus was maximum (95%) at the concentration level of 0.5 mg/l BAP and 0.2 mg/l NAA. Austin *et al*.[21] XU *et al*.[22] and Steinitz *et al*.[23] reported the *in vitro* propagation success of many solanaceae members by using the basal medium containing auxin type of growth hormones. Paulsamy *et al*.[24] reported the callus formation in leaf discs for a shola species *Berberis tinctoria*. Earlier Molinar *et al*.[25] also reported that 0.5mg/l BAP and NAA actively initiate the callus formation in *Berberis trifoliata.*

The BAP at 0.5 mg/l in the basal medium was found to be the optimum concentration at which 90% calli initiate shoots (Table 3). The importance of BAP for shoot proliferation was already discussed and documented well.[26,27]

It was observed that a maximum of 75% (leaf) shoots subcultured on the medium containing the NAA at 0.1 mg/l produced roots and 80% (stem) shoots produced maximum number of roots. The number of roots produced was also higher (5/shoots) in the subculture of the medium containing the same concentration of 1 mg/l (Table 4). Ray[28] already reported that the NAA concentration at the level of 1 mg/l initiates roots effectively for many endangered medicinal plants. The preliminary studies by Molinar *et al*.[25] also confirmed the requirement of NAA at the rate of 1 ml/l in the basal medium for the high degree of root initiation of the shola element, *Berberis trifoliata.*

It is concluded that for the effective *in vitro* regeneration of the shola medicinal herb *C. pedata* var. *glabra*, the basal medium is standardised with the hormone concentration as follows: 0.5 mg/l of BAP and 0.2 mg/l NAA for effective shoot formation; 0.5mg/l NAA for effective root initiation. The massive production of plantlets through this method will be helpful to meet the demand of this bio resource at one side. At the other end, by replanting the plantlets in the shola habitats of Nilgiris, it will enhance the population of this species. Subsequently, the severe habitat protection under practice in the sholas of Nilgiris will ensure the species conservation effectively. However this fact will be confirmed only after knowing the survivability rate of seedlings through proper experiments. This high regeneration system would provide an effective strategy for the conservation and proliferation of this medicinal plant species on large scale for commercial purpose.

CONCLUSION

The protocol given in the present study is simple, gives an efficient way for *in vitro* propagation of *C. pedata* var. *glabra* to meet the industrial requirement. It shows 100% development of leaf and stem per explants in better quality and quantity.

REFERENCES

1. Coste A, Viasa L, Halmagyi A, Dellu C and Coldea G. Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. Plant Cell Tiss. Org. Cult, 2011; 106: 279-288.
2. Coppede JS, Pina ES, Paz TA, Fachin AL, Marins MA, Bertoni BW, Franca SC and Pereira AMS. Cell cultures of *Maytenus ilicifolia* Mart. are richer sources of quinine-methide triterpenoids than plant roots in nature. Plant Cell Tiss. Org. Cult, 2014; 118: 33-43.
3. Piekoszewska A, Ekier H and Zubek S. Arbutin production in *Ruta graveolens* L. and *Hypericum perforatum* L. *in vitro* cultures. Acta Physiol. Plant, 2010; 32: 223-229.
4. Praveen N and Murthy HN. Effects of macroelements and nitrogen source on biomass accumulation and with anolide-A production from cell suspension cultures of *Withania somnifera* (L.) Dunal. Plant Cell Tiss. Org. Cult, 2011; 104: 119-124.
5. Pawar KD and Thengane SR. Influence of abiotic elicitation on production dipyranocoumarins in suspension cultures of *Calophyllum inophyllum* L. Acta Physiol. Plant, 2011; 33: 2149-2158.
6. Khare CP. Indian medicinal plants. Springerscience, New Delhi, 2007.
7. Patil SG and Honrao BK. Journal of Economic and Taxonomic Botany, 2000; 24(3): 688-694.
8. Rajendran V, Rathinambal V and Gopal V. A preliminary study on antiinflammatory activity of *Cayratia pedata* leaves on Wister albino rats, Scholars research library. Der Pharmacia Lettre, 2011; 3(2): 433-437.
9. Murashige T and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol, 1962; 15: 473-497.
10. Dode LB, Bobrowski VL, Braga EJB, Seixas FK, Schuch MW. *In vitro* propagation of *Ocimum basilicum* L. (Lamiaceae). Acta Scientiarum. Biological Sciences Maringa, 2003; 25(2): 435-437.

11. Sebastin P, Karamthanam PV and Mathew MM. Somatic embryogenesis and plant regeneration in *Phyllanthus emblica* L. Plant Cell Biotech. And Mole. Biol, 2005; 6: 155-158.
12. Karuppusamy S and Pulliah T. *In vitro* shoot multiplication of *Bupleurum distichophyllum* Wight – A native medicinal plant of Southern India. Plant Tissue. Cult. and Biotech, 2007; 17(2): 115-124.
13. Jamwal M, Singh B, Sharma N, Kumar R, Sharma A, Sharma RM, Wali VK and Parmar AM. In vitro regeneration of Grape (Vitis vinifera L.) cv. Perlette. World J. Agri. Sci, 2013; 9(2): 161-166.
14. Khanam N and Sharma GK. Rapid in vitro propagation of Aloe vera L. with some growth regulators using lateral shoots as explants. World J Pharma. Pharmac. Sci, 2014; 3(3): 2005-2018.
15. Riberio IG, Gayer CRM, Castro TCD, Coelho MGP and Albarello N. Compact callus cultures and evaluation of the antioxidant activity of *Hovenia ducis* Thunb. (Rhamnaceae) under in vivo and in vitro culture conditions. J. Medi. Plants Res, 2015; 9(1): 8-15.
16. George EF and Sherrington PD. Plant propagation by tissue culture. Exegetic, Ltd., Eversley, Florida, 1984.
17. Tripathi L and Tripathi. Role of Biotechnology in medicinal plants. Trop. J. Pharm. Res, 2003; 2(2): 243-253.
18. Thrope TA. and Patel KR. Clonal propagation: adventitious buds. In: Cell Culture and somatic cell genetics of plants, Academic press, New York, 1984; 1: 49-60.
19. Ahmed AMN, Ibrahim DA and Uzun SP. *In vitro* micropropagation of *Vitis vinifera* L. In kurdistan region of IRAQ. J. University of Zakho, 2015; 3(1): 55-66.
20. Chalupa V. *In vitro* propagation of willows (*Salix sp*.), European mountain ash (*Sorbus aucuparia* L.) and black locust (*Robinia pseudoacacia* L.). *Biologica. Pi*, 1983; 25: 305-307.
21. Austin S, Lojkowska E, Ehlenfeldt MK, Kelman A. and Helgeson JP. Fertile interspecific somatic hybrids of *Solanum*: A novel source of resistance to *Erwinia* soft rot. *Phytopathol,* 1988; 78: 1216-1220.
22. Xu YS, Murto M, Dunckley R, Jones MGK and Pehu E. Production of asymmetric hybrids between *Solanum tuberosum* and irradiated S. brevidens. Theor. Appl. Genet, 1993; 85: 729-734.

23. Steinitz B, Kusek S, Tabib Y, Paran I and Zelcer A. Pepper (*Capsicum annuum* L.) regenerants obtained by direct somatic embryogenesis fail to develop a shoot. In vitro Cell Dev. Biol. Plant, 2003; 39: 296-303.
24. Paulsamy S, Padmavathy S and Vijayakumar KK. Conservation of an endemic medicinal plant, *Berberis tinctoria* Lesch. in Nilgiris through micropropogation. Ancient sci. life, 2004; 24(1): 22-26.
25. Molinar JF, Mackay WA, Wall MM and Cardenas M. Micropropogation of Agarita (*Berberis trifoliata* Moric.). Horticul. Sci, 1996; 31(6): 1030-1032.
26. Das N, Misra M and Misra AN. Sodium chloride salt stress induced metabolic changes in pearl millet callus: Free solutes. J. Plant Physiol, 1990; 137: 244-246.
27. Subbaiah MM and Minocha SC. Shoot regeneration from stem and leaf callus of *Eucalyptus tereticornis*. Plant Cell Reports, 1990; 9(7): 370-373.
28. Ray MK. Current advances in mycorrhization in micropropagation. In vitro Cell. Dev. Biol. Plant, 2001; 37: 158-167.