ISSN: 0975-766X
Research Article
Available Online through
www.ijptonline.com

IN VITRO STUDIES ON CALLUS INDUCTION AND SUSPENSION CULTURE BIOMASS YIELD FROM PERGULARIA DAEMIA FORSSK.CHIOV

P.Vinoth kumar[1]*, N.Ramesh[2] and S.Siva Subramaniyan[3]
[1*, 2,] Department of Biotechnology, J.J college of Arts and science, Pudukottai, India,
[3] Department of Botany, J.J college of Arts and science, Pudukottai, India.
E.mail ID: *vinothkumarphd@gmail.com*

Received on 06-08-2010 ***Accepted on 20-08-2010***

Abstract

Callus induction were initiated from stem and leaf explants of *Pergularia daemia* on Murashige and Skoog (1962) medium containing different concentration of 2,4-D (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l), α-NAA (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l), IAA (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l), Kinetin and BAP. Callus induction was observed in 1.5 mg/l of 2 4-D and 1.0 mg/l of α-NAA supplemented medium for leaf and stem explants. The harvested cell biomass extracts were compared with extracts from leaves and stem of naturally growing *Pergularia daemia* plants. HPLC analysis of the extracts showed that the larger amount of Kaempferol present in the culture of undifferentiated cells.

Key words: *Pergularia daemia*, Kaempferol, Callus.

Introduction

Pergularia daemia [Forssk.chiov] (Asclepidaceace) commonly known as `Uttamani`, `Velipparuthi` in India, grows in various parts of southern and northern India. The plant is perennial slender foetid-smelling climber. The plant is used to treat jaundice, by the folk people of pudukkottai district. Its leaves are also used for the treatment of heaminthiasis, hemorrhoids, leprosy and strongly recommended for the malarial intermittent fever (Kirtikar and Basu, 1984). The extract of the plant is useful in uterine and menstrual disorder and

facilitating parturition (Prajapathi, *et al*., 2003). The roots are reported to show central nervous system depressant activity (Lokesh *et al*., 2009).

Plant cell suspension culture is an effective alternative way that could be used for the secondary metabolite production of desirable medical compounds from plants, bio-technological approach, especially plants tissue cultures are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra Rao and Ravi Shankar, 2002). There are number of studies on *Pergularia daemia* active principles, pharmacological activity and phytochemistry (Kumar and Mishra, 1997; Suresh Kumar and Mishra, 2005).This studies shows that the significant important of this plant in production of total kaempferol. Therefore, the objective of the presented investigation was to develop a procedure for the callus induction.

Material and Methods

Explant collection

Pergularia daemia (Forssk) chiov plants raised in pots containing soil and farmyard manure (FYM) used in the ratio1:1. The mother plants were obtained from medicinal plant garden (MPG), J.J College of Arts and Science, Pudukkottai, Tamil nadu, India.

Sterilization of explants

Small young twigs were collected from two months old healthy plants cut into 1 - 1.5 cm nodal segments with axillary buds. Young leaves were also used as explants for culture. These explants were washed thoroughly in running tap water to remove the adhering particles and then treated 5% solution of Teepol for 3 minutes. Then rinsed with running tape water for ten minutes. The cleaned explants were surface sterilized with 20% hydrogen peroxide for 4 minutes followed by 4 – 5 times rinsed in sterile distilled water to remove the surface. After trimming the cut ends surface sterilized explants were planted on the culture medium.

Preparation of nutrient stock solutions

The basal medium used was MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and solidified with 0.6% agar. There were placed into conical flasks, each containing 25 ml basal medium with exogenous hormone. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. The cultures were incubated under fluorescent lights with 1500-2000 lux for 16 hours at a temperature of 25 ± 1°c and 80 ± 10 relative humidity. Each experiment had 20 replicates and repeated at least three times data were collected up to six weeks of culture.

α-NAA and 2,4-D as auxins and BAP, Kinetin as cytokinins were added into MS medium to test their effect on callus formation (Table: 1). The concentration of phytohormone varied from 1.0 - 3.0 mg/l. MS basal medium containing 2.0 mg/l BAP and 0.5 mg/l α-NAA without agar were used for suspension culture. About 30 mg callus tissues were transferred from solidified medium into 150 ml conical flasks containing 50 ml of liquid medium.

At 2, 4, 6, 8, 10, 12, 14, 16 and 18 days the suspension cultured callus was harvested by filter and measured fresh weights and dry weights. Fresh weights of cells/callus were taken after removing the excess of moisture on the surface using blotting paper. Dry weight of callus was determined by drying in a hot air oven at 60°C for 24 hr.

The active components extractions from the callus and leaf samples were analyzed as prescribed by (Markham, 1982). The dried callus cells and dried leaf tissue (about 1.0 g each) were powdered and the active principles were extracted with 75% ethanol. The dried extract was mixed with n-butanol and water 2:1 ratio. Then the layer of n-butane evaporated under vacuum and the residue washed with petroleum ether. After filtration, the concentrated extract in methanol was used for separation by HPLC study.

Result and Discussion

Based on the studies on *Pergularia daemia* concentration of cytokinins and auxins were tested. While BAP and α-NAA induced the highest 92% response. Initially callus was initiated from stem and leaf explants of

Pergularia daemia on basal medium MS medium supplemented with 2,4-D at different concentrations (1.0 - 3.0 mg/l). The proliferation efficiency of callus of leaf explants was significantly higher than that of stem explants for 4 - 5 weeks incubation of culture. The maximum induction rate was recorded as 94.2% in leaf explants and 87.1% in stem explants on MS medium with 1.0mg/l 2,4-D and 0.5 mg/l BAP. The callus nature was delicate with pale yellow in colour (Fig.1).

As stem or leaf explants were cultured on MS medium free of growth regulators no calli were induced (Table.1). However, MS medium with single BAP induced callus in either Stem or leaf explants, the callus was turned brown. As high concentration of 2 4-D in combination with BAP and α-NAA in medium was relatively more suitable for subculture. The well grown callus were selected for subculture on MS medium with 2.0 mg/l 2 4-D in combination with 0.5% mg/l BAP or 1.0 mg/l α-NAA to test their growing state.

In 2,4-D supplemented medium the callus was pale yellowish green in colour. But in α-NAA and KN supplemented medium the callus was light green, less hard and compact. In BAP medium, more biomass yield was achieved than of Kinetin. The yield of fresh biomass was high on medium with 2,4-D 141.2 gfw/l and 8.02 gdw/l followed by α-NAA 114.6 gfw/l and KN 94.2 gfw/l and IBA 85.5 gfw/l (Table.2). The result of biomass yield from combination effect of auxins 2,4-D and α-NAA with BAP. About 2.0 g of actively growing calli were inoculated in conical flasks each containing 30 ml of solid medium. The combination of 2,4-D, α-NAA and BAP showed more callus biomass yield than the combinations of auxin with Kinetin. The yield of fresh biomass in BAP supplemented medium was about three times more than that in Kinetin supplemented medium. Dry biomass was about one time higher in BAP than Kinetin supplemented medium. On the basis of yield of both fresh and dry biomass, BAP has greater effect than Kinetin. Based on the above results, the PGR combinations of 2,4-D - 2.0 mg/l, α-NAA -1.0 mg/l and BAP- 0.5mg/l which yielded maximum biomass. This combination

Table 1: The callus induction from stem and leaf on *Pergularia daemia.*

S.No.	PGR (mg/l)				Rate of induction % ± S.D		Growing State
	2,4-D	BAP	KIN	α-NAA	Stem	Leaf	
1	1.0	0.5	0	0	15.5 ± 2.1	16.8 ± 2.2	Pale, compact hard.
2	1.5	0.5	0	0	50.8 ± 1.8	55.8 ± 2.0	Pale, compact hard.
3	2.0	0.5	0	0	26.2 ± 2.0	31.1 ± 3.2	Pale, compact hard.
4	2.5	0.5	0	0	28.3 ± 2.5	30.8 ± 3.8	Pale, compact hard.
5	3.0	0.5	0	0	35.8 ± 1.4	39.2 ± 1.3	Pale, compact hard.
6	1.0	0	0	1.0	78.5±1.7	76.7±2.5	Yellowish, loose sponge.
7	1.5	0	0	1.0	67.5 ± 2.0	53.8 ± 4.1	Yellowish, loose sponge.
8	2.0	0	0	1.0	37.5 ± 3.5	40.0 ± 1.8	Yellowish, loose sponge.
9	2.5	0	0	1.0	49.2±1.8	61.7±2.5	Yellowish, loose sponge.
10	3.0	0	0	1.0	50.0 ± 2.0	55.8.± 5.0	Yellowish, loose sponge.
11	1.0	0	0.5	0	52.1 ± 2.8	58.2 ± 1.6	Compact, hard granular.
12	1.5	0	0.5	0	48.2 ± 1.1	50.1 ± 0.5	Compact, hard granular.
13	2.0	0	0.5	0	36.3 ± 1.7	46.5 ± 1.4	Compact, hard granular.
14	2.5	0	0.5	0	49.2 ± 2.7	52.1 ± 1.9	Compact, hard granular.
15	3.0	0	0.5	0	51.6 ± 3.1	56.1 ± 1.8	Compact, hard granular.
16	1.0	0.5	0	1.0	70.5 ± 1.2	73 ± 2.2	Pale green, light compact.
17	1.5	0.5	0	1.0	68.2 ±2.0	71.6 ± 0.8	Pale green, light compact.
18	2.0	0.5	0	1.0	87.1 ±2.1	94.2 ±1.5	Pale green, light compact.
19	2.5	0.5	0	1.0	52.1 ±1.1	57.3 ±1.6	Pale green, light compact.
20	3.0	0.5	0	1.0	59.0 ±1.0	56.5 ±2.4	Pale green, light compact.

PGR:Plant growth regulators,2,4-D:2,4-Dichlorophenoxocy acetic acid,BAP:6-benzyl amino purine,KIN:Kinetin,α-NAA: Alpha naphthalene acetic acid.

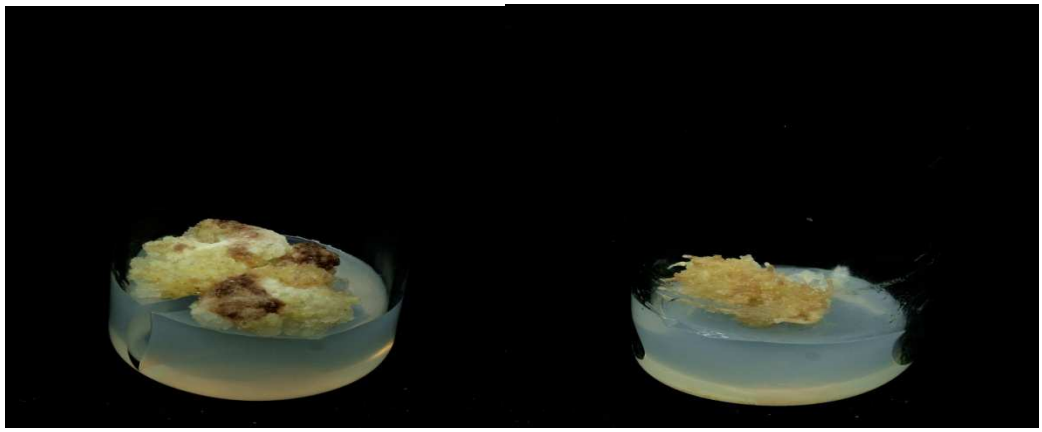
Table 2: The effect of auxins and cytokinins on *Pergularia daemia* callus culture.

Auxins							Cytokinins			
PGR mg/l	**2,4-D**		**α-NAA**		**IBA**		**BAP**		**KIN**	
	f.w	d.w	f.w	d.w	f.w	d.w	f.w	d.w	f.w	d.w
1.0	120.56	8.72	79.16	5.12	68.5	4.41	109.7	6.4	90.8	2.21
1.5	132.1	9.01	91.89	6.18	80.21	6.72	121.6	4.3	96.5	3.81
2.0	141.25	8.02	114.64	7.00	85.51	5.21	136.1	7.5	94.21	6.21
2.5	110.57	6.54	81.26	5.29	79.6	4.81	110.7	2.3	105.7	5.8
3.0	97.52	3.28	76.56	4.39	71.5	3.21	106.2	8.5	98.2	3.4

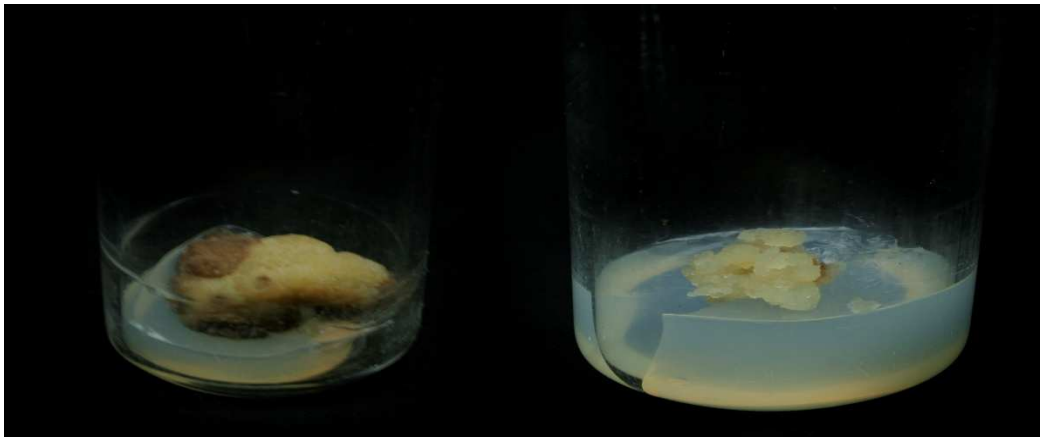
f.w: fresh weight g/l; d.w: dry weight g/l; PGR: Plant growth regulators was used for further studies. In order to determine the active principles kaempferol present callus was extracted by using methanol and water and compared with leaf extract of *Pergularia daemia* through HPLC analysis. The results of chromatogram of callus samples showed most of the compounds present in leaf extract chromatogram.

The precondition of the suspension culture is to obtain well grown callus. Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture (Gang *et al*., 2003). Subsequently plant growth regulators were added into MS medium to test their effects on callus formation of cotyledon and hypocotyls explants of *Pergularia daemia.* The result revealed that auxins play an important role in the callus induction and different types of auxins had various effects (Baskaran *et al*., 2006). The 2,4-D is superior to α-NAA in callus induction of *Pergularia daemia.* Further more, the cytokinins facilitated the effect of auxin in callus induction (Rao *et al*., 2006). The leaf and stem responded differently to auxins especially to single α-NAA supplemented medium of *Pergularia daemia*. In this study,

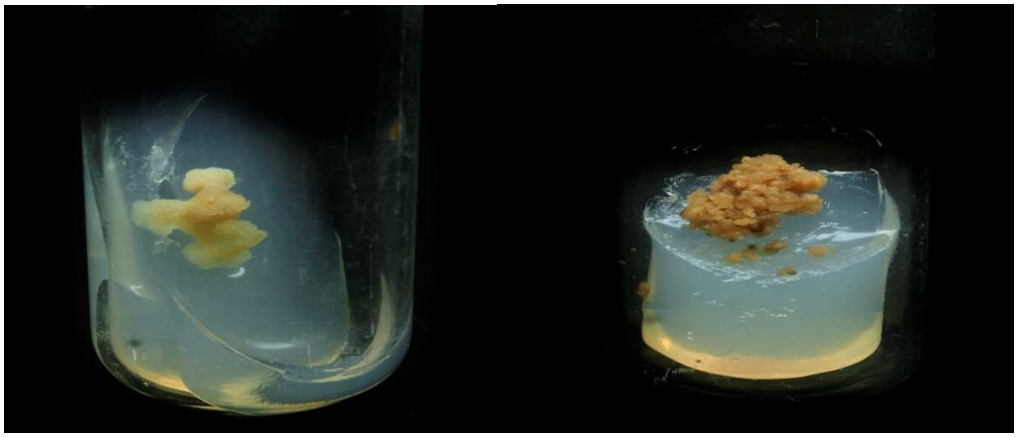
Figure.1: Callus Formation on *Pergularia daemia* Forssk.Chiov.



A B



C D



E F

A→ Pale compact and hard induced with MS medium Containing o.5 mg/l BAP and 1.5 mg/l 2, 4-D.

B→ Yellowish, loose and sponge callus induced with MS medium containing 1.0 mg/l NAA and 1.0 mg/l 2, 4-D.

C→ Compact, hard and granular callus induced with MS medium containing 0.5 mg/l KN and 1.0 mg/l 2, 4-D.

D→ Pale green and light compact callus induced with 1.0 mg/l NAA, 0.5 mg/l BAP and 1.0 mg/l 2, 4-D.

E→ Pale green and light compact callus induced with 1.0 mg/l NAA, 0.5 mg/l BAP and 2.0 mg/l 2, 4-D.

F→ Callus cultured in MS medium containing 2.0 mg/l 2, 4-D.

The callus induced from medium with 2,4-D and BAP was light compact, but they were transferred to new medium with α-NAA and BAP they become light yellowish, loose and were more suitable for suspension culture.

Numerous research reports exist in the literature about the effects of plant growth regulators on secondary metabolites of *in vitro* cultures. The enhance alkaloid production in several plant species for berberine in *Coptis japonica* (Nakasawa *et al*., 1986).

In summary, efficient callus was induced on MS medium with 2.0 mg/l BAP and 0.5 mg/l of 2,4-D and was subculture on MS medium with 2.0 mg/l BAP and 0.5mg/l α-NAA. This is the successful attempt of production of secondary metabolites of *Pergularia daemia.* Where, production levels can be manipulated with appropriate PGRS. Further studies will be directed towards large scale production, testing the efficiency of secondary metabolites through animal cell lines and exploring market potential.

Conclusion

The MS medium contains various plant growth regulators like 2,4-D, BAP, KIN, α-NAA and IBA using the range between 1.0-3.0mg/l. Callus induction was recorded and the maximum callus induction was observed in the 2,4-D 2.0mg/l combination with 0.5mg/l BAP and 1.0 mg/l α-NAA.

Acknowledgements

Authors are thankful to J.J College of arts and science for providing the facilities for me. I would like to thank Dr.N.Ramesh encourage me during the project period.

References

1. P.Baskaran, B.Rajeswari, N.jeyabalan, 2006, Development of an *in vitro* regeneration system in sorghum (*Sorghum bicolor* L. mocunch) using root transverse thin cell layers (t TCLS). Turn.J.Bot, 30:1-9.
2. Y.Y.Gang, G.S. DH, D.J. SHI,M.Z. Weng, D.LIX,2003,Establisement of *invitro* regeneration system of the Atrichum mosses.Acta Bot.sin 45(12):1475-1480
3. Kirtikar, K.R and Basu, B.D. Indian medicinal plants, vol-III, (second ed), International Book Distributors, Dehradun 1983:pp.1615-1617.
4. S.R.Kumar, S.H.Mishra, 1997, Screening of anti-inflammatory and hepatoprotective activities of alantolactone isolated from the roots of *Inula racemosa*, *Indian Drugs* 34:pp.571-575.
5. T.Lokesh Nikajoo, 2009, Central nervous system depressant activity of *Pergularia daemia*, 1: 119-124.
6. MarkhamK.R, Techniques of flavonoid identification, Academic Press, London, 1982.
7. T.Murashige, E.Skoog, 1962, A revised medium for rapid growth and bioassays with tobacco tissue culture, Physiol Plant, 15:473-497.
8. K.Nakagawa, H.Fukui, M.Tebata, 1986, Hormonal regulation of berberine production in cell suspension cultures of *Thaijcdvum minus* plant cell Rep 5:69-71.
9. Prajapathi, N.D, Purohit S.S, Sharma A.K, Kumar T,A hand book of medicinal plants. Agrobios, India, 2003.
10. S.Ramachandra Rao, G.A.Ravishankar, 2002, Plant cell cultures; chemical factories for secondary metabolites.Biotechnol.Adv, 20:101-153.

11. A.Q. Rao, S.S.Hussain, M.S.Shahzad, S.Y.A.Bokhair,M.H.Raza,A.Rakha Majees ,A.A.Shahid,T.Saleemz Husnain,S.Riazuddin,2006,Somatic embryogenesis in wild relatives of cotton (*Gossypium spp*) J.Zhejiang Univ.(Science B),7(4):291-298.
12. S.V.Suresh Kumar, S.H.Mishra, 2005, Hepatoprotective activity of rhizomes of C*yperus rotundus* Linn.aganist carbon tetrachloride induced hepatotoxicty, Indian Journal of Pharmaceutical sciences, 67(1): 84-88.

Corresponding author*

Email ID: *vinothkumarphd@gmail.com*

Contact no: 09943451023.