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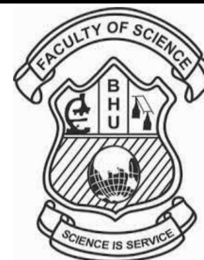

Enhancement of Secondary Metabolites by Elicitation in cell cultures of *Pergularia daemia* Forsk.

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Abstract: **Plant secondary metabolites are used in dyes, cosmetics, pharmaceuticals, and agricultural industries. These secondary metabolites are produced in various plant parts. Due to their various applications, several procedures have been developed in plant biotechnology to increase their production. In the present study, *Pergularia daemia* Forsk. (Family- *Asclepiadaceae*) have been used for tissue culture studies and elicitation experiments. UV radiation was used for plant pigment production and pigmented callus was grown and extracted at the ages of 2, 4, 6, and 8 weeks for further spectrophotometric analysis. Four-week-old callus estimated a higher amount of cyanidin-3-glucoside (314.52 ± 0.05 µg/g dw). As the plant pigments are known for their antioxidant potentials, cell cultures were examined for their activities using the Ferric ion-reducing antioxidant potentials and DPPH method. In FRAP activity, pigmented, non-pigmented and whole plant showed reductions of 250.00 ± 7.55, 325.00 ± 3.33, and 263.33 ± 4.35 µg/g DW respectively. In DPPH method, pigmented callus exhibited a lower IC_{50} value (0.06 mg/ml) as compared to non-pigmented and whole plant methanolic extracts (0.065 and 0.8 mg/ml respectively). Their total phenolic and flavonoid contents were also compared to standard flavonoid and phenolics.**

Index terms: Pergularia daemia, anthocyanins, Asclepiadaceae, antioxidant, elicitors, secondary metabolites.

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1. INTRODUCTION

Pergularia daemia Forsk. (Fam. Asclepiadaceae) is a perennial herb, that commonly grows wildly in India. *P. daemia* is used for its anti-venom, antiseptic, and anti-helminthic properties along with the treatment of gastric ulcers, and uterine and menstrual problems (Dutta and Ghosh 1947, Singh et al., 1980, Elango et al., 1985). Leaves are used as an ailment for anaemia, leprosy, arthritis, haemorrhoids, amenorrhea, dysmenorrhea, infantile diarrhoea, body pain, asthma, bronchitis, and whooping cough (Arseculeratne *et al.,* 1985; Gill and Akinwumi, 1986; Bruneton, 1997). Boils and sores are treated with Latex. Stem is used for the treatment of colds, malarial fever, and fever treatment (Selvanayahgam *et al.,* 1994; Singh and Zaher, 1998). Due to these benefits, this plant has been selected for tissue culture experiments (Kiranmai *et al.,* 2008). Tissue culture is an excellent tool for large-scale production of useful metabolites throughout the year without being affected by weather or temperature. By changing the

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culture condition, medium concentration, and induction of elicitation we can enhance the concentration of desired product in the callus (Simões *et al.,* 2012). Several experiments have been conducted on elicitation for secondary metabolite enhancement. Elicitors are the compounds that stimulate physiological abnormality in plants. This may be exogenous (pathogenic origin or environmental stress related) or endogenous (compound released from the action of pathogen attack). These elicitors trigger the defence system and enhance the production of commercially important secondary metabolites (Sharma et al., 2011).

Elicitors can be classified in two types physical and chemical. Based on nature, they are of biotic and abioitic (Radman et al., 2003). Biotic elicitors are produced from the plant itself or pathogenic-derived. Abiotic elicitors are differentiated into physical and chemical compounds. Common examples of abiotic elicitors are metal ions (calcium, silver, cadmium, oxalate) and radiation (UV rays, X rays; Staskawicz et al., 1991, Vasconsuelo et al., 2007). In biotic elicitors, fungal or bacterial cell wall composites such as polysaccharide, pectin, chitosan, cellulase, mannan, lipopolysaccharide, and glutathione were listed. The elicitors induce the production of several secondary metabolites in medicinal plants such as phtoalexins in *Glycine max* and *Nicotiana tabacum,* forskolin production in *C. forskolin*, shikonin in *Lithospermum erthrorhizon*, catharanthine and ajmalicine in *Catharanthus roseus* (Nef-Campa et al., 1994, Rijhwan and Shanks et al., 1998, Namdeo et al., 2002).

As *P. daemia* is already known for its biological potential, elicitation experiments will be conducted in tissue callus in order to enhance the utility of callus extracts for in various purposes. Previously flavonoids and pigments were produced from tissue culture experiments using UV – radiation in several cases (Jiao et al., 2015; Gai et al., 2016; Huang et al., 2016).

II. MATERIALS AND METHODS

A. Materials

Young nodal explant was excised from a 6-month-old plantlet from the University of Rajasthan campus, Jaipur, India (January-February 2022); verified by Herbarium, Department of Botany, University of Rajasthan (Herbarium Sheet no. 20147).

The media and chemicals for phytochemical analysis chemicals were obtained from SRL (Sisco Research Laboratory), Pvt. Ltd, Mumbai, India.

B. Culture medium

Murashige and Skoog's medium (MS; 1962) supplemented with 2,4D (2 mg/L) and Kn (0.5 mg/l) was used for callus induction (Kiranmai et al., 2008).

C. Composition and preparation of media

MS medium with inorganic salts, vitamins, and growth hormones was prepared and pH was adjusted to 5.8, followed by autoclaving at 121ºC for 20 min.

For inoculation of explants, leaf and internode explants were aseptically excised from seedlings, cut into appropriate sizes (1 cm), and placed in a sterilized MS medium. Leaf and stem explants were inoculated in the sterilized medium. The callus was further subcultured in a similar medium for further biochemical and phytochemical analysis.

D. Exposure to UV-B radiation

Cotton plugs from culture flasks were replaced with parafilm which is 85% transparent to UV-B radiation (≥300 nm). The flasks were exposed for UV radiation (15 min; 1.2 m, 40 W UV B-313; Simões et al., 2012). After UV exposure callus was provided 25 μ mol m^{-2} s^{-1} fluorescent light. This experiment was repeated for 3 days (Mahdood and Sarropoulou 2024). The experiment was repeated three times per week, and fifteen replicates were made per experiment. Callus samples without any UV-treatment was taken as control.

For pigment extraction, fresh callus tissue of approx. 5g was mixed in 10 ml of 0.1% (v/v) HCl-methanol mixture. The solution was filtered and centrifuged at 10,000 rpm (5 min). The supernatant was used for further phytochemical analysis (Takahama et al., 2013).

E. Isolation and identification of anthocyanin pigments

To characterize chemically the anthocyanin in *P. daemia* callus line, freshly harvested tissue was separately extracted using 0.1% HCl-methanol. The acidic methanol extracts thus obtained were concentrated at room temperature, applied directly onto a Whatman filter paper No. 3 as well on cellulose and Silica Gel G 254 chromatography plates, and resolved using different solvent systems (butanol: acetic acid: water, 4:1:5 or 6:1:2 and chloroform: methanol: formamide, 16:3.8:0.2). The discrete anthocyanin bands obtained from the two extracts were cut and eluted with methanol, dried *in vacuo* and subjected to spectrophotometry to determine the absorption spectra in the visible range. The methanolic solution was measured for absorbance at 535 nm (UV/VIS spectrophotometer LABCARE LB-925; Ayu et al. 2018)

The amount of anthocyanin content was measured using a standard curve for red onion anthocyanin extracted with the same procedure mentioned above (Fossen et al., 2003). For the calculation of anthocyanin contents, 5 gm callus was extracted in 0.1% HCl-methanol (40 rpm at RT for 15 min).

Using red onion extract (0.25-3 g/10 ml concentrations) as a reference solution a standard curve of cyanidin-3-glucoside was prepared. Anthocyanin content (mg/g fresh weight) was calculated. Five replicates were used for all experiments. Data was analysed using ANOVA.

F. Estimation of total phenolics and total flavonoid contents

A standard calibration curve of gallic acid (10-500 mg/mL) was prepared, and phenolic content was measured using the FC reagent (Folin-Ciocalteau method; Latif et al., 2013). Optical density was measured at 750 nm. For the extract sample, 1 g of dry weight extract, was treated with FC reagent, and phenolics were expressed in mg of gallic acid equivalents (mg GAE/g dw of extract). Experiments were carried out in triplicate and the results were analysed statistically. For total flavonoid estimation, the AlCl3 spectrophotometric method is used (Shraim et al., 2021). A standard graph is prepared using 10-100 mg/mL of quercetin solution. On plotting 1gm dw of test extract in standard graph, the flavonoid contents were estimated. The values were expressed as milligrams of quercetin equivalents per gram dry weight (mg QE/g dw). Experiments were carried out in triplicate.

G. Antioxidant activity by DPPH technique

Oxidation of DPPH (1,1-diphenyl-2-picrlyhydrazyl) radical was conducted for the determination of the antioxidant properties of extracts (Baliyan et al., 2022). Standard concentrations (0.8, 0.6, 0.4, 0.2, 0.1 mg/mL) were prepared in methanol, subsequently, DPPH was added at a 2 mg/10 mL concentration. Optical density was measured after 30 min of incubation (517 nm; Varian type Cary PCB 150 Water Peltier System). Data was recorded in triplicate and processed using EXCEL. The % inhibition can be calculated using the following equation:

$$\% \text{ Inhibition} = 1 - (\text{OD Sample} / \text{OD Control}) \times 100$$

where OD Sample is the optical density of the test sample, and OD Control is the optical density of the test control.

F. Ferric ion reducing antioxidant potentials method

For calculation of antioxidant power by the Ferric ion reducing antioxidant potential method, ascorbic acid and extract of suitable concentration (62.5-1000 µg/mL) was prepared in 1.0 mL absolute ethanol. The resultant solution was mixed with potassium ferricyanide (2.5 mL, 1 % w/v) and phosphate buffer (2.5 mL, 0.2 M, pH 6.6). By following the standard protocol described by Spiegel *et al.* Absorbance was measured at 700 nm (Spiegel *et al.* 2020).

Statistical analysis

Experiments were performed in triplicate and tissue culture experiments in quintuplicate or multiples of five. Data analysis was done on mean ± SE (SPSS 16.0; IBM Corporation, IL, USA)

III. RESULTS AND DISCUSSION

A. Callus induction

Leaf and stem explants produced a mass of friable callus on MS medium supplemented with 2,4 D (0.1-7 mg/L) within 30 days of incubation (Stem culture Figure 5 A, Leaf culture 5 B). As induction of the callus was maximum (100 %) from the leaf explant at 2,4-D (2

mg/l) and Kn (0.5 mg/l), the leaf callus was chosen over stem for the experiment with elicitation. Leaf explant gave maximum response to the selected hormone concentration. Callus was profuse and white friable (Table 1, Figure 5 C).

Table 1. General appearance, growth, and colour comparison of callus

Age of callus	Consistency of callus		Growth index		Moisture content (in %) *	
	P	NP	P	NP	P	NP
2 week	Fragile	Fragile	1.29	0.69	87.06	87.68
4 week	"	Compact	2.31	1.65	78.31	78.28
6 week	"	"	4.38	5. 54	74.07	74.67
8 week	"	"	6.68	5.64	80.18	71.71

P= pigmented callus

NP=non-pigmented callus

After UV radiation treatment, purple-red coloured cell clusters appeared spontaneously in one of the cell line cultures (Figure 5 D). This sector was selectively excised and subcultured on the fresh medium.

Age of callus	Morphology of callus		Growth in relation to substrate		Colour	
	P	NP	P	NP	P	NP
2 week	Irregular lobed	Irreg ular lobed	No pene-tration	Penetrate into the medium	Light purple	White
4 week	"	"	"	"	Purple	Yellow
6 week	"	"	"	"	Purple brown	Yellow green
8 week	"	"	"	"	Brown	Green

The frequency of pigmented cells gradually increased with each subculture cycle. On continuous agitation, a uniformly stained, dark purple pigmented callus was generated (Figure 5E, 4F for 6-week and 8-week-old callus respectively).

The dried white and pink callus was extracted in ethanol and used for phytochemical evaluation. Growth index of purple callus was more (5.64) as compared to the non-pigmented callus (6.68 in 8 weeks old callus). However, moisture contents were similar (Table 2).

Table 2. Moisture content and growth index comparison of callus

Moisture content = Final dry weight of tissue-Initial dry weight of the tissue/Initial dry weight of tissue. P= pigmented callus; NP=non-pigmented callus

B. Phytochemical evaluation

A four-week-old callus was used for preliminary phytochemical evaluation. Total flavonoids and total phenolics levels of pigmented callus were higher (5.45 ± 0.19 mg GAE/g dw and 17.16 ± 0.21 mg QE/g dw) as compared to white callus (5.03 ± 0.00 mg GAE/g dw and 12.91 ± 1.16 mg QE/g dw, respectively).

Table 3. Antioxidant activities of pigmented and non-pigmented callus by DPPH method

Nature of extract	Total phenolic content	% Inhibition (DPPH method) (Concentration in mg/ml)					
		IC_{50}	0.1	0.2	0.4	0.6	0.8
NP	5.03±0.0	0.06	77.7	77.8	82.6	84.5	86.2
P	5.45±0.1	0.06	80.0	83.6	87.3	90.0	90.95
WP	7.68±0.5	0.08	61.7	63.1	70.2	72.2	72.7
Quercetin		0.04	62.4	60.5	93.3	93.82	94.7

% Inhibition=1- (Absorbance of the sample/Absorbance of the control) × 100

Total phenolics were expressed in mg GAE/g dw

Similar results were observed in antioxidant potentials where IC_{50} value of pigmented callus was lower (0.060 mg/ml) than the white callus (0.065 mg/ml). Coloured calli demonstrate better antioxidant activity (% inhibition 90.04) than the whole plant (% inhibition 72.25; Table 3; Fig 1)

In FRAP method, significantly higher levels of antioxidant activity were observed at 1000 µg concentration in pink callus (323.33 ± 3.33 mg AAE/g) as compared to the control callus (250.00 ± 7.55 mg AAE/g; Table 4; Fig 2). For further bioactivity, pigmented callus was harvested on 2, 4-, 6-, 8- and 10-week-old age.

Figure 1. Antioxidant activities of pigmented and non-pigmented callus by DPPH method

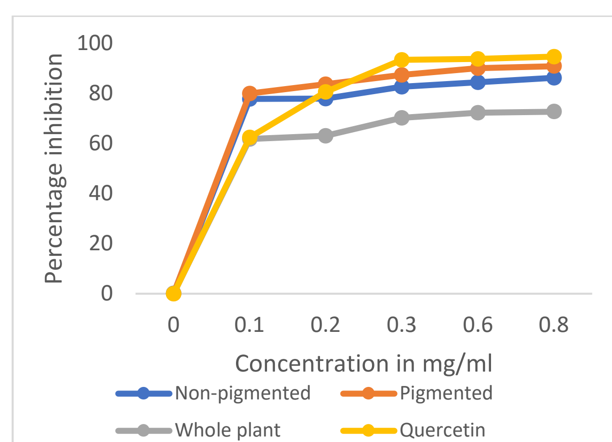


Figure 2. Antioxidant activities of pigmented and non-pigmented callus by FRAP method

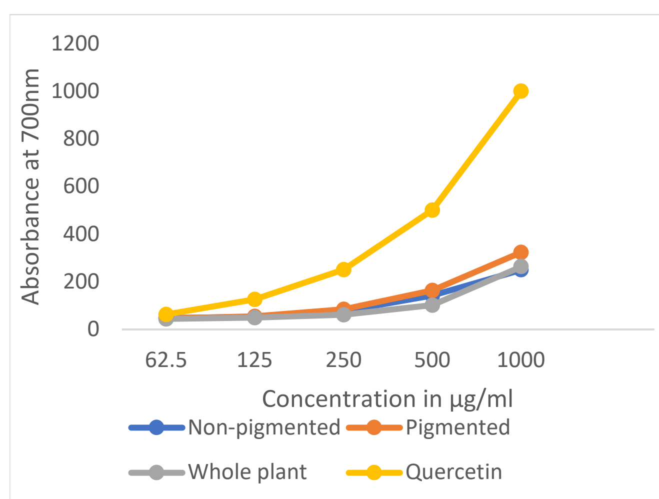


Table 4. Antioxidant activities of pigmented and non-pigmented callus by FRAP method

Nature of extract	Total flavonoids	Antioxidant activity in mg AAE/gdw (FRAP method) (Concentration in µg/ml)				
		62.5	125	250	500	1000
NP	12.91 ± 1.16	48.33 ± 1.66	51.66 ± 1.66	75.00 ± 4.56	141.66 ±7.65	250.00± 7.55
P	17.16 ± 0.21	45.00 ± 5.78	53.33 ± 6.61	83.33 ± 3.33	163.33 ±1.66	323.33± 3.33
Whole plant	13.75 ± 1.25	43.33 ± 1.66	48.33 ± 1.66	60.00 ± 7.64	101.66 ±2.79	263.33± 4.35
Ascorbic acid	-	62.5	125	250	500	1000

mg AAE/g = mg Ascorbic acid equivalent/g extract; Total flavonoids were expressed in mg GAE/g dw

On compound isolation fresh purple pigmented cells were harvested at 4 weeks-old age, extracted in HCl-MeOH and concentrated. The dried extract was applied on silica gel TLC plates using butanol: acetic acid: water (4:1:5/6:1:2) and chloroform: methanol: formamide (16:3.8:0.2) solvent systems using a standard marker of red onion peel extract. Developed chromatograms were air dried and examined under UV light. Positive spots for anthocyanins, plates were sprayed with ρ-anisaldehyde reagent (0.5 ml ρ-anisaldehyde in 50 ml glacial acetic acid 1 ml 97% conc. H_2SO_4; Bidkar et al., 2021).

For confirmation of anthocyanins, butanol: acetic acid: water (4:1:5) as a solvent system were used exhibiting 4 discrete bands with R_f values (0.12, 0.28, 0.30 and 0.36), out of which the upper band (R_f 0.36) coinciding with to red onion peel was isolated and confirmed using

previous experiments on red onion (cyanidine-3-glucoside). Later, the major purified compound was isolated by PTLC, dissolved in methanol, crystallized and UV maximum was taken at 535 nm. However, due to poor yields other spots could not be isolated (Delazar et al., 2010).

C. *Quantification of compound*

For the confirmation, extracts of onion peel and pigmented callus showed absorption maxima (λ max) at 535 nm, overlapping each other i.e. the characteristics peak absorbance of cyanidin-3-glucoside (Fig. 4). On quantification, callus tissue exhibited appreciable levels of cyanidin quantified in 4 weeks-old callus (314.52 ± 0.05 mg/g fw) followed by 2 weeks-old callus (274.33± 0.11 mg/g fw). The anthocyanin level followed a sigmoid pattern where, after 4 weeks, there was a regular decline in the in the total levels (74.23 ± 0.02, 52.69 ± 0.08, 41.88 ±0.06 mg/g fw for 6-, 8- and 10-weeks age; Table 5; Fig 3).

Table 5. Quantification of isolated compounds in plant callus and *in vivo* plant

Isolated compounds	Concentration of isolated compounds (µg/g dw)					
	Age of callus in weeks					
	2	4	6	8	10	Whole plant
Kaempferol	36.9± 0.37	45.8± 0.00	35.66 ± 0.17	38.2 ± 0.11	35.6 ± 0.58	45.3± 0.11
Quercetin	35.8 ± 0.11	42.6 ± 0.00	24.1 ± 0.15	28.4± 0.58	28.7± 0.58	42.3± 0.66
Cyanidin-3-glycoside	274.3 ± 0.11	314.5 ± 0.05	742 ± 0.02	52.6 ± 0.08	41.8± 0.06	Nil

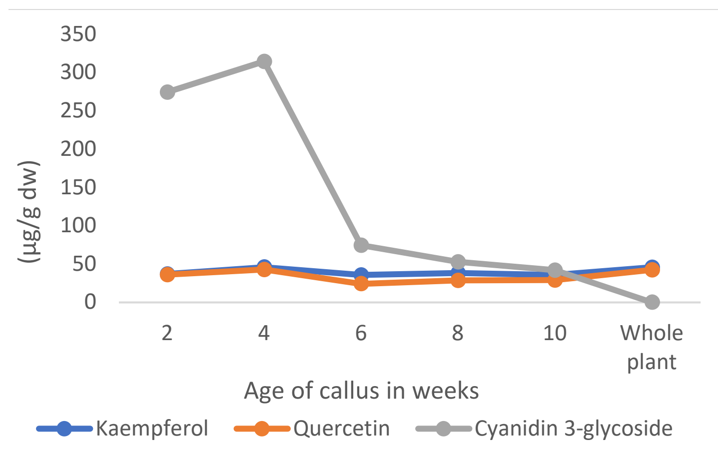
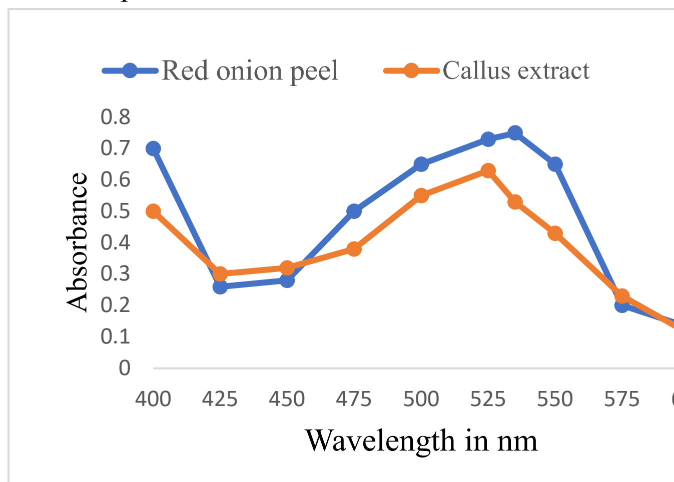


Figure 3. Antioxidant activities of pigmented and non-pigmented callus by FRAP method

Figure 4. Comparative analysis of wavelength in onion peel extract and callus extract.



DISCUSSION

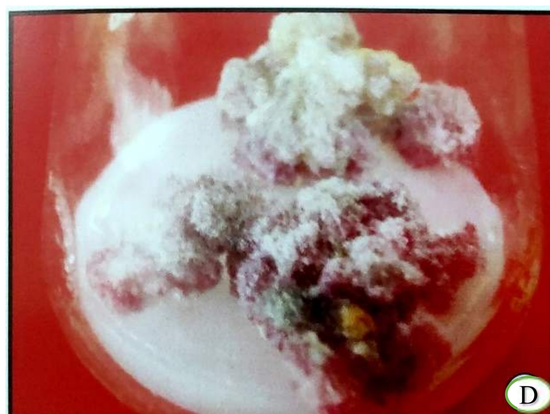
Tissue culture techniques provide an excellent opportunity for the extraction of valuable plant metabolites under controlled conditions throughout the year. These cell cultures provide valuable biological products under optimized conditions, without being affected by environmental factors. Secondary metabolites like alkaloids, flavonoids and terpenoids used for several therapeutic, pharmaceutical industry, paint manufacturing, food processing, cosmetic industry and agricultural purposes (Espinosa-Leal et al., 2018). For large scale production of these secondary metabolites phyto-fermentation or *in vitro* cell cultures were now popular worldwide.

In the current study, *P. daemia* widely popular as indigenous medicine, cell cultures have been generated. As from previous studies callus cultures were reported for the presence of α-amyrin, β-amyrin, β-sitosterol, lupeol, lupeol acetate, oleanolic acid, kaempferol and quercetin (Pancholi, 2023). For the first, time elicitation experiment has been reported so far. Using UV rays, cyanidin 3-o-glucosides were isolated and identified from callus cultures of *P. daemia*. Cyanidin pigments were compared with red onion extract using TLC plates. Higher amount of cyanidin pigment were reported in 4 weeks old callus and their antioxidant activity was comparable to standard kaempferol and Quercetin. As the plant pigments are known for their antioxidant potentials, cell cultures were examined for their activities using Ferric ion- reducing antioxidant potentials method and the DPPH method. Antioxidant activities of the pigmented callus showed higher potentials those of the whole plant extracts. In the DPPH method of antioxidant activity, % inhibition in at 0.8 mg/ml is 90.95 in pigmented callus comparable to quercetin (94.71% inhibition). On quantification, a higher amount of Cyanidin-3-glycoside pigment was reported in 4 weeks old callus tissue (314.52 ± 0.05 µg/g dw).

CONCLUSION

In vitro callus generation is of immense importance due to the commercial exploitation potential of secondary metabolites. The efficiency of secondary metabolites production depends on many factors such as type of explant, medium supplements, growth factors, plant growth hormones and elicitors.

In the present work, *in vitro* pigmented callus was developed by tissue culture protocol for *P. daemia* using UV ray elicitation procedure. On phytochemical estimation, the pigmented callus showed the presence of cyanidin 3-glucosides. The pigmented callus had a higher number of antioxidants, flavonoids, and phenolics compared to normal leaf callus. By discovering of new secondary metabolites and to increasing their biochemical contents, elicitation method was proved effective. Chemical industry depends on plant-based metabolites for large-scale production. Therefore, this study will prove important for future prospect and their evolution in research studies. Further for current aspect more elicitation experiments will be conducted for targeting drought resistant compound synthesis.



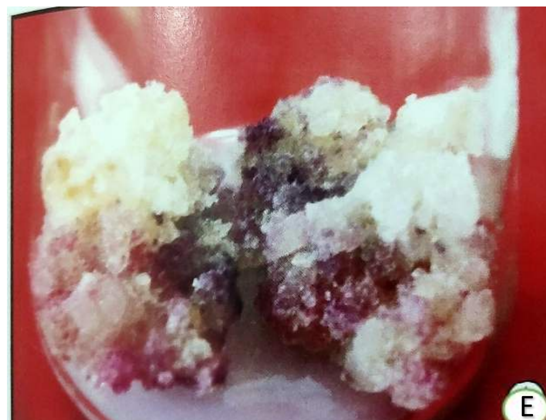


Figure 5. A- Callus initiation from stem explant; B- Callus initiation from leaf explant;

C- 4 weeks old untreated callus; D- 4 weeks old callus after UV treatment;

E- 6 weeks old treated callus; F- 8 weeks old treated callus.

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