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In vitro techniques to accelerate flavonoid synthesis in some Euphorbiaceae members

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ABSTRACT: In the present investigation, an attempt has been made to accelerate the flavonoid synthesis in *Baliospermum montanum* (Wild.) Muell-Arg., *Dryptes roxburghii* (Wall.) Huresawa and *Codiaeum variegatum* (L.) Bl, members of Euphorbiaceae family. Internodal explants were used to induce callus. Maximum growth of callus was observed in Baliospermum montanum and *Codiaeum variegatum* at 2mg/L and 3mg/L of 2,4-D respectively whereas *Dryptes roxburghii* showed maximum growth of callus at 3mg/L of BAP. Suspension culture was obtained from well developed callus. Three elicitors were used to induce flavonoid synthesis namely phenylalanine, copper sulphate and *Pseudomonas aeruginosa.* Among three, phenylalanine was found to be the best elicitor and maximum production of flavonoid was observed in *Baliospermum montanum*.

KEYWORDS: Baliospermum montanum, Dryptes roxburghii, Codiaeum variegatum, callus, suspension culture, Elicitor and flavonoid.

1 INTRODUCTION

The Euphorbiaceae is one of the largest family in dicotyledons, and has significant economic importance. The family has a cosmopolitan distribution with five subfamilies, 49 tribes, 317 genera and about 8,000 species. The family Euphorbiaceae is generally distinguished by the presence milky sap, the unisexual flowers, superior ovary and generally trilocular, axile placentation, collateral ovules, pendulous with ventral raphe and usually carunculate. Euphorbiaceae classification is difficult because of the variability in morphology, genetics and complexity in habitat. The family consists of species of great economic importance, the source of rubber and medicine (*Hevea*); staple starch source (*Manihot*) and fruits (*Phyllanthus emblica*); seed oils (*Ricinus, Vernicia*); insecticides, Waxes (*Euphorbia antisyphilitica*), edible seeds (*Caryoclendron orinocense*), leafy vegetable (*Cnidoscolous aconitifolius*).

Secondary metabolites are phytochemicals produced by plants which does not play any primary role in growth, photosynthesis and reproduction. Due to its diversity, Phytochemicals can be used as taxonomic characters in plant classification. Flavonoids are a specific class of phenolic plant phytochemicals represented by over 5000 compounds, subdivided Into 13 categories that include anthocyanidins, catechins, flavonols, and flavones [1]. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization [2]. Many have antiallergic, antiviral actions and some of them provide protection against cardiovascular mortality [3] & [4].

Dietary intake of phytochemicals may promote health benefits, protecting against chronic degenerative disorders, such as cancer, cardiovascular and neurodegenerative diseases. Majority of foods, such as whole grains, beans, fruits, vegetables and

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herbs contain phytochemicals. These phytochemicals, either alone or in combination, have tremendous therapeutic potential in curing various ailments [5]. Because of the wide spectrum of medicinal properties of phytochemicals, it is being commercially important to produce phytochemicals on large scale.

Study has revealed that phytochemicals plays important role in the immune system of plants, so by exposing plants to different environmental conditions, the phytochemicals production can be induced. There are different methods employed for phytochemical production, such as by adding precursor, using elicitor (biotic/ abiotic), metabolic engineering, biotransformation and mutagenesis. In this paper, flavonoid is induced by using its precursor (phenylalanine), by adding elicitor (biotic – dead cells of *Pseudomonas aeruginosa* and abiotic –copper sulphate).

2 MATERIALS AND METHODS

2.1 COLLECTION OF PLANT MATERIAL

Baliospermum montanum (Wild.) Muell-Arg,, *Dryptes roxburghii* (Wall.) Huresawa were collected from Sirsi, Western Ghats of Karnataka and *Codiaeum variegatum* (L.) Bl. was collected from Lal Bagh Botonical garden, Bangalore. Plants are being maintained in the Department of Molecular Biology, Bangalore University, Bangalore.

2.2 CALLUS INDUCTION

Internodal explants of *Baliospermum montanum*, *Dryptes roxburghii* and *Codiaeum variegatum* were inoculated on Murashige and Skoog basal media (MSBM) consisting of vitamins, 3% sucrose and 0.8% agar supplemented with 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine at the concentrations of 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L were used for callus initiation and proliferation. MSBM without any plant growth regulators (2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine) was used as control. These cultures were allowed to grow upto their maximum growth age (6 weeks). The pH of media was adjusted to 5.8 before autoclaving at 121ºC for 20 min. All cultures were maintained at 25 ± 2ºC in growth chamber with fluorescent light (1500 lux), 16 hrs light and 8 hrs dark photoperiod.

2.3 SUSPENSION CULTURE

Cell Suspension of *Baliospermum montanum*, *Dryptes roxburghii* and *Codiaeum variegatum* were obtained from callus tissue (6 weeks old) developed from internodal explants. One gram of friable callus was excised in Petri dish containing Whatman No.1 filter paper. Callus was slightly mashed and carefully transferred with sterilized forceps to each of 250ml Erlenmeyer flasks containing 50ml liquid MSBM, supplemented with 0.5ml/L of 2, 4-D alone. They were subcultured after every 14 days, the ratio innoculum to fresh medium was 1:6. The flasks were kept on a gyratory shaker at 100 rpm to a photoperiod of 16 hours, with fluorescent light (1200 lux) and a temperature of 25ºC.

2.4 VIABILITY TESTS

Viability was determined by the MTT assay. One ml sample of 6 week old cell suspensions was placed in micro-centrifuge tubes, centrifuged at 1000 rpm, and 100 µl of MTT was added to the sample. MTT protocol was based on Tisserat and Manthey method [6]. 1% MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution was used to test the incubation conditions of 30min at room temperature or 37°C. Viability was determined by counting the coloured cells in a hemacytometer. Control tests of viability were done with suspension cultures fixed in 70% ethanol.

2.5 ELICITOR TREATMENT

The first type of experiment: Phenylalanine (25-100 µg/ml) was added in the 6 week old cell suspensions. The plant cells were harvested after 24, 48 and 72 hours respectively and the liquid media was used to estimate total content of flavonoid.

The second type of experiment: Copper sulphate (CuSO4) was added in flasks containing 6 weeks suspension cultures to obtain a final copper concentration of 20 and 40 µM respectively. The plant cells were harvested after 24, 48 and 72 hours respectively.

The third type of experiment: The bacterial cultures (*P. aeruginosa*) were maintained on Mueller-Hinton Agar (MH). Cultures that maintained on agar slants were transferred to 100 ml liquid medium in 250-ml flasks and incubated at room temperature. The bacterial liquid cultures were kept on a rotary shaker (90 rpm). The culture was collected after reaching

stationary phase (48 hrs). Flasks which contain bacteria were then autoclaved and the solution obtained was stored at 4˚C for future use. The pH was adjusted to 5.8 before autoclaving at 121˚C and a pressure of 1.04 kg/cm^2 for 20 min. The dead cells of *P. aeruginosa* (1, 2, 3 µg/ml) was added in 6 weeks old suspension culture.

2.6 EXTRACTION

For the extraction of flavonoids, plant cells from suspension cultures were oven dried at 45°C and pulverized. Powdered samples (1.0 g) was soaked in 100 ml of methanol for 72 hours and filtered through a Whatman No. 1 filter paper. The filtrates were concentrated using a rotary evaporator

2.7 TOTAL FLAVONOID ESTIMATION

Aluminium chloride colorimetric method [7] with few modifications was used to estimate total flavonoid content. The 1mL of plant cells methanol extract was mixed with 1ml of methanol, 0.5 mL aluminium chloride (1.2%) and 0.5mL potassium acetate (120mM). The mixture was allowed to stand for 30 min at room temperature, and then the absorbance was measured at 415nm. Quercetin was used as standard. Flavonoid is expressed in terms of quercetin equivalent (mg/g of extracted compound)

2.8 STATISTICAL ANALYSIS

Each of the tests was carried out in triplicate and the results are expressed in Mean ± STD

3 RESULTS

3.1 CALLUS INDUCTION

Callus proliferation was observed from the internodal explants of *Baliospermum montanum* (Wild.) Muell-Arg., *Dryptes roxburghii* (Wall.) Huresawa and *Codiaeum variegatum* (L.) Bl within a week of inoculation and the highest frequency of callus was observed after 5 weeks on MS medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. Maximum growth of Baliospermum montanum, *Codiaeum variegatum* callus were observed at 2mg/L and 3mg/L of 2,4-D respectively whereas *Dryptes roxburghii* showed maximum growth at 3mg/L of BAP.

3.2 VIABILITY TESTS

The MTT assay showed 88, 80, and 82% of cell viability of 6 weeks old cell suspension culture of *Baliospermum montanum* (Wild.) Muell-Arg,, *Dryptes roxburghii* (Wall.) Huresawa and *Codiaeum variegatum* (L.) Bl. respectively.

3.3 FLAVONOID ESTIMATION AFTER ELICITOR TREATMENT

The results are presented in Table -1

Fig 1: Callus proliferation: 1- Baliospermum montanum (Wild.) Muell-Arg, 2- Dryptes roxburghii (Wall.) Huresawa and 3- Codiaeum variegatum (L.) Bl

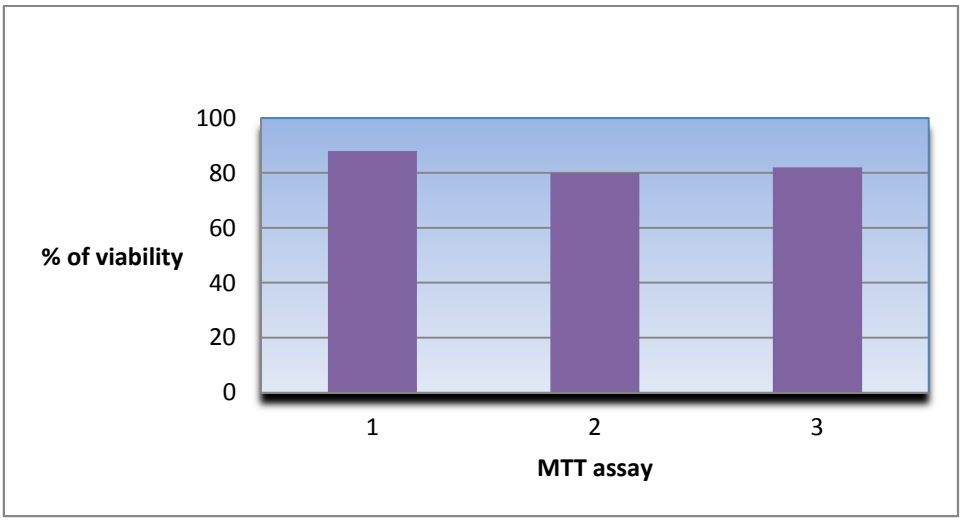

Fig 2: MTT assay: 1- Baliospermum montanum 2- Dryptes roxburghii and 3- Codiaeum variegatum

Table 1- Effect of elicitors on flavonoid synthesis

Type of experiment	Treatment of duration (hrs.)	Concentration of elicitor	Total content of flavonoid mg/g (Bm)	Total content of flavonoid mg/g (Cv)	Total content of flavonoid mg/g (Dr)
Phenyalanine	24	Control	44.08 ± 0.06	32.64 ± 0.24	22.38± 0.04
		25 µg/ml	48.48 ± 0.37	35.4 ± 0.20	27.16± 0.02
		50 µg/ml	50.50 ± 0.40	38.12 ± 0.10	29.65 ± 0.03
		75 µg/ml	55.44 ± 0.38	40.93 ± 0.08	31.83 ± 0.02
		100 µg/ml	47.03 ± 0.05	34.76 ± 0.06	25.14 ± 0.50
	48	Control	47.22 ± 0.08	33.72 ± 0.13	24.55± 0.03
		25 µg/ml	48.34 ± 0.23	36.91± 0.03	28.76± 0.02
		50 µg/ml	51.74 ± 0.06	39.66 ± 0.09	31.26 ± 0.02
		75 µg/ml	54.77 ± 0.23	43.25 ± 0.13	33.54 ± 0.02
		100 µg/ml	52.15 ± 0.14	41.12 ± 0.09	30.65 ± 0.02
	72	Control	54.40 ± 0.22	42.25 ± 0.07	32.13± 0.03
		25 µg/ml	**57.12 ± 0.09**	**45.33± 0.14**	**35.26 ± 0.02**
		50 µg/ml	53.45 ± 0.14	43.85± 0.04	34.47± 0.01
		75 µg/ml	52.45 ± 0.09	41.65 ± 0.11	33.36 ± 0.02
		100 µg/ml	49.89 ± 0.04	39.89 ± 0.08	30.64 ± 0.02
CuSO4	24	Control	45.22 ± 0.09	37.93± 0.03	24.48± 0.02
		20 µM	**49.74 ± 0.13**	**43.51± 0.13**	**31.59 ± 0.02**
		30 µM	43.76 ± 0.08	38.22 ± 0.09	29.65 ± 0.03
		40 µM	42.49± 0.04	35.28 ± 0.20	27.83± 0.03
	48	Control	43.45 ± 0.08	36.8± 0.03	23.47± 0.01
		20 µM	45.41 ± 0.02	38.25± 0.12	25.35± 0.03
		30 µM	48.23 ± 0.09	40.30 ± 0.07	22.14 ± 0.03
		40 µM	44.31 ± 0.14	35.28 ± 0.20	20.24 ± 0.04
	72	Control	40.15 ± 0.05	28.80± 0.07	20.933± 0.03
		20 µM	42.3 ± 0.13	31.29± 0.09	22.83± 0.02
		30 µM	46.61 ± 0.19	37.81 ± 0.02	25.45 ± 0.02
		40 µM	43.29 ± 0.09	35.38 ± 0.08	23.65 ± 0.02

P. aeruginosa	24	Control	45.52 ± 0.21	37.16± 0.03	28.49± 0.03
		1 µg/ml	47.75 ± 0.20	39.5± 0.04	30.09± 0.06
		2 µg/ml	51.23 ± 0.18	43.59 ± 0.02	32.84 ± 0.02
		3 µg/ml	49.71± 0.08	44.65 ± 0.02	33.74 ± 0.02
	48	Control	48.51 ± 0.11	42.46± 0.03	32.7± 0.01
		1 µg/ml	52.42 ± 0.31	45.15± 0.44	33.94± 0.04
		2 µg/ml	53.57 ± 0.10	46.44 ± 0.17	34.18 ± 0.01
		3 µg/ml	**55.93± 0.04**	**48.37 ± 0.03**	**35.15 ± 0.03**
	72	Control	51.71 ± 0.10	46.84± 0.02	34.64± 0.02
		1 µg/ml	47.9 ± 0.02	44.72± 0.16	32.15± 0.03
		2 µg/ml	43.77 ± 0.07	43.66 ± 0.02	33.53 ± 0.02
		3 µg/ml	41.66± 0.07	41.37 ± 0.03	30.64 ± 0.02

n=3, Data is presented as Mean ± STD, Bm- Baliospermum montanum, Dr- *Dryptes roxburghii*
Cv- *Codiaeum variegatum*

4 DISCUSSION

Secondary metabolites are non nutritive phytochemicals which are produced at different developmental stages, under stress conditions and plays important role in giving protection against pathogen attack. When these phytochemicals are ingested by humans, it enhances their resistance power. Flavonoids, widespread plant secondary metabolites, are of immense economic functions such as potential drugs, food nutraceuticals and industrial materials, while their importance was also manifested by the larger and larger demands [8]. Therefore; different methods are being employed to accelerate the phytochemical production. Many successful attempt of elicitor treatment has been reported, *Cephalocereus senile[9], Andrographis paniculata[10], Morinda citrifolia[11], Citrus hystrix[12], Marsilea quadrifolia[13].*

In the present investigation, three elicitors have been used namely phenylalanine, CuSO4 and *P. aeruginosa* to accelerate the flavonoid production in the selected medicinal plants from Euphorbiaceae family. The production of flavonoid varies with type, concentration and duration of elicitor used. Among all three elicitor, phenylalanine was found to be best and the maximum production of flavonoid was observed in *Baliospermum montanum.*

All three selected elicitors showed acceleration in uniform pattern. Phenylalanine showed maximum elicitation of flavonoid at 25µg/ml and 72hrs in Baliospermum montanum (57.12 ± 0.09), *Codiaeum variegatum* (45.33± 0.14) and *Dryptes roxburghii* (35.26 ± 0.02). The mechanism of elicitation is well known. Flavonoid belongs to phenypropanoid group of compounds which are derived from aromatic amino acid (phenylalanine) and end product of krebs cycle (acetyl Co-A.) [14] and [15]. Conjugation of malonylCoA and coumaroyl-CoA molecules to chalcones catalyzed by chalcones synathase is the first committed step of flavonoid synthesis [16]. Chalcones are converted to flavanones by the action of chalcone isomerase (CHI). Flavanones are precursors of all classes of flavonoids [17].

CuSO4 showed maximum elicitation of flavonoid at 20 µg/ml and72 in Baliospermum montanum (49.74 ± 0.13), *Codiaeum variegatum (*43.51± 0.13) and *Dryptes roxburghii (*31.59 ± 0.02). This agrees with the findings of Cristina and Constantin [18]. Similary, the literature is reported by Tumova [19] in *Ononis arvensis* suspension cultures where flavonoid is elicitated after 24 hrs using nickel, cobalt and in callus by silver. Similar results was obtained by Jeong [20] in hairy roots cultures of Panax ginseng, the addition of 20µM of NiSO4 resulted in an increase in gingeng saponin content to about 1.2 times compared with the control levels.

P.aeruginosa showed maximum elicitation at 3 µg/ml and 48hrs in Baliospermum montanum (55.93± 0.04), *Codiaeum variegatum (*48.37 ± 0.03) and *Dryptes roxburghiie(*35.15 ± 0.03). Savitha [21] accounted the betalain production in 7 days by treating the hairy root cultures of *Beta vulgaris* with *Penicillium notatum* (0.25 % concentration) *by* 2.2 fold. Buitelaar [22] Reported 15 % increase in thiophene accumulation in hairy root cultures of *Tagetus patula* by using *P. expansum.* Maojun [23] reported enhanced puerarin content using *Penicillium citrinum* in *Pueraria thomsonii* cell suspension culture.

5 CONCLUSION

Callus was well developed from intermodal explants of Baliospermum montanum, *Codiaeum variegatum* and *Dryptes roxburghii.* Suspension culture was obtained from callus which showed cell viability up to 88, 80, and 82% of *Baliospermum montanum, Dryptes roxburghii* and *Codiaeum variegatum* respectively. The present investigation revealed that Elicitor

treatment accelerates the production of flavonoid in the selected members of Euphorbiaceae and among three elicitors, phenylalanine was found to be best and maximum elicitation was observed in *Baliospermum montanum*.

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