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In vitro Callogenesis and Phytochemical Screening of Harsingar (Nyctanthes arbor-tristis) a Multipotent Medicinal Tree

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Abstract: *Nyctanthes arbor-tristis* belongs to family Oleaceae is a small sacred ornamental tree. This plant has several medicinal properties. Different parts are used traditionally for treatment of various diseases like sciatica, chronic fever, skin disease and posses properties like anti-inflammatory, hepatoprotective, antidiabitic, antioxidant etc. The plant based traditional knowledge has become a recognized tool in search for new sources of drugs. The present work is based on developing a protocol for callus induction from nodal explants of *N. arbor-tristis*. Among various PGRs 2,4-D shows maximum callus induction. Phytochemical analysis of natural and *in vitro* raised plants showed the presence of bioactive substances like flavonoids, alkaloids, terpenoids in different types of extracts.
Key words: *Nyctanthes arbor-tristis*, *in vitro* regeneration, BAP, TDZ, 2,4-D, Kn, phytochemical, Callus.

Introduction:

Over centuries, cultures around the world have learned how to use plants to fight illness and maintain health. These readily available and culturally important traditional medicines form the basis of an accessible and affordable health-care regime and are an important source of livelihood for indigenous and rural populations[1]. *Nyctanthes arbor-tristis* is an important medicinal plant belongs to family Oleaceae, commonly known as Parijata (Sanskrit) and Harsingar (Hindi), Night Jasmine (English). It is a small tree with its fragrant flowers found wild in the forests of Central India and Sub-Himalayan regions; cultivated in gardens in many parts of India[2]. The bright orange corolla tubes of the flowers contain a saffron-yellow colouring matter, which was formerly used for dyeing silk[3]. Traditionally the flowers are used as stomachic, expectorant, ophthalmic purpose, skin diseases[4,5], the stem bark is given in rheumatic joint pain, malaria, bronchitis[4,6], leaves are used for treatment of various diseases like sciatica, chronic fever, as a laxative, diaphoretic and diuretic[7] and seeds are used as anthelmintics and in alopecia[8]. The plant posses anti-inflammatory[9], hepatoprotective[10], antidiabitic and antioxidant[11], antibacterial[12], antileishmanial[13] activities.

Many young seedlings of *N. arbor-tristis* die due to excess of phenolic compound in the pericarp and poor seed germination[14,15] as a result of which natural population of this plant is depleting. Increasing global inclination toward herbal medicines, resulting in obligatory demand for huge raw material[16]. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional methods in the industrial production of bioactive plant metabolites[17].

Plant growth regulators are one of the most important factors affecting cell growth, differentiation and metabolite formation[18]. To produce cell dry mass as well as secondary metabolites from medicinal plants, it is important to establish the optimal culture conditions (chemical and physical environments) for the plant species used. The individual levels of auxin and cytokinin in the media used influenced the growth and regulation of cell metabolism[19]. Callus tissues are a good biosource of genetic variability. By manipulating different growth conditions, PGRs etc. it can be used for regeneration of complete plants, initiation of cell suspension cultures and production of various secondary metabolites[20].

The present study aimed to find phytochemical constituents through preliminary analysis from different parts *viz.* natural and *in vitro* leaves and stem of *N. arbor-tristis* along with callogenesis studies in different concentrations of PGRs.

Material and Methods:

Plant material:

Natural plant was procured from the nursery of Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV), Jabalpur. Plants were identified by Institutional Botanist and voucher specimen (No. 22420). The nodal segments were aseptically inoculated on to MS[21] media supplemented with cytokinins Kinetin (Kn) and BAP[22] and the degree of callusing was studied in different concentrations of cytokinins and auxins. The *in vitro* raised nodal explants were used to initiate callus on semi-solid MS media.

The formula for calculating callus induction is shown:

$$\text{Callus induction} = \frac{\text{Number of explants with callus}}{\text{Total number of explants inoculated}} \times 100$$

The natural and *in vitro* leaf, stem and callus material were dried in shade, powdered and stored in air-tight container for further preliminary phytochemical studies.

Preparation of crude extracts:

Approximately 5 gm of sample was weighed and dissolved with solvents *viz.* petroleum ether, methanol, diethyl ether, ethyl acetate and water separately and was allowed to stay for 24 hours. After incubation the sample was filtered by Whattman filter paper, the filtrate was used for phytochemical analysis.

Phytochemical tests:

The phytochemical analysis was carried out to determine the presence of following bioactive compounds using the standard qualitative procedures[23,24].

Anthocyanins and Anthocyanidins:

About 2 ml of aqueous extract was added to 2ml of 2N HCl and ammonia results in appearance of pink red colour which turns blue violet indicates the presence of anthocyanins and anthocyanidins.

Anthracene glycosides:

Sample (5 ml) was added to ammonium hydroxide (25 %) gives red colour indicates the presence of anthracene glycosides.

Coumarins:

Formation of yellow colour on adding 3ml NaOH (10 %) to 2ml of extract indicates the presence of coumarins.

Flavonoids:

Jone's Test: To small amount of sample dissolve in 1 ml of acetone, 2 ml of 10% aq $K_2Cr_2O_7$ and 6 ml of 6 M H_2SO_4. A blue green colour indicates the presence of flavonoids.

Tannins:

About 1% lead acetate was added 2ml of extract. A yellowish precipitate shows the presence of Tannins.

Emodins:

Red colour indicates the presence of emodins when NH_4OH (2 ml) and benzene (3 ml) was added to the extract.

C**arotenoids:**

Presence of carotenoids was showen by development of blue green colour after adding conc. HCL: Phenol (1:1).

Saponin:

Froth test: Froth formation by shaking 1 ml solution of sample in water in a tube reveals saponin.

Sterols and Terpenoids:

Libermann-Buchard test: Samples were treated with few drops of acetic anhydride, boiled and cooled. Conc. H_2SO_4 from the sides of the test tube was added shows a brown ring at the junction of two layers and the upper layer turns green which shows the presence of steroids and formation of deep red colour indicates the presence of terpenoids.

Salkowski test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of conc. H_2SO_4, shaken and allowed to stand. Appearance of red colour at the lower layer indicates the presence of sterols and yellow colour at the lower layer indicates the presence of terpenoids.

Alkaloids:

Meyer's test: To 1ml of each of the sample solution few drops of Meyer's reagent (potassium mercuric chloride solution) was added. Formation of cream white precipitate indicates the presence of alkaloids.

Wagner's test: To few ml of each of the sample solution, Wagner's reagent (iodine in potassium iodide) was added, which resulted in the formation of reddish brown precipitate indicating the presence of alkaloids.

Phenols:

Ferric Chloride test: Extracts were treated with few drops of 5 % acidified ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Cardiac glycosides:

Kellar Kiliani test: Conc. H_2SO_4 (1 ml) was taken in a test tube then 5ml of extract and 2ml of glacial acetic acid with one drop of ferric chloride were added which results in formation of a blue colour.

Carbohydrates:

Molisch's test: Treat the test solution with few drops of alcoholic alpha napthol. Add 0.2 ml of conc. H_2SO_4 slowly through the sides of the tube, a purple to violet ring appears at the junction.

Proteins:

Xanthoprotein test: Yellow colour developed when extracts were treated with few drops of conc. nitric acid. indicating the presence of proteins.

Amino acids:

Ninhydrin test: To 1 ml of sample boiled with 0.1% acetone solution of nynhydrin, appearance of violet colour shows the presence of amino acids.

Starch:

Extracts were treated with lugol solution (1g iodine + 2g potassium iodide) and water, appearance of blue colour shows the presence of starch.

Table 1: Callusing from *in vitro* regenerated shoots of *Nyctanthes arbor-tristis* (Values are Mean ± SE)

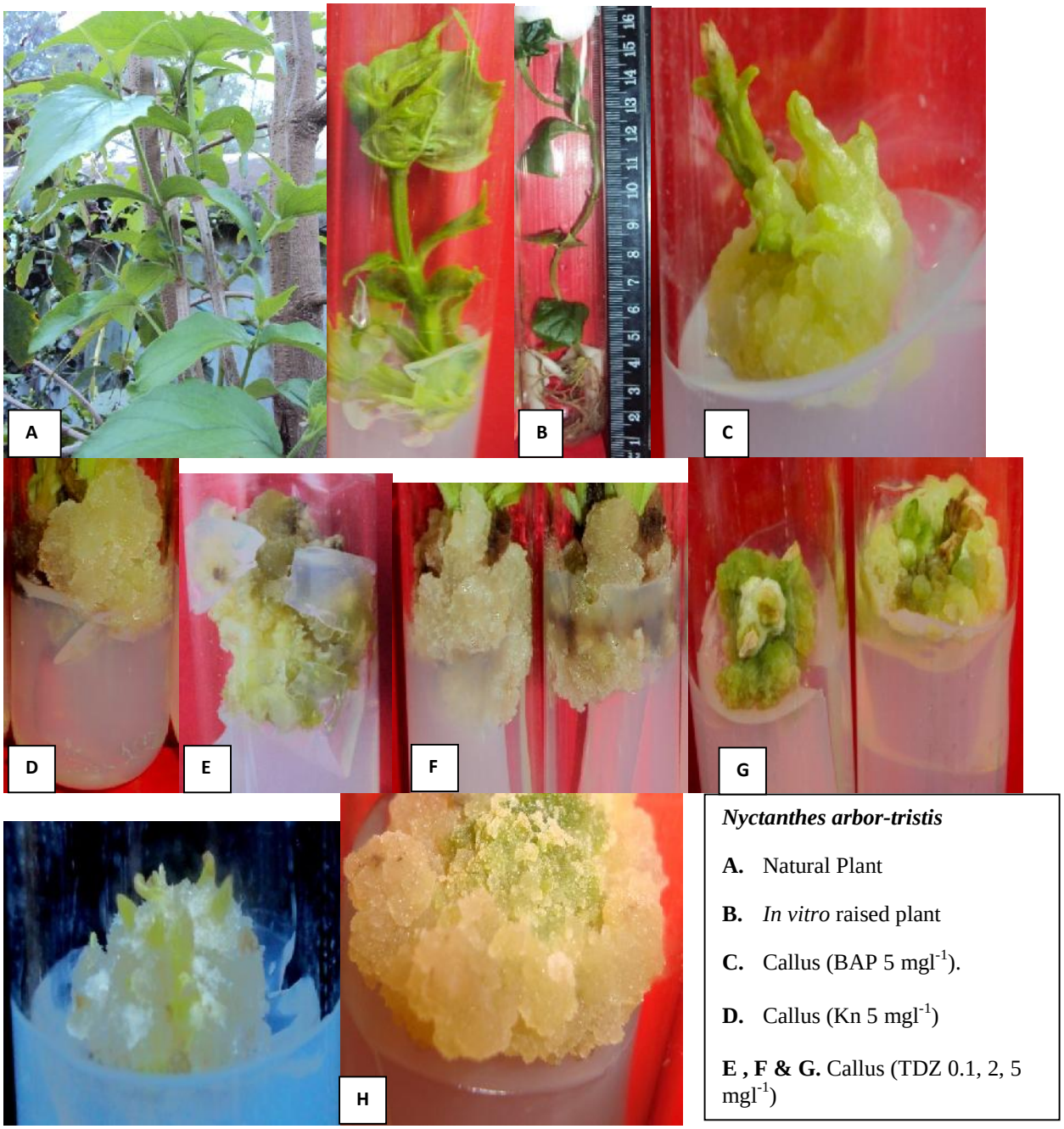
S/N.	PGR's	Callus Induction	Degree of callusing	Nature of callus	Colour
1.	BAP				
	0.1	-	-	-	-
	0.5	-	-	-	-
	1.0	58.33±7.855	+ +	Hard, shiny	Light green
	2.0	69.44±4.537	+ +	Hard, shiny	Light green
	5.0	80.55±2.266	+ + +	Hard, shiny	Light green
2.	Kn				
	0.1	-	-	-	-
	0.5	-	-	-	-
	1.0	-	-	-	-
	2.0	38.88±2.266	+	Soft, shiny	Light yellow
	5.0	55.55±0.756	+ +	Soft, shiny	Light yellow
3.	TDZ				
	0.1	36.10±2.266	+	Soft	Cream yellow
	0.5	44.44±4.536	+	Soft	Cream yellow
	1.0	61.11±6.000	+ +	Soft	Green
	2.0	63.88±2.266	+ + +	Medium	Yellow
	5.0	88.83±2.266	+ + +	Hard	Green yellow
	2,4-D				
	0.1	86.10±2.266	+ + +	Soft sticky	Green yellow
	0.5	91.66±3.922	+ + +	Soft sticky	Pale yellow
	1.0	94.44±2.266	+ + +	Soft sticky	Cream yellow
	2.0	49.99±3.513	+	Soft	yellow
	5.0	58.33±3.926	+ +	Soft	yellow

BAP = Benzyl amino purine, Kn = Kinetin, TDZ= Thidiazuron, 2,4-D= Dichlorophenoxyacetic acid, += Low, + += Moderate, + + + = Profuse, SE= Standard Error.

Table 2: Phytochemical screening:

S/N.	Tests	Natural Leaf					Natural Stem					*In vitro* Leaf					*In vitro* stem					Callus				
		ME	**PE**	**DE**	**EE**	**WE**	**ME**	**PE**	**DE**	**EE**	**WE**	**ME**	**PE**	**DE**	**EE**	**WE**	**ME**	**PE**	**DE**	**EE**	**WE**	**ME**	**PE**	**DE**	**EE**	**WE**
1.	**Anthocyanine & Anthocyanidine**	+	-	-	+	-	+	-	+	-	-	+	-	-	+	-	+	-	-	-	-	+	-	-	+	-
2.	**Anthracene glycoside**	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3.	**Coumarins**	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
4.	**Flavonoid**	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	+
5.	**Tannins**	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-
6.	**Emodin**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7.	**Saponins**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8.	**Steriods**																									
	Salkowski test	-	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Libbermans Burchad	-	+	-	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	+	-	+	+	-	-
9.	**Terpenoids** Libbermans Burchad	-	+	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
10.	**Alkaloids**																									
	Mayers	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	+	-	+	-	-	-	+
	Wagners	-	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11.	**Phenolics**	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.	**Cardiac Glycosides** (Keller-Kiliani test)	-	-	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	-	-
13.	**Carbohydrates** (Molish's Test)	-	-	-	-	+	-	+	+	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-	+
14.	**Fatty acids**	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-
15.	**Proteins** (Xanthoprotein test)	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16.	**Amino acids**	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-
17.	**Starch**	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-

*Methanolic Extract (ME), Petroleum Ether Extract (PE), Diethyl ether extract (DE), Ethyl acetate extract (EE), Water extract (WE).Positive test (+), Negative test (-).



Nyctanthes arbor-tristis

A. Natural Plant

B. *In vitro* raised plant

C. Callus (BAP 5 mgl^{-1}).

D. Callus (Kn 5 mgl^{-1})

E , F & G. Callus (TDZ 0.1, 2, 5 mgl^{-1})

Results and Discussion:

Callogenic studies were done by inoculating nodal explants on MS medium supplemented with different concentrations of cytokinins and auxins. Profuse degree of callus induction was observed in MS supplemented with auxin 2,4-D (0.5mgl^{-1} and 1.0mgl^{-1}), soft and sticky cream yellow callus were obtained. Whereas callus obtained in various concentrations of BAP (1.0mgl^{-1}, 2.0mgl^{-1}, 5.0mgl^{-1}) are green in colour and shiny hard in texture. Whereas Callus on different concentrations of TDZ (0.1mgl^{-1}, 0.5mgl^{-1}, 1.0mgl^{-1}, 2.0mgl^{-1} and 5.0mgl^{-1}) ranges from soft to medium and colour also varies from green to yellow.

Phytochemical studies show the presence of secondary metabolites *viz* flavonoids, alkaloids, coumarins, steroids and terpenoids whereas saponins and emodin are absent similar results were obtained by Suresh[25] and Phani[26]. Phenolics are detected in natural leaf and stem whereas lacks in *in vitro* leaf, stem and callus. Similarly protein is also detected in natural leaf only.

Plant growth regulators *viz.*, auxins and cytokinins play a profound role in cell division, callus induction and regeneration[27]. In the present study 2,4-D showed maximum callus induction followed by TDZ. Lower concentrations of 2,4-D (0.1 mgl^{-1},0.5 mgl^{-1},1.0 mgl^{-1}) shows more profuse callusing which gradually decreases with further increase in concentration of 2,4-D (2.0 mgl^{-1}, 5.0 mgl^{-1}). Similar results were obtained in *Tridax procumbens* L[28] and in *Dendrobium*[29]. TDZ on the other hand gives a vice versa pattern of callus induction similar results are also observed in *Solanum tuberosum* L[30].

Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and fortuitously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products[31]. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites, the phytochemical screening of *in vitro* and nature grown *N. arbor-tristis* reveale the presence of different types of bioactive compounds like alkaloids, coumarins, flavonoids and steroids. Therefore the present study gives a suitable protocol for establishment of callus culture which further paved the way for the setup of suspension cultures which further opens the path for various physiological, molecular, biochemical studies and production of a wide range of secondary metabolites which are present in *N. arbor-tristis.*

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