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Original Article

Callus Induction and Elicitation of Total Phenolics in Callus Cell Suspension Culture of *Celastrus paniculatus* – willd, an Endangered Medicinal Plant in India

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ABSTRACT

Celastrus paniculatus - Willd belonging to the family Celastraceae is a vulnerable medicinal plant in India. It has been employed as a stimulant, nervine tonic, rejuvenant, sedative, tranquilizer and diuretic. It is also used in the treatment of leprosy, leucoderma, rheumatism, gout, paralysis and asthma. Because of its high pharmaceutical application, this plant species were over exploited and now considered under threatened species. So the highlight of the present investigation is the induction of callus by using different concentration of various phytohormones such as 2, 4-D (0.5 - 3.0 mg/l) and NAA (0.5 - 2.5 mg/l). In order to ensure the presence of the bioactive compounds preliminary phytochemical screening of the various extracts of callus were performed. Finally elicitation of total phenolics were done in callus cell suspension cultures by using elicitors such as jasmonic acid, salicylic acid and copper sulphate. Among the applied elicitors jasmonic acid showed superiority. To our knowledge, this is the first report of the elicitation of secondary metabolites especially total phenolics from callus cell suspension cultures of *Celastrus Paniculatus*.

Keywords: *Celastrus paniculatus*, Callogenesis, Bioactive constituents, Elicitors, Total phenolics.

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INTRODUCTION

Celastrus paniculatus Willd belongs to family Celastraceae is a large, woody, climbing shrub, distributed almost all over India is known for its ability to improve memory.[1] **Botanical name:** *Celastrus paniculatus* Willd Family: Celastraceae Synonym: Celastrus dependens Vernacular names: Hindi – Malkangani; English – Staff tree; Kannada – Kariganne; Tamil – Valuluvai; Telugu –Malkangani. Botanical description: *Celastrus paniculatus* Willd. is a climbing or scrambling shrub, with erect branches; the young shoots and branches are pendulous. Leaves – glabrous, broadly ovate or obovate, a cuminate or acute. Flowers – unisexual, yellowish-green, borne in terminal, pendulous panicles (flowering throughout the year). It has a fast gaining importance in the primary healthcare systems as well as in herbal drug formulations.[2] This plant has much more importance in Indian system of medicines such as Ayurveda, Folk, Siddha, and Unani to cure many diseases such as leprosy, leucoderma, skin diseases, paralysis, depression, arthritis, asthma and cancer.[3] Many pharmacological studies proved that the antioxidant,anti-inflammatory and anticancerous activity of this plant.[4-6] The phytochemical analysis revealed the presence of alkaloid such as celastrine, paniculatin, celapagine and celapanine[7] which possess anticancerous activity. The objective of the present investigation is to enhance the production of secondary metabolites especially phenolics in callus cell suspension culture. The different elicitors such as Jasmonic acid, Salicylic acid and copper sulphate were used for the present study. The elicitors were added with varing concentrations (25, 50, 100 and 250 µM) and incubated for 24, 48 and 72 hrs to check the enhanced production of total phenolics.

MATERIALS AND METHODS

Plant material

Approximately 10-year-old plant of *Celastrus paniculatus* maintained in the Botanical garden, University of Calicut were used as explants source. The collected plant material were identified and authenticated by Dr, A K Pradeep , Assistant professor, Department of Botany, University of Calicut. University of Calicut was used as explant source. Juvenile shoots obtained from freshly emerged sprouts were collected. The nodal segments as well as midrib (3–5 cm) were used as explants.

Surface sterilization

Endophytic microflora is a major cause of contamination; hence in order to prevent the chance of contamination surface sterilization were done. Explants were first washed under running tap water for about 30 min followed by treatment with 2.5% NaOCl for 10 min and subsequently washed with sterile distilled water for 4-5 times. The explants were further surface sterilized with 0.1% $HgCl_2$ for 10 min. Explants were finally rinsed (5–6 times) with sterile double-distilled water to remove any traces of the surfactants.

Nutrient media and culture conditions

MS medium[8] supplemented with 3.0% sucrose and 0.8% Agar-agar (Hi-Media, India). The pH of the medium was adjusted to 5.8 and then autoclaved for15 min at 121^{0}C. The cultures were maintained at 24°C ± 2°C under 12-h photoperiod (provided by fluorescent lamb). Each treatment was carried out with 12 culture bottles with single explant per bottle and each experiment repeated as twice. The response in various media was expressed in terms of mean number of callus fresh weight. Callus morphology were also recorded.

Callogenesis

The leaf explants of *Celastrus paniculatus* were used for callus induction. The callus was induced in MS medium fortified with various concentrations of 2,4-D (0.5 - 3.0 mg/l) alone and a combination with optimum concentration of 2,4-D (1.5 mg/l) with varying concentrations of NAA (0.5 - 2.5 mg/l).The percentage of callus induction was recorded. The callus was observed every 10 days and was subcultured at an regular interval of 15-20 days. The culture bottle was maintained under aseptic conditions.

All the experiments were repeated twice with a minimum of 12 replicates. Each bottle contains single explant and all the results were expressed as the mean ± SD for all experiments.

Preliminary phytochemical screening

The powdered samples of callus extract of *Celastrus paniculatus* were screened for phytochemical constituents using standard procedures (Harborne 1998).

Extract from callus

The dried callus were ground into a course powder, 10gram each of the powder was mixed with 40 ml of chloroform, petroleum ether, ethyl acetate and methanol in 250 ml of conical flask and was kept at room temperature for 24 hrs. The suspension was filtered through Whatman's filter paper and collected in large Petri plates. These were allowed to dry completely in water bath set at 40 ± 0.2°C for 30 min. Dried extracts were scraped out by using scalpels and was collected in pre weighed vials separately. Extracted powders were made available to use as per requirements by re suspended in the above solvents used for various analysis.

Cell suspension culture

The six week old callus from *in vitro* culture was transferred into liquid MS medium supplemented with the same hormonal combination without agar. The cell suspension culture was grown for 25 days with 110 rpm in shaker at room temperature.

Elicitors

Various elicitors such as Jasmonic acid, Salicylic acid and copper sulphate were used for the elicitation of secondary metabolites in cell suspension culture. The various concentrations of the elicitors such as 25, 50, 100 and 250 µM were added on the 25th day of callus cell suspension culture.

These cultures were incubated at 25 ± 2^{0}C in gyratory shaker at 110 rpm under dark condition. The effects of different concentrations of the elicitors were recorded for 24, 48 and 72 hrs of treatment duration and all the experiments were repeated thrice.

Determination of total phenolic content

Total phenolic content of the callus cell suspension culture of *Celastrus paniculatus* was determined by Folin- Ciocalteau method using gallic acid as standard.[9] The total phenolic content of the extracts were expressed as gallic acid equivalents.

RESULTS AND DISCUSSION

Callus induction

MS medium modified with various levels of 2,4-D (0.5-3.0 mg/l) alone and a combination with NAA(0.5-2.5 mg/l) resulted callus formation from leaf explants. The callus response was slow at the initial stages but the callus exhibited good growth within 25 days and covered the entire explants. MS medium with 1.5 mg/l of 2, 4-D was the best concentration for the callogenesis and showed 88% callus response (Table 1). In the leaf explants, the callus developed from the adaxial as well as abaxial surface. The callus developed on the optimal concentration of 2, 4-D was friable, shiny and cream coloured. The combination of optimum concentration of 2, 4-D with varying concentrations of NAA favoured the callus development. The same combination exhibited the organogenic nature of callus (Figure 1).

Qualitative phytochemical screening

The preliminary phytochemical screening of different bioactive compouds was found to vary depending on the polarity of the solvents. The screening was performed by using standard procedures and is summarized in the Table 2.

In the present investigation, the qualitative test for most of the extracts showed significant indication about the presence of the metabolites. In comparison to the other extracts, the methanolic extract of *Celastrus paniculatus* showed majority of phytoconstituents such as phenols, glycosides, aldehyde, alkaloids, tannins, terpenoid, flavanoids, and saponinns. The ethyl acetate extract showed the presence of glycosides, phlobotannins, alkaloids, tannins, steroids and flavanoids. The petroleum ether extract showed positive results to only few bioactive compounds, such as phlobotannins, glycosides and terpenoids. The chloroform extract showed the presence of compounds such as reducing sugar, flavanoids, steroids, glycosides and aldehyde. The similar results were reported by[10] using different extracts of *Celastrus paniculatus* seeds.

Effect of Jasmonic acid on total phenolics

Jasmonic acid (JA) is a naturally occurring growth regulator found in higher plants. Several physiological roles have been described for this compound (or a related compound, methyl jasmonate) during plant development and in response to biotic and abiotic stress. The results obtained in jasmonic acid treated cell suspension culture samples with respect to total phenolics are shown in Table 3.

After 24 hrs of treatment the total phenolics were maximum in 250 µM of jasmonic acid was 43.18 ± 0.42 µg/g, which is higher than control. In 48 hours of treatment, the maximum response in terms of total phenolics was obtained in the same concentration, 104.51 ± 0.33 µg/g. Whereas in 72 hrs of treatment duration 50 µM of jasmonic acid was observed maximum response of total phenolics was 106.82 ± 0.11 µg/g. Many workers reported that jasmonic acid has been used to modulate the production of various secondary metabolites in plant tissue culture techniques[11-15] reported that the enhanced secondary metabolite production is usually associated with rapid, transient increase in the activity of key enzymes of the phenylpropanoid pathway such as phenylalanine ammonia lyase and chalcone isomerase.

Effect of salicylic acid (SA) on total phenolics

Salicylic acid (SA) is a hormone-like substance that plays an important role in the regulation of plant growth and development[16-18] The results obtained in salicylic acid treated cell suspension culture samples with respect to total phenolics are shown in Table 4.

After 24 hrs of treatment duration, the total phenolics were maximum in 100 µM of salicylic acid with a yield of 56.24 ± 1.63 µg/g. Whereas in the case of 48 and 72 hrs of treatment duration the optimum concentration inducing high increase in total phenolic concentration was 250 µM which was 92.23 ± 1.55 µg/g and 99.23 ± 0.27 µg/g respectively.

SA has attained more attention because of its involvement in plant defense mechanisms, such as establishment of systemic acquired resistance (SAR),[19] induction of pathogenesis related (PR) proteins[20] as well as hypersensitive response.[21] The protective effect of SA against abiotic stress factors such as toxic metals,[22] heat stress,[23] low temperature[24,25] and oxidative damage[22,26] has been demonstrated. SA has been reported to induce salinity tolerance in tomato,[27] maize,[28] carrot[29] and wheat.[30] It has also been used to enhance in vitro regeneration in several plant species.[31-33]

Effect of Copper sulphate on total phenolics

The metals like copper, cobalt, iron, manganese and zinc are essential for plant life but are required in a very small or trace amounts[34,35] Among them, copper salts such as copper sulphate and copper chloride were successfully used as abiotic elicitors in number of plant cell culture.[36]

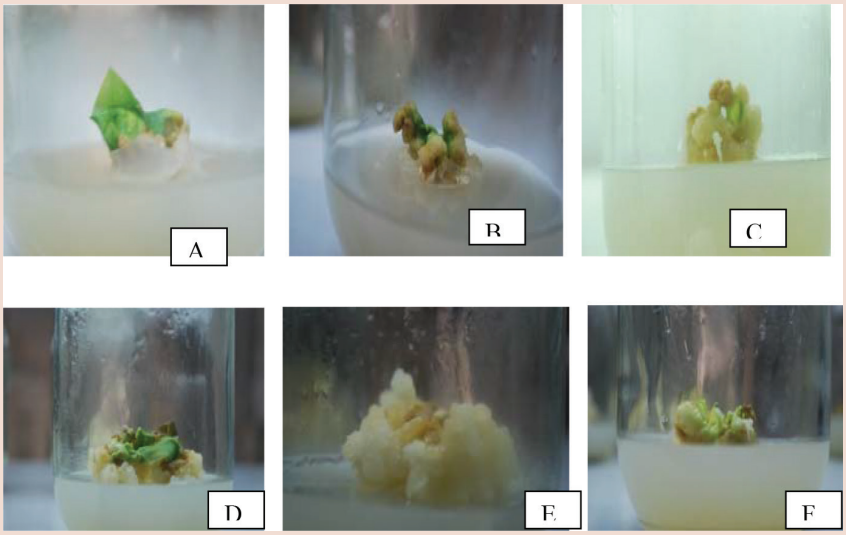

Figure 1: The various developmental stages of callus proliferation from leaf explants *celastrus paniculatus* A-E) Callus initiation from leaf explants on MS medium supplemented with different concentrations of 2,4-D (0.5 – 2.5 mg/l) alone and a combination of optimum concentration of 2,4-D and varying concentrations of NAA(0.5 - 2.5 mg/l). Creamy, friable callus were obtained. F) Organogenic callus and initiation of shoot formation on MS medium supplemented with 1.5 mg/l of 2,4-D with 1.0 mg/l of NAA.

Table 1: Effect of 2, 4-D and NAA for callus induction from leaf explants of *Celastrus paniculatus*

Hormone conc. (mg/l)	Callus Response (%)	Callus morphology
2,4-D		
0.5	32.40 ± 2.1	Whitish green
1.0	58.20 ± 1.40	Whitish green, hard
1.5	88.10 ± 2.38	Cream, friable
2.0	70.34 ± 1.93	Cream,white, friable
2.5	64.40 ± 1.23	Pale green, friable
2,4-D+NAA		
1.5 + 0.5	60.10 ± 1.20	Pale green, hard
1.5 + 1.0	96.42 ± 2.03	Cream,friable.
1.5 + 1.5	80.10 ± 1.60	Shiny cream,friable
1.5 + 2.0	79.23 ± 1.62	Cream,hard
1.5 + 2.5	72.43 ± 0.38	Cream,friable

Table 2: Preliminary phytochemical screening of bioactive compounds present in different extracts of *Celastrus paniculatus*

Phytochemical constituents	Petroleum ether extract	Chloroform Extract	Methanol extract	Ethyl acetate extract
Reducing sugars	–	+	–	–
Anthraquinones	–	–	–	–
Saponins	–	–	+	–
Flavanoids	–	+	+	+
Steroids	–	+	+	–
Terpenoids	+	+	–	+
Tannins	–	–	+	+
Alkaloids	–	–	+	+
Aldehyde	+	+	+	+
Phenols	–	–	+	–
Glycosides	+	+	+	+
Phlobotannins	+	–	–	+

The results obtained in copper sulphate treated cell suspension culture samples with respect to total phenolics are shown in Table 5.

After 24 hrs of treatment duration, the total phenolic concentration was maximum in 250 µM CuSO4 treated sample, which was 96.30 ± 1.22 µg/g. In the 48 hours treatment, 100 µM CuSO4 was found to be most effective with respect to total phenolic production with yielded 88.83 ± 1.12 µg/g. Whereas in the 72 hrs treated sample, 250 µM CuSO4 was observed to be optimal concentration and yielded 67.38 ± 1.77 µg/g of total phenolics.

Copper performs very important physiological and biochemical processes include photosynthesis, respiration, conversion of nitrogen compounds, transport of carbohydrates and also it is a constituent of the protein component of several enzymes in plants, mainly those participating in electron flow, catalyzing redox reactions in mitochondria, chloroplasts, cell wall and cytoplasm of plant cells.[37,38] reported that the addition of 40 µM CuSO4 to the medium significantly increased the embryoids production from wheat anther cultures.[39] studied the effect of different concentrations of CuSO4 on callus culture of two cultivars of barley and found that medium containing 50 µM copper regenerated significantly more plants. Total and phenol content increased at high concentration of Cu and Zn.[40]

CONCLUSION

From the present investigation it was concluded that MS medium modified with various levels of 2,4-D (0.5-3.0 mg/l) alone and a combination with NAA (0.5-2.5 mg/l) resulted callus formation from leaf explants. Phytochemical screening of the different callus extracts of *Celastrus paniculatus* contained many bioactive constituents which includes reducing sugars, anthraquinones, phenols, flavanoids, alkaloids, steroids, terpenoids, phlobotannins, aldehydes/ketones, glycosides, saponins and tannins. The presence of these bioactive constituents suggests potential source for useful drugs, and enhancers of the health status. These findings of phytochemicals were good enough to reflect its importance. The comparative enhancement in phenolic content by elicitors was the point of interest in

Table 3: Effect of Jasmonic acid on phenolic production in *celastrus paniculatus*

Treatment Duration	Concentration of Elicitor	Total phenolics in µg/g
24 hrs	Control	3.54 ± 0.25
	25 µM	9.44 ± 0.27
	50 µM	34.61 ± 0.61
	100 µM	39.25 ± 0.46
	250 µM	43.18 ± 0.42
48 hrs	Control	22.45 ± 0.63
	25 µM	9.49 ± 0.37
	50 µM	42.78 ± 0.47
	100 µM	69.31 ± 0.31
	250 µM	104.51 ± 0.33
72 hrs	Control	9.30 ± 0.28
	25 µM	84.20 ± 0.38
	50 µM	106.82 ± 0.11
	100 µM	32.30 ± 0.62
	250 µM	30.40 ± 0.28

Table 4: Effect of Salicylic acid on phenolic production in *celastrus paniculatus*

Treatment Duration	Concentration of Elicitor	Total phenolics in µg/g
24 hrs	Control	5.23 ± 0.27
	25 µM	7.39 ± 1.24
	50 µM	24.67 ±1.52
	100 µM	56.24 ± 1.63
	250 µM	9.24 ± 1.55
48 hrs	Control	7.22 ± 1.59
	25 µM	5.38 ± 1.44
	50 µM	22.53 ± 1.63
	100 µM	43.62 ± 1.54
	250 µM	92.23 ± 1.55
72 hrs	Control	6.26 ± 1.73
	25 µM	7.22 ± 1.39
	50 µM	23.56 ± 1.83
	100 µM	49.62 ± 1.30
	250 µM	99.23 ± 0.27

the present investigation. The obtained result revealed that jasmonic acid is the most effective in eliciting in total phenolic production of callus cell suspension cultures of *Celastrus paniculatus*.

ABBREVIATIONS USED

l2,4-D: 2,4 Deoxyribonucleic acid; **NAA:** Naphthalene acetic acid; **$CuSO_4$:** Copper sulphat; **$HgCl_2$:** Mercury chloride; **NaOCl:** Sodium hypochlorite.

Table 5: Effect of Copper sulphate on phenolic production in *celastrus paniculatus*

Treatment Duration	Concentration of Elicitor	Total phenolics in µg/g
24 hrs	Control	6.77 ± 1.43
	25 µM	6.54 ± 1.40
	50 µM	38.72 ± 1.26
	100 µM	40.20 ± 1.26
	250 µM	96.30 ± 1.22
48 hrs	Control	8.30 ± 1.34
	25 µM	28.83 ± 1.25
	50 µM	49.20 ± 1.59
	100 µM	88.83 ± 1.12
	250 µM	38.26 ± 1.30
72 hrs	Control	5.30 ± 1.52
	25 µM	4.21 ± 1.39
	50 µM	6.92 ± 1.27
	100 µM	33.29 ± 1.53
	250 µM	67.38 ± 1.77

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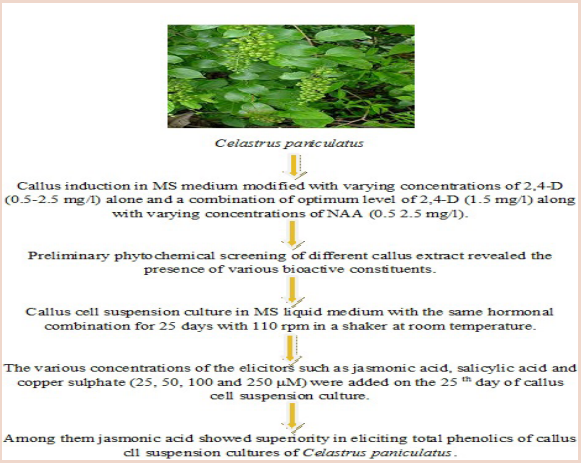
PICTORIAL ABSTRACT



SUMMARY

- *Celastrus paniculatus* is an endangered medicinal plants in India.
- The optimum level of 2,4-D (1.5 mg/l) along with the combination of 1.0 mg/l of NAA resulted maximum callus response from the midrib of the leaf explants.
- The preliminary phyto¬chemical screening of the different callus extracts of *celastrus paniculatus* contained many bioactive constituents which includes reducing sugars, anthraquinones, phenols, flavanoids, alkaloids, steroids, terpenoids, phlobotannins, aldehydes / ketones, glycosides, saponins and tannins.
- The present study revealed that jasmonic acid is the most effective in eliciting in total phenolic production of callus cell suspension cultures of *Celastrus paniculatus*.

ABOUT AUTHORS

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