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Research Article

QUERCETIN IN CALLUS CULTURES OF *CLITORIA TERNATEA* LINN.

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ABSTRACT

Callus cultures of leaf, root and hypocotyledon of *Clitoria ternatea* were developed independently in MS medium supplemented with 1mgl^{-1} each of 2,4-dichlorophenoxy acetic acid (2,4-D), benzyl adenine (BA), Indole-3-acetic acid (IAA), and Kinetin. Three months old calli were subjected to phytochemical screening which showed the presence of alkaloids, amino acids, flavonoids, carbohydrates, phenolic compounds, tannins, saponins, mucilage and proteins. Quercetin, a flavonoid having pharmacological functions was quantitatively estimated by Reversed – Phase HPLC in natural leaf and callus cultures of leaf, hypocotyledon and root. Leaf callus was found to content the highest amount of Quercetin, i.e. 1.21% w/v as compared to other callus while natural leaf extract showed 1.25%w/v of Quercetin.

Keywords: *Clitoria ternatea*, callus cultures, HPLC, Querceti

INTRODUCTION

Clitoria ternatea L. (Fabaceae) is a vigorous, strongly persistent, herbaceous perennial legume. It is used as a brain tonic to promote memory & intelligence [1]. The plant is considered useful for eye infections, skin diseases, urinary troubles, ulcers and its extract is used in a rejuvenating recipe to treat neurological disorders [2]. Its roots are used in Ayurvedic Indian Medicine; they are bitter, refrigerant, laxative, diuretic, anthelmintic and tonic and are useful in dementia, hemicranias, burning sensation, leprosy, inflammation, leucoderma, bronchitis, asthma, pulmonary tuberculosis, etc [3]. Leaf juice is used as nasal drops in headache. Flavonoids are a ubiquitous group of poly – phenolic substances which are present in most plants.

A few research work is reported on tissue culture studies of *Clitoria ternatea* which includes, direct shoot regeneration and multiplication is reported [4], indirect shoot organogenesis, rooting and transplantation [5], embryo formation, i.e. somatic embryogenesis [6], embryo induction, maturation and germination [7]. Tessereau et al have reported the callus initiation is reported by using different hormone combinations in MS Medium supplemented with 2,4-D, NAA, BA. And after callus initiation, growth of callus was assessed by calculating the growth score (G), Cryopreservation by Encapsulation - Dehydration Method of the germinated somatic embryos is reported [8]. Total protein analysis and Electrophoresis analysis done on leaves and seeds of *Clitoria ternatea* [9], mitotic study for the assessment of ploidy was done [10]. However, no data has been reported for the separation of flavonoid Quercetin from the methanol extracts of natural leaf and callus extracts of leaf, hypocotyledon and root callus cultures.

In the present work an attempt has been made to develop calli from different parts of the plant and to find out the potential of the cultures for the biosynthesis of Quercetin and determination of its content in natural leaf and different cultures using RP - HPLC.

Materials and Methods

The explants (leaves and seeds) of ***Clitoria ternatea*** Linn. were collected from the plant growing in the Herbal Garden, Jamia Hamdard University, New Delhi, India. The identification of the plant species (*Clitoria ternatea* Linn.) was done by Dr. H.B. SINGH (Head, Raw Materials Herbarium and Museum, NISCAIR, Pusa Road, New - Delhi) and the voucher specimen of the plant was deposited in Herbarium of NISCAIR (Ref. NISCAIR/RHMD/Consult/-2009-10/1320/123).

The immature leaves and seeds were surface sterilized by washing with very dilute detergent solution or teepol and washed with double distilled water and then treated with different concentrations (0.05, 0.1%) and contact time (5, 6, 8 and 10 mins.) of mercuric chloride and with different concentrations (0.1, 0.2%) of sodium hypochlorite for contact time (5, 7, 8 and 9 mins.).

Under aseptic conditions, the explants (leaves and seeds) were transferred to a sterile beaker and washed four to five times with sterile double distilled water to remove the traces of the chemical sterilants. The surface sterilized seeds were then transferred aseptically into the sterile petri-plates containing cotton and filter paper beds and were kept in a B.O.D. Incubator (Yorko, Delhi) in light and dark cycle (16 hr light and 8 hr dark) at 25 ± 2°C for germination.

The surface sterilized leaves (1 mm length) were transferred into the tubes containing MS medium supplemented with various combinations and concentration of plant hormones as given below:

1) MS + 2,4-D + IAA (1ppm each)
2) MS + 2.4-D + Kinetin (1ppm each)
3) MS + 2,4-D + IAA + Kinetin (1ppm each)
4) MS + 2.4-D + 6BA (1ppm each)
5) MS + 2,4 –D + NAA (1ppm each)
6) MS + 2,4-D + IAA + Kinetin + 6BA (1ppm each)

Similarly, seedlings of germinated seeds and their different parts (roots, cotyledon and hypocotyledon) were transferred with sterile forceps in culture tubes containing MS medium supplemented with various combinations and concentration of plant hormones as given above.

After initiation of callus on leaf explant in 20-22 days, they were separated aseptically from the mother explants and the calli initiated on hypocotyledon and root in 26-28 days and were transferred to the same medium on which they were initiated in order to develop an independent calli. The growth was observed for further 3 weeks.

The developed calli were chopped down and transferred aseptically in MS medium supplemented with the similar hormonal combinations which showed excellent results in the previous experiment. During the maintenance, the sub-culturing was routinely done after every three weeks.

In order to find out the presence/absence of different types of primary and secondary metabolites, general chemical tests were carried out as reported by Zafar et al [11]. The extracts of natural leaf, leaf callus A, B, C, root callus A, B, C, hypocotyledon callus A, B and C (Table 1) were tested for the detection of different metabolites.

Analysis of Quercetin by RP-HPLC method

Quantitative analysis of Quercetin is done by a modified method of Rajalakshmi et al [12].

a) Preparation of mobile phase

The mobile phase was prepared by mixing Methanol and 0.5% OPA (pH 3.8) in the ratio of 50:50 v/v. The mobile phase was degassed by sonication and filtered through 0.45-µm membrane filter under vacuum just before the HPLC analysis.

b) Preparation of stock and standard dilutions

Standard stock solution of Quercetin (100 µg/ml) was prepared as follows:

5 mg of drug was weighed accurately and dissolved in 50 ml of HPLC grade methanol. The prepared solution was stored at 4°C protected from light. Working standard solutions were freshly obtained by diluting the stock standard solutions with mobile phase during the time of analysis.

Linearity

Solution of different concentration 1, 2, 4, 8, and 16 µg/ml were prepared from stock solution by diluting with mobile phase. Calibration curve was constructed between the peak area and concentration. The linearity was evaluated by linear regression analysis, that was calculated by least

square regression method using Microsoft excel 2007 software.

c) Sample preparation

Leaf extract and Callus Extracts:

1 gm each of powdered leaf and calli (leaf A and B, hypocotyledon A and B and root A and B) dried at 60° was extracted with 25 ml of methanol separately, filtered and filtrates were concentrated. The final volume was made upto 10 ml. 1 ml solution was taken out from this and filtered through 0.2 µm membrane filter (Gelman Science, India). The samples were analyzed by HPLC for the quantitative estimation of Quercetin by a Shimadzu model HPLC equipped with quaternary LC-10A VP pump, variable wavelength programmable UV/VIS detector, SPD-10AVP column oven (Shimadzu), SCL 10AVP system controller (Shimadzu), Rheodyne injector fitted with a 20 L loop. Class-VP 5.032 software was used for the routine drug analysis.

Results

In the present investigation, callus cultures of *Clitoria ternatea* Linn. was developed from seedling and leaf explants in order to study the production of different secondary plant metabolites and content of Quercetin.

The seeds of *Clitoria ternatea* were germinated aseptically by using two different sterilants; mercuric chloride and sodium hypochlorite of different concentrations at different contact times i.e. 0.05, 0.1 & 0.2% respectively. 0.05% concentration of mercuric chloride showed 100% germination within 4 to 5 days and 0.1% concentration of sodium hypochlorite showed 80% germination.

The germinated seedlings were then transferred to the sterilized culture tubes containing MS medium supplemented with different hormonal combinations and concentrations. The initiation of callus was observed on cotyledon, hypocotyledon, whole seedling as well as on root. All the hormone combinations (MS + 2,4-D + IAA, MS + 2,4-D + Kinetin, MS +2,4-D + IAA + Kinetin, MS + 2,4–D + 6BA, MS + 2,4 – D + NAA and MS + 2,4 – D + IAA + Kinetin + 6BA) showed initiation of callus except one hormone combination, i.e. 2,4 - D + NAA, which shows no initiation of callus from germinated seeds of *Clitoria ternatea*.

Later on, the calli were independently transferred to MS medium containing same hormonal combinations and concentration in which they were initiated to study their growth pattern.

The development of callus was vigorous on MS + 2,4-D + 6BA (1ppm each) and on MS + 2,4-D + IAA + Kinetin + 6BA (1ppm each) of root and hypocotyledon callus. The calli obtained from hypocotyledon and root were subcultured at regular interval of 3 weeks. After development the different calli were maintained upto 120 days. It was observed that the root callus developed on MS + 2,4-D + 6BA (1ppm each) showed yellowish green coloured growth which later on turned to light brown (Fig. 1) and the hypocotyledon callus which was creamish green soft callus turned to yellowish green (Fig. 2).

The callus on MS + 2,4-D + IAA + Kinetin + 6BA (1ppm each) showed yellowish coloured callus which later on turned to light brown and yellowish green soft callus turned to light green in colour on the same media of root and hypocotyledon respectively.

The explants (leaves) of *Clitoria ternatea* Linn. were surface sterilized with 0.1 & 0.05 concentration of mercuric chloride and 0.1 & 0.02 concentration of sodium hypochlorite with different contact time. With 0.1 & 0.05 concentration of mercuric chloride no contamination but browning of explant was observed. With 0.1 concentration of mercuric chloride at 7 minutes contact time no contamination and no browning was observed, whereas, both the concentrations of sodium hypochlorite showed contamination in the explants (leaves).

MS medium supplemented with all the hormonal combinations mentioned in material and methods showed excellent initiation and growth of callus on leaf except with 2,4 – D + NAA (1ppm each).

Then independent calli were developed on the same hormonal combinations. The independent leaf calli grew vigorously on MS + 2,4-D + IAA+ Kinetin (1ppm each) and on MS + 2,4-D + 6BA (1ppm each) but the calli showed slow growth on other hormonal combinations. The developed calli were then maintained further upto 120 days. It was observed that the callus developed on MS + 2,4-D + Kinetin (1ppm each) showed yellow coloured callus which later on turned to light brown in colour.

The growth of callus on MS + 2,4-D + IAA + Kinetin (1ppm each) was found to be satisfactory, the callus turns to light brown coloured compact mass from greenish yellow coloured

callus. While the callus on MS + 2,4-D + 6BA (1ppm each) (Fig. 3) turned dark brown coloured nodular callus in the 5th or 6th passage of sub-culturing, whereas, callus on MS + 2,4-D + IAA + Kinetin + 6BA (1ppm each) shows dark brown coloured compact mass of cells in the 5th and 6th passage of subculture.

The qualitative chemical tests were performed for the presence/absence of various plant constituents in natural leaves and different calli samples, i.e. Leaf callus, root callus and hypocotyledon callus of different hormonal combinations. It was found that the natural leaf, leaf callus, root callus and hypocotyledon callus contains alkaloids, amino-acids, flavonoids, carbohydrates, phenolic compounds, tannins, saponins, mucilage and proteins. The steroids, coumarins, anthraquinone glycosides and cardiac glycosides were found to be absent in all the above samples. (Table1).

Quantitative analysis of Quercetin carried out by RP – HPLC method. HPLC method was employed for its quantification in different callus and leaf extract samples as reported by Rajalakshmi et al [12]. The chromatogram showed the retention time of 2.625 mins. with standard Quercetin (Fig. 4), that was less than the earlier reported retention time (3.273 mins.). The chromatographic peak of the Quercetin is different and is showing in Fig. 4. The chromatogram of standard and the different extracts showed sharp and uniform peak with nonsignificant variations in retention time. The quantity of Quercetin in natural leaf extract (Fig. 5), leaf callus A (Fig. 6), hypocotyledon callus A (Fig. 7), root callus A (Fig. 8), leaf callus B (Fig. 9), hypocotyledon callus B (Fig. 10) and root callus B (Fig. 11) of *Clitoria ternatea* Linn. was found to be 1.25%, 0.9%, 0.81%, 0.94%, 1.21%, 0.88% and 0.85% w/v of Quercetin respectively (Table 2).

Discussion

In the present investigation, the biogenetic ability of the biomass developed *in vitro* for production of Quercetin and its quantitative estimation is reported for the first time and the maximum amount of Quercetin was found to be present in leaf extract followed by leaf callus B. However, further work to increase the content of Quercetin in callus and suspension cultures is under progress using abiotic and biotic elicitors.

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