Research Article

In vitro enhanced accumulation of polyphenols during somatic embryogenesis in *Plantago ovata* Forsk

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ABSTRACT

***Plantago ovata* Forsk (common isabgul) is much known for its medicinal importance. It is a potential source of bioactive compounds like polyphenols which serve as natural antioxidant having an ability to quench cytotoxic free radicals that are preventive to many diseases. In present investigation, the accumulation of polyphenolic compounds was studied during *in vitro* somatic embryogenesis in *Plantago ovata*. Calli of three different developmental stages during somatic embryogenesis were selected for this purpose and total polyphenol content was determined spectrophotometrically using Folin-Ciocalteu reagent. The embryogenic calli were found to contain highest level of polyphenolics. Further, the effect of exogenous additives like casein hydrolysate and coconut water on the accumulation of total polyphenol was also included into the study. 42 days old calli were treated with different concentrations of casein hydrolysate (1 gL^{-1}, 2 gL^{-1}, 3 gL^{-1}) and coconut water (5%, 10%, 15%) and used for total polyphenol content determination. 2 gL^{-1}casein hydrolysate and 5% coconut water produced the best result. So the study shows a greater promise in advancing the use of *Plantago ovata* for producing phenolic compounds in a larger scale.**

INTRODUCTION

Plantago ovata Forsk, the common isabgul has gained importance throughout the world for its wide variety of medicinal properties. It is a bushy annual herb indigenous to Asia, Mediterranean region and North Africa. In India it is cultivated in Gujarat and Rajasthan. *Plantago ovata* has recently claimed economic importance for its immense use in agricultural, pharmaceutical, cosmetic and food industries worldwide. The seed husk of *Plantago ovata* are used as laxatives [1]. The mucilage contained in the seed coat is used as a stabilizer in ice cream; chocolate etc [2]. *Plantago ovata* is a rich source of different kinds of biologically active compounds among which polyphenols are of great importance. In recent years polyphenols have gained much attention due to their beneficial implications on human body. Polyphenols represent a large group of heterogeneous bioactive compounds produced in many medicinal and vegetable plants during their secondary metabolism. The position of hydroxyl or phenol group in the polyphenolic compounds are believed to determine their antioxidant properties, especially against peroxyl and superoxide radicals [3]. A broad spectrum of polyphenolic compounds includes simple molecules like phenolic acids to large polymers like tannins. Most important beneficial aspect of polyphenolic compounds in human health is their antioxidant activities and free radical scavenging abilities which have been well explored during recent years [4]. Several studies have documented the ability of polyphenols in quenching cytotoxic reactive oxygen species (ROS) as well as their role in metal ion chelation [5]. Polyphenols are also known to have antiulcer [6], anticarcinogenic, antimutagenic [7], anti-inflammatory [8] and antiviral [9] activities. Accumulation of polyphenolic compounds in embryogenic tissues during somatic embryogenesis has been previously reported in many plants [10, 11]. Previous studies have shown that polyphenolic compounds increases with the age of callus tissue in chick pea [12]. Phenolic compounds are intermediates of phenylpropanoid pathway and their production is regulated by the differential expression of basic genes [13, 14]. Phenolic compounds can be induced by several abiotic stresses like ethylene, methyl jasmonate, temperature, light and wounding [15]. Therefore studying effect of external additives like casein hydrolysate and coconut water on total polyphenol content during *in vitro* somatic embryogenesis in *Plantago ovata* would be of utmost importance. Casein hydrolysate and coconut water are widely used additives in plant tissue culture experiments. Casein hydrolysate is a rich source of amino acids and coconut water, the liquid endosperm of coconut is known to contain cytokinins. Therefore, both casein hydrolysate and coconut water may play a role in inducing the expression level of basic genes involved in polyphenol biosynthesis. The present investigation was therefore intended to determine the effect of exogenous additives on total polyphenol content during *in vitro* somatic embryogenesis in *Plantago ovata*.

MATERIALS AND METHOD

Tissue culture: Seed sterilization

Seeds of *Plantago ovata* were procured from Gujarat (India).*Plantago ovata* seeds were surface sterilized in 10% sodium hypochlorite (commercial bleach) solution for 20 min with vigorous shaking and washed four to five times (each 5 min) with sterile distilled water to remove excess bleach. Seeds were then imbibed overnight in sterile distilled water. Following imbibition the seeds were inoculated aseptically in germination medium

containing 3% (w/v) sucrose (SRL, Mumbai, India) and 0.9% (w/v) agar (SRL, Mumbai, India) for germination.

Callus induction following the additive supplementation

Callus cultures were established using seedlings without roots as explants (~2-3 cm) from 10 day old germinated plants. Callus was initiated on MS (Murashige and Skoog) medium [16] containing 1 mgL^{-1} 2,4-D (2,4-dichlorophenoxy acetic acid) and 0.5 mgL^{-1} kinetin (6-furfuryl amino purine) as plant growth regulators and incubated over a period of 21 days (1^{st} passage). The pH of the medium was adjusted within 5.6-5.8 (using 1N KOH) prior to autoclaving. The cultures were maintained in Plant Growth Chamber (GC-300TLH, Lab companion, Korea) at a temperature of 22°C-25°C and a relative humidity of 55%-60% under Philips fluorescent day light tubes emitting 32 x 10^8 mole s^{-1} m^{-2} for 16/8 hrs duration in light/dark photoperiods. The calli were subcultured on the same medium with similar combination and concentration of plant growth regulators for another 21 days (2^{nd} passage) to increase callus mass. Each culture with specific plant growth regulator combination was replicated five times.

To study the effect of additives on total polyphenol content in *Plantago ovata*, 42 days (2^{nd} passage) old calli were transferred to MS medium containing 0.5 mgL^{-1} NAA (α-naphthalene acetic acid) and 1 mgL^{-1} BAP (6-benzyl amino purine) along with different concentrations of external additives like casein hydrolysate (Sigma Chemical, USA) and filtered coconut water (obtained from green and unripe coconut fruit) and incubated over a period of 21 days (3^{rd} passage). 1 gL^{-1}, 2 gL^{-1} and 3gL^{-1} casein hydrolysate and 5%, 10% and 15% coconut water were used to treat the calli. Casein hydrolysate was weighed and added to the MS medium (w/v) along with 0.5 mgL^{-1} NAA and 1 mgL^{-1} BAP , made upto volume and heated in microwave oven to dissolve. Coconut water was first filter sterilized and added to the MS medium (v/v) along with the same plant growth regulator combination as in case of casein hydrolysate. A set of embryogenic calli without any additive supplementation was maintained as control in both the cases. The culture medium and the specific plant growth regulator combination and concentration had been chosen following the standardized tissue culture method reported in *Plantago ovata* by Das (Pal) and Sen Raychaudhuri (2001).

Extraction of total polyphenols from callus

Total polyphenol was extracted from 1^{st} (21 days), 2^{nd} (42 days) and 3^{rd} passage (63 days) calli during somatic embryogenesis to identify the developmental stage at which maximum accumulation of polyphenolic compounds occurred. The calli showing maximum polyphenol accumulation were then treated with exogenous additives and total polyphenol extraction was done from the treated samples.

100 mg of plant tissue (calli) from each of the samples was finely crushed and homogenized in presence of liquid nitrogen and dissolved in 1ml of 50% HPLC grade aqueous ethanol (Merck, Germany). The mixture was then subjected to ultrasonication for 20 minutes followed by centrifugation at 10,000 rpm for 5 minutes to separate the clear supernatant. The supernatant was collected in a fresh tube and volume was made up to 1 ml with 50% aqueous ethanol (HPLC grade). Five samples were used for the extraction of total polyphenols in this case.

Spectrophotometric determination of total polyphenol content by FCR method

Total polyphenol content of control and treated samples were determined by Folin-ciocalteu reagent (FCR) method as discussed by Singleton with minor modifications [17]. 50µl plant extract from each sample was mixed with 250µl of Folin-ciocalteu reagent and 750µl of 10% sodium carbonate (Na_2CO_3) solution. The mixture was shaken well and incubated at room temperature for 30 minutes in dark. Following incubation absorbance was determined at 760 nm in a HITACHI UV-VIS (U-2800, Tokyo, Japan) spectrophotometer. The content of total polyphenol in each sample was expressed as µg Gallic Acid Equivalent (GAE)/ g fresh weight of tissue by comparing with the gallic acid calibration curve which was constructed beforehand with standard gallic acid.

Preparation of gallic acid calibration curve

In this study gallic acid was used as a standard polyphenolic compound to express total polyphenol content in the extracted samples. A gallic acid calibration curve was prepared for this purpose by taking a range of standard gallic acid (Sigma Aldrich, USA) concentrations from 0 to 50µg/ml. Each of the standard gallic acid solutions were reacted with Folin-ciocalteau reagent and sodium carbonate using above mentioned procedure and absorbances were recorded at 760 nm.

Statistical analysis:

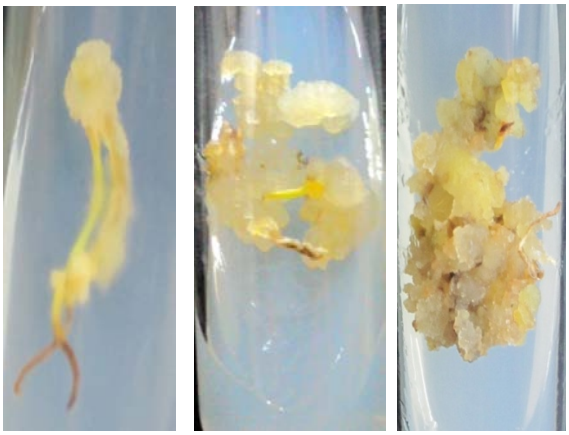
All experimental results were represented as mean values ± SEM (standard error of the mean). The data were analyzed by an analysis of variance (ANOVA) and means were compared using Student's t test using Microsoft Office Excel. All the experiments were replicated five times. Differences in the data at $p \leq 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The present study was carried out to identify the developmental stage during *in vitro* somatic embryogenesis of *Plantago ovata* at which maximum accumulation of polyphenolic compounds occured and effect of exogenous additives on the accumulation of total polyphenols during somatic embryogenic development.

Tissue culture

Plantago ovata seeds were found to germinate within 7-10 days of inoculation in agar-sucrose medium. Initiation of callus from entire explants without the shoot tip was observed within 21 days of culturing on MS medium. The calli were soft, friable and light green in color at the first passage. Subculture increased the mass of calli in successive passages. The color and texture of the calli changed with passage of time. Calli were found to be nodular, compact and yellowish brown in color at the second and third passage. Somatic embryo formation was observed at the third passage (~60-65 days) when MS medium was supplemented with 0.5 mgL^{-1} NAA and 1 mgL^{-1} BAP (Figure 1).



A B C

Figure 1: Callus of *P. ovata* at different developmental stages A: 1st Passage callus (21 days), B: 2nd Passage callus (42 days), C: 3rd Passage callus (63 days)

Application of plant additives to the culture medium has been attempted by many researchers to improve growth and differentiation of *in vitro* plant cultures [18]. For our study we have used casein hydrolysate and coconut water as plant additives. Casein hydrolysate serves as an excellent source of amino acids [19] and coconut water is known to contain diphenyl-urea having cytokinin like activity [20]. The addition of casein hydrolysate and coconut water along with 0.5 mgL^{-1} NAA and 1 mgL^{-1} BAP in MS media showed a positive influence in increasing callus mass. Similar observations have been reported by earlier studies in our laboratory [21]. Both casein hydrolysate and coconut water induced profuse callusing as compared to control (embryogenic calli without any additive supplementation). Calli of treated samples were more compact, nodular and brown in color (Figure 2 & 3).

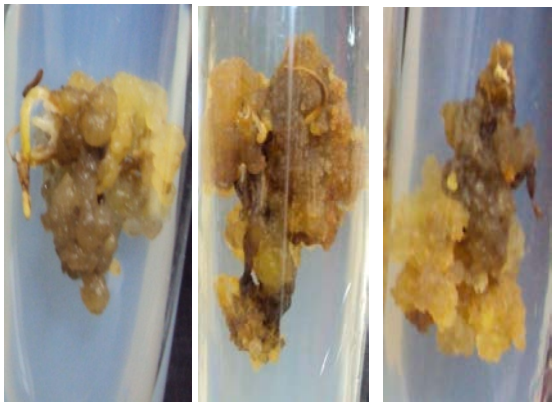


Figure 2: Callus of *P. ovata* treated with different concentrations of casein hydrolysate (CH) A: 1 gL^{-1} CH, B: 2 gL^{-1} CH, C: 3 gL^{-1} CH

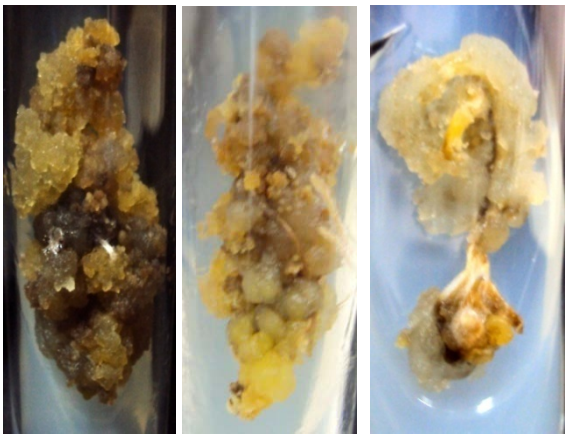


Figure 3: Callus of *P. ovata* treated with different concentrations of coconut water (CW)
A: 5% CW, B: 10% CW, C: 15% CW

Gallic acid calibration curve

The gallic acid calibration curve (Figure 4) was found to be linear across the concentration range assayed (0 to 50µg/ml). The linear regression equation of the gallic acid calibration curve was Y= 0.004X with a correlation coefficient of R^2= 0.995. The total polyphenol content for each of the extracts was determined in terms of µg Gallic Acid Equivalent (GAE)/ g fresh weight of callus tissue with reference to the gallic acid calibration curve.

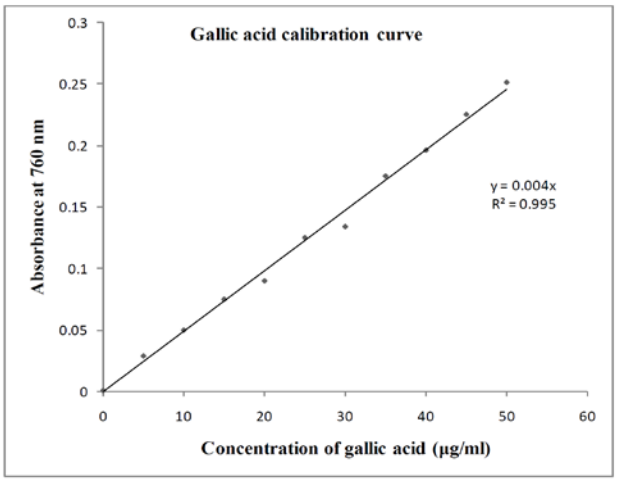


Figure 4: Gallic acid calibration curve

Spectrophotometric determination of total polyphenol content during somatic embryogenesis in *Plantago ovate*

Polyphenolic compounds are one of the most fascinating molecules in the field of medicinal plant research due to their antioxidant properties. Accumulation of polyphenolic compounds in embryogenic cultures of many plants has been previously reported by different groups of researchers [22]. Association of polyphenolic compounds with somatic embryo formation is well established [23]. The present study documented accumulation of polyphenolic compounds in the embryogenic cultures of *Plantago ovata*. Amount of total polyphenol was found to increase with developing stages of the calli. Highest polyphenol accumulation was observed in the embryogenic calli (~60-65 days) of *Plantago ovata* (Figure 5). The third passage calli contained 244±7.23 µg of total polyphenol/g fresh weight of the callus tissue. For this reason 42 days old calli were selected for treatment with external additives.

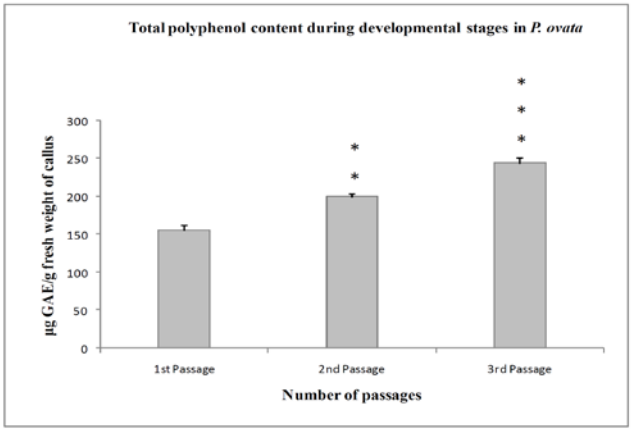


Figure 5: Total polyphenol content in different developmental stages during somatic embryogenesis in P. ovata. Each value is the mean with standard error of five replicates. Asterisks indicate significant differences at $p < 0.05$ (*) or $p < 0.01$ (**) or $p < 0.001$ (***) between the means.

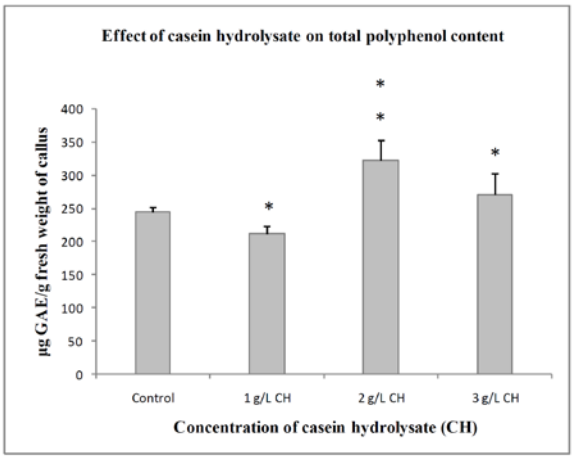


Figure 6: Effect of casein hydrolysate on total polyphenol content in P. ovata. Each value is the mean with standard error of five replicates. Asterisks indicate significant differences at $p < 0.05$ (*) or $p < 0.01$ (**) or $p < 0.001$ (***) between the mean of the sample as compared to the mean of the control.

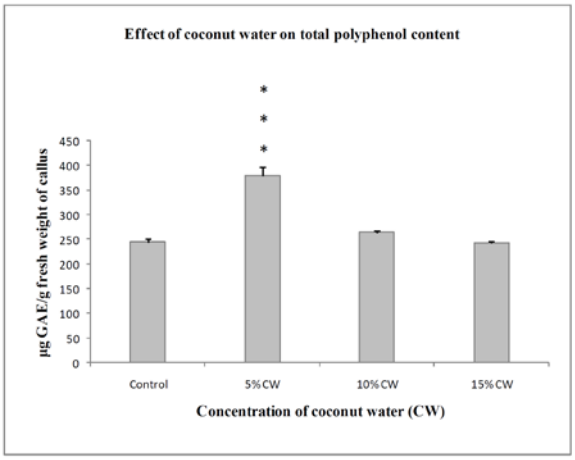


Figure 7: Effect of coconut water on total polyphenol content in P. ovata. Each value is the mean with standard error of five replicates. Asterisks indicate significant differences at $p < 0.05$ (*) or $p < 0.01$ (**) or $p < 0.001$

(***) between the mean of the sample as compared to the mean of the control.

Effect of additives on total polyphenol content in *Plantago ovata*

Plantago ovata has always been well explored by researchers for phytochemical screening. Presence of different polyphenolic compounds in different species of *Plantago* has been reported in a number of studies [24-26] but enhancement of total polyphenol content in this species has not yet been established. Total polyphenol content greatly contributes to the antioxidant properties of many medicinal plants as evident from previous reports [27]. Effect of different organic additives and physical factors on total polyphenol content in plants has been extensively studied before [28-30]. In the present study we have attempted to enhance the total polyphenol content in *Plantago ovata* by supplementing exogenous additive to the embryogenic culture medium with an objective to develop an *in vitro* culture with enhanced amount of polyphenolic compounds. The supplementation of casein hydrolysate and coconut water along with 0.5 mgL^{-1} NAA and 1 mgL^{-1} BAP led to an increase in total polyphenol content. In case of casein hydrolysate total polyphenol content decreased when the additive was used in lower concentration (1 gL^{-1}). Lower concentration of casein hydrolysate might trigger a negative signal in the polyphenol synthesis pathway or it might be due to the decrease in the amount of one or more individual phenolic compounds which led to an overall decrease in total polyphenol content. Similar observations have been reported when plants are exposed to lower doses of salinity and gamma stresses [31, 32]. But in case of 2 gL^{-1} casein hydrolysate a sharp increase in total polyphenol content was observed. Cultures treated with 2 gL^{-1} casein contained 322.5±29.77 µg of total polyphenol /g fresh weight of callus tissue. Samples treated with 3 gL^{-1} casein hydrolysate was found to contain greater amount of phenolic compounds than control but lesser than 2 gL^{-1} casein hydrolysate. Hence 2 gL^{-1} was considered as the best among other concentrations of casein hydrolysate in enhancing total polyphenol in *Plantago ovata*. Coconut water was also proved to be effective in enhancing total polyphenol in *Plantago ovata*. 5% coconut water was found to be maximally effective in enhancing phenolic compounds. Cultures supplemented with 5% coconut water contained 379.5± 17.49µg of total polyphenol /g fresh weight of callus tissue. But phenolic content was found to decrease with increasing concentrations of coconut water. 10% coconut water produced more phenolic compound than control but less than 5% coconut water. According to Das (Pal) and Sen Raychaudhuri (2001) higher concentration of coconut water enhances callus mass in *Plantago ovata* however, the authors did not study total polyphenol content. The increased callus mass might caused greater accumulation of total phenolics as compared to control. 15% coconut water was not found to be effective. Higher concentration of coconut water might cause degradation of polyphenolic compounds by different enzymes leading to an overall decrease in total polyphenol content. Embryogenic cultures supplemented with casein hydrolysate and coconut water were intensely brown in color. This may be attributed to the enhanced accumulation of phenolic compounds with callus ageing [33, 34] and the role of polyphenolic compounds in tissue browning [35, 36].

CONCLUSION

The results documented in the present investigation clearly indicated accumulation of

polyphenolic compounds during somatic embryogenesis in *Plantago ovata*. In our investigation we have used casein hydrolysate and coconut water in different concentrations as external additives to enhance production of callus as well as total amount of polyphenolic compounds. Both the additives were proved to be effective in increasing the amount of total polyphenols in *Plantago ovata.* In the present article 2 gL^{-1} casein hydrolysate and 5% coconut water gave best results in enhancing callus mass and total polyphenol content. However, 5% coconut water was found to be more effective as compared to 2 gL^{-1} casein hydrolysate in enhancing total polyphenol content in *Plantago ovata.* The present article therefore would enrich new insights in the field of herbal formulations in biotechnological research.

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