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In vitro regulation of diosgenin biosynthesis in *momordica charantia* cell suspension cultures by feeding precursors and elicitation

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ABSTRACT

Plant cell culture provides an alternative means for producing secondary metabolites. In this study, experiments were carried out to study the effect of cholesterol as precursor and salicylic acid and sodium nitroprusside as elicitors on the enhancement of diosgenin production in the cell suspension culture of *Momordica charantia.* Callus was induced from seeds when inoculated on Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of auxins and cytokinins to initiate cell suspension culture. The maximum diosgenin recovery was estimated in 6 week old callus (7.54 mg/gdw) which was significantly superior then the rest of time treatment and the treatment doses of salicylic acid (0.05 mM) gave highest diosgenin recovery (8.98 mg/gdw) in comparison to Sodium nitroprusside (8.53 mg/gdw) and control (7.61 mg/gdw).

ntroduction

Plants form an important part of our everyday diet and their nutraceuitcal values have been intensively studied for past decade. In addition to essential primary metabolites, plants are also able to synthesize a wide variety of low molecular weight compounds known as secondary metabolites. They have an important role in the interaction of the plant with its environment. The production of these compounds is often low and depends greatly on the physiological and developmental stage of the plant (Caldentey and Inze , 2004 and Dixon, 2001). Among various secondary metabolites, diosgenin is one of the promising compounds, which have attracted lot of researchers, have been reported in a few higher plant species (Liu et al., 2005). It has been reported that diosgenin can be absorbed through the gut and plays an important role in the control of cholesterol metabolism (Roman et al., 1995). Diosgenin is generally used as starting material for partial synthesis of oral contraceptives, sex hormones and other steroids (Zenk, 1978), which has been a boon specially from plant-based precursors because of the increasing demand for corticosteroids, contraceptives, sex hormones and anabolic steroids (Hall and Walker, 1991). Plant cells and tissue cultures hold great promise for controlled production of a myriad of useful secondary metabolites on demand. The current yield and productivity cannot fulfill the commercial goal of a plant cell-based bioprocess for the production of most secondary metabolites. In order to stretch the boundary, recent advances, new directions and opportunities in plant cell-based bioprocessing have been critically attempted.

Finding the biosynthetic pathway of a natural drug is the key issue for its industrial production. Even a limited increase in production rate may lead to a drastic effect at a commercial level with increased yield of a specific compound using key precursor (Patel, 1991). Exogenous supply of a biosynthetic precursor to culture medium can also increase the yield of the desired product (Staba, 1985). Attempts to induce or increase the production of plant metabolites by supplying precursor or intermediate compounds have been successful in many cases (Moreno et al., 1993, Whitmer et al., 1998).

An elicitor is the compound that induces regulation of secondary metabolic genes, which are often associated with signal transduction thereby triggering the formation of bioactives (Kolewe et al., 2008). Successful elicitation is influenced by many factors such as the type of elicitor and its concentration, time of elicitation, growth phase of the cell culture and the nutrient medium composition.

The elicitor can be regarded as a stress factor involved in the interactions e.g. plant-microorganism, plant-pesticide, plant-heavy metal, plant-UV irradiation. Due to the chemical defensive reactions, elicitors increase the activity of certain enzymatic systems for a short period of time and these systems catalyze the formation of stress substances similar to the particular secondary metabolites.

Elicitors include natural hormones, specific chemicals, nutrients and many fungi-derived compounds, classified as biotic and abiotic elicitors (Eilert, 1987; Verpoorte et al.,1991 and Roberts and Shuler,1997)

Momordica charantia L., Variety - VNR Katahi of family Cucurbitaceae commonly known as ‘karela’ in India and ‘bitter gourd’ in English. It is cultivated throughout India. The fruits are used as vegetable. Fruits contain protein, fat, carbohydrate, mineral matter and moisture. It is rich in vitamin like riboflavin, thiamine, ascorbic acid etc. The entire plant has medicinal properties and used for preparation of indigenous medicine (Chopra et al., 1956).

Thus present investigation deals with in vitro regulation of diosgenin from M. charantia cell cultures by feeding precursors and elicitors (salicylic acid and sodium nitroprusside).

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Experimental

All plant parts were collected, cleaned and oven dried at 100ºC for 15 min to inactivate the enzymes and then at 250C till constant weight was achieved and then powdered. The voucher specimen of experimental plant was deposited in Herbarium at Department of Botany, University of Rajasthan, Jaipur (RUBL* No. 20391).

Establishment of Tissue Cultures

MS medium (Murashige and Skoog, 1962) was used for initiation and maintenance cultures of M. charantia. Seeds were surface sterilized with HgCl2 solution, rinsed thrice with sterile distilled water. Explants were inoculated on Laminar flow hood fitted with ultraviolet light lamp (Thermodyne), in the flasks containing culture medium aseptically. Cultured flasks were incubated in culture chamber with temperature maintained at 25 ± 1°C and 1200 lux light was provided for 16 h. The callus culture was established by using various treatment doses of hormones. These calli were maintained for about six months with frequent subculturings at time interval of 4-6 weeks. Growth index (GI) was calculated after 2, 4, 6 and 8 weeks of fresh subculturing to record the growth pattern * University of Rajasthan, Botanical Herbarium, Jaipur

Diosgenin

Extraction

Callus tissues were powdered weighed and defatted, separately in soxhlet apparatus in petroleum ether for 24 h on a water bath. Each mixture was hydrolyzed with 15% ethanolic HCl (1g/5 mL: w/v) for 4 h by refluxing on water bath (Tomita et al., 1970). Each hydrolysate was filtered and filtrate extracted thrice with ethyl acetate. The ethyl acetate fractions of each sample was pooled and washed to neutrality by repeated washings with distill water, dried in vacuo, reconstituted in chloroform , filtered , dried again and weighed. Each test sample was replicated thrice. Thin glass plates coated with silica gel (250 mμ thick) were dried at room temperature, thereafter kept at 100 0C for 30 min to activate. The freshly prepared activated plates were used for qualitative as well as quantitative analysis.

Qualitative Analysis

The crude diosgenin extract of each sample was examined on tlc, along with the reference standard of Diosgenin. The plates were developed in a solvent system of chloroform, hexane and acetone (23:5:2), air dried and sprayed with 50% sulphuric acid (Bennett and Heftmann , 1962) and anisaldehyde reagent (composed of 0.5 mL of anisaldehyde , 1mL of conc. sulphuric acid and 50mL of acetic acid), separately and heated to 100 0C until the characteristics colors developed. The fluorescence response as well as permanent black zones was recorded. The times required for the initial appearance of a colour reaction, the initial colour in day light and after heating for 10 min. and the colour in UV light (360nm) were recorded. A combination of other solvent systems such as benzene and ethyl acetate (85:15 (Heble et al., 1968) and acetone and benzene (1:2 (Khanna and Jain,1973) were also used but solvent system of chloroform, hexane and acetone (23:5:2) was comparatively better than other solvent system. Three replicates were run and Rf values were calculated.

Quantitative Analysis

Preparative thin layer chromatography (PTLC)

Ptlc was used to isolate diosgenin from crude steroidal sapogenin extract on silica gel G plates by using solvent mixtures of chloroform, hexane and acetone (23:5:2). The spots were marked on tlc by spraying with anisaldeyde reagent, to one of the columns on each plate and spots corresponding to the standard diosgenin were marked and scrapped separately from the unsprayed plates/column. The ptlc was repeated until about 20mg of the substance was obtained. Co-tlc of crystallized isolated substance along with reference marker (standard diosgenin) was carried out to test the purity of isolated compounds. Such chromatograms were also visualized by spraying a solution of antimony trichloride in conc. HCl (Kadkade et al., 1976). After ptlc the diosgenin was crystallized from methanol-acetone (Kaul and Staba., 1968) and examined for mp, mmp and infra –red spectral studies.

Spectrophotometry for Steroidal sapogenins- The spectrophotometric method (Sanchez et al., 1972) was followed to estimate quantitatively the levels of diosgenin in respectively in each sample. It includes the preparation of regression curve of the standard diosgenin from there stock solution (1mgL-1) prepared in chloroform, from which different concentrations (20μg to 200μg) were made and applied on silica gel G plates with fine applicators separatively. The plates were then developed along with a parallel run of blank in an organic solvent of chloroform, hexane and acetone (23:5:2), which were later on dried and exposed to iodine vapours. The resultant dark yellowish spots as also the spots corresponding in the blank were marked and the plate were heated to 100 0C for 15 min to remove iodine. Each of the marked spot was scrapped along with the adsorbent, transferred to separate test tube and eluted with 5 ml of methanol. The mixture was then centrifuged, the supernatant transferred to separate test tube and evaporated to dryness. To the dried residues, 4ml of 80% methanolic sulphuric acid was added and left at room temperature for about 2 hours by intermittent shaking. Optical density of the reaction mixture was read at 405nm against a blank solution (80% methanolic sulphuric acid). Three replicates of each concentration were taken and average optical density was calculated. A regression curve between various concentrations and their respective optical density was computed which followed the Beer's law.

Each of the crude extract of plant and tissue samples were dissolved in 5 ml of chloroform and applied (0.1ml) on silica gel G plates along with standards as marker, developed in solvent mixture of chloroform, hexane and acetone (23:5:2) which were later on dried and exposed to iodine vapours. The resultant dark yellowish spots and corresponding spot of the standard authentic samples were marked on each plate, scrapped, eluted with methanol, dried and then treated with 80% methanolic sulphuric acid as above. The concentration of diosgenin in each samples were worked out referring to their optical densities in the standard curve and the results were calculated on dry weight basis. Three replicates of each sample were taken and their mean values calculated

Precursor Feeding

An experiment was conducted in which three treatment doses of cholesterol which is a precursor of diosgenin (0.025, 0.05 & 0.1mM) were premixed with liquid MS media supplemented with 2 mgL-1 of 2,4-D, separately. Later, six month old established calli were transferred to the liquid MS media which was supplemented with three treatment doses of cholesterol. The MS media without cholesterol served as control. The liquid cultures were grown on reciprocal shakers (125 rpm; 5 cm/strokes). The tissues grown in different media were harvested at regular time intervals of 2, 4, 6 and 8 weeks after subculturing. The various tissue samples obtained were dried at 100o for 20 min to inactivate the enzyme activity and

later at 60o C till a constant weight was achieved. The calli were powdered and subjected to qualitative and quantitative estimation for diosgenin as mentioned previously.

Elicitation

The liquid MS medium supplemented with 0.5 mgL-1 BAP and 2 mgL-1 NAA was prepared. 3gm of callus was transferred to each flask and the liquid cultures were grown on reciprocal shakers (125 rpm: 5 cm/strokes) for 6 days, on the 1st day Liquid MS media was supplemented with three treatment doses (0.025, 0.05 & 0.1mM) separately with each of salicylic acid (SA) and sodium nitroprusside (SNP), which are abiotic elicitor and the cultures were again kept on same shaker for exactly 24 h. The tissue grown in different media were harvested. The various tissues obtained were dried at 100 0 C for 20 min to inactivate the enzyme and later at 60 0 C until constant weight was achieved. The calli was powdered and subjected to qualitative and quantitative estimation for diosgenin as mentioned previously.

Results and Discussion

Callus Cultures

Callus was formed on MS medium supplemented with various concentrations of auxins and cytokinins. Best response was on medium supplemented with BAP: 2, 4-D (2.0mgL-1 each).

The callus cultures were maintained for 6 months by periodic subculturings. Callus was harvested at the time intervals of 2, 4, 6 and 8 weeks of fresh subculturing to determine growth index (GI). The GI value on fresh weight basis depicted a sigmoid pattern from 2 to 8 weeks.

Use of precursors to transform biosynthetically in to the desired product has been advocated (Staba, 1985; Endress, 1994; Kamal and Mehra, 1995). Cholesterol is one of the intermediate compounds in biosynthetic steps leading to formation of diosgenin. It was reported (22) less than 1% diosgenin from various species of Dioscoera and 1 to 2.5% from D. deltoidea when grown on media supplemented with cholesterol and 2,4- D (26, 17, 27) due to incorporation of precursor during biosynthesis of diosgenin. In the present investigation taking different concentration along with control, the diosgenin content in callus cultures was highly significant. The two treatment doses of cholesterol (0.05M, 0.1mM) gave significant diosgenin recovery (6.82 mg/gdw, 6.56 mg/gdw).

Taking time treatment individually the maximum cholesterol recovery was estimated in 6 week old callus (7.54 mg/gdw) which was significantly superior then the rest of time treatment. The interaction between treatment doses of cholesterol and time treatments showed a maximum diosgenin recovery in 6 week old calli obtained from 0.05 mM each (8.34 mg/gdw). This was at par with 0.1mM each of cholesterol (8.12 mg/gdw) fed medium and minimum in 2 week old callus (3.41 mg/gdw) obtained from control (Fig. 1)

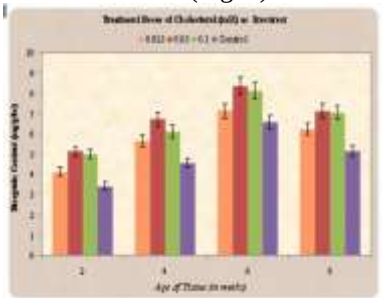

Fig. 1 .Effect of Precursor feeding on Diosgenin content of *M. charantia* Static Cultures

In the present findings six month old maintained callus cultures of *M. charantia* were grown for different time period on different treatment doses of cholesterol fed medium. Diosgenin recovery in the tissue was highly significant in comparison to control.

As stated earlier that biosynthetic capacity of diosgenin *in vitro* tissue cultures gradually decreases after few months, the present investigation suggests that transferring the callus on cholesterol supplemented medium could enhance the diosgenin biosynthesis.

Secondary product formation can be triggered in cell culture due to environmental stress. Elicitation of cell cultures offers a novel approach for rapid accumulation of certain secondary metabolites, which can improve the efficacy of product via induction of various enzymes or by providing additional biosynthetic pathway. Such treatment could lead to substantial change in cellular metabolism. Treatment of cell cultures by biotic elicitors resulted in increased biosynthesis of flavonoids, stilbenes, terpenoids, steroids, alkaloids, insecticides etc (Wolters and Eilert, 1983; Verpoorte *et al*., 1991; Kamal *et al*., 1995).

Enhanced metabolite yield by elicitation is a result of a complex interaction between the elicitor and the cultured plant cell. In this process number of enzymes are involved which may or may not induce various biosynthetic pathways (Hahlbrock and Scheel ,1989). SA and SNP acts as signal compound in the induction of plant defense mechanism thereby increasing the production of secondary metabolites which supports the previous studies (Kamal *et al*., 1995). Moreover, SA and SNP might also affect the transient transcription and translation of enzymes needed for the biosynthetic pathway of the metabolite of interest (Memelink *et al*., 2000), which is also in confirmation with the observation (Verpoorte *et al*., 1999) that certain biosynthetic pathways are inducible at the gene level by external signals and such signals can induce complete pathway.

The diosgenin recovery data of 6 week old callus cultures (control) of *M. charantia* and callus samples obtained from three treatment doses of salicylic acid 0.025 mM, 0.05M, 0.1mM along with control were observed. The treatment doses of SA and SNP both at dose of 0.05 mM gave highest diosgenin recovery (8.98 and 8.53 mg/gdw respectively) in comparison to control (7.61 mg/gdw) (Fig. 2)

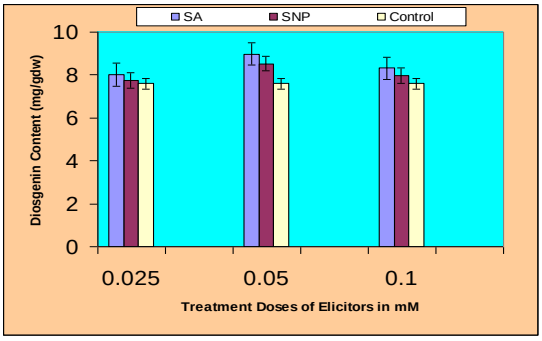

Fig. 2. Effect of various Elicitor treatment on Diosgenin content of M. charantia Suspension Cultures

Production of secondary metabolites in tissue culture can also be enhanced by use of elicitors (Eilert , 1987; Verpoorte et al, 1991). Dixon (1986) reported that concentration of elicitor plays an important role in accumulation of secondary metabolite. It was reported (Rokem and Goldberg, 1984) that 70% increase in diosgenin content in Dioscorea deltoidea using Rizopus arrhizus homogenate. SA acts as a disease- resistance signal

compound in the induction of a plant defence mechanism (Kamal et al ., 1995).

In the present study, six- month- old callus cultures of M. charantia were fed with different doses of SA and SNP in the medium. Diosgenin recovery in the tissue was highly significant (1.18 times in SA and 1.12 times in SNP) in comparison to control.

Increase in the diosgenin production shows confirmation with the previous studies (Rokem and Goldberg, 1984) and also with the other findings (Kamal et al ., 1995) that SA and SNP might be acting as a signal compound in the induction of plant defence-mechanism thereby increasing the production of secondary metabolites and diosgenin in particular in the present investigation.

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