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"EFFECT OF BIOTIC & ABIOTIC ELICITORS ON THE PRODUCTION OF DIOSGENIN IN TISSUE CULTURE OF LYCIUM BARBARUM"

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Shortened version of the Title for the running head: Effect of Elicitors on Diosgenin Production.

ABSTRACT

Callus cultures of Leaf explants of Lycium barbarum were established and growth indices were measured on Basal MS media supplemented with Auxin (1.5 mg/l 2, 4D; 1mg/l IAA) and Cytokinin (0.5 mg/l Kinetin) up to10 weeks. Callus with maximum Growth Index (08 weeks) was used for further elicitation and quantification procedures. Effect of various elicitors of biological and chemical origin was studied for elicitation of diosgenin in optimized and established callus cultures of Lycium barbarum. Chemical elicitors salicylic acid and cobalt chloride were used in optimized concentrations of 2.5, 12.5 and 37.5 mg/100 ml of media respectively. Biotic (Yeast Extract) elicitor was used in varying concentration of 2.5, 5 and 7.5 ml /100 ml of media for elicitation in callus culture. Diosgenin production was monitored on established callus cultures for 24, 48 and 72 hours post elicitation. After elicitation the diosgenin produced was identified and separated using thin layer chromatography. Quantification of diosgenin so produced was done using spectro photometric method. Significant increase in Diosgenin content was observed with increasing concentrations of all elicitors. Yeast Extract elicitor was most potent elicitor as it increased the diosgenin content

2.45 folds as compared to the control one. Cobalt Chloride was the weakest elicitor in diosgenin production.

Keywords: Callus Culture, Diosgenin, Elicitors, Growth Index, TLC

1. Introduction

Lycium barbarum or Goji berry are woody, deciduous, perennial plants belonging to family Solanceae. They grow 1- 3 meter height above ground level. It covers large surface area, conserve moisture and also protect soil erosion in barren desert lands. The plant is well adapted morphologically and physiologically for extreme dry environmental conditions of Indian arid zone. It is drought resistant and prefers light sandy soil.

The plant produces orange red ellipsoid berries 1-2 cm length carrying 10-60 tiny yellow seeds compressed with a curved embryo. *Lycium barbarum* has been prevalent in Chinese medicine about 600 years ago. It has been claimed that barks of *Lycium barbarum* contains valuable pharmaceutical compounds that contribute in treatment of various chronic and systemic diseases.

It has crucial role in immunity improvement, eyesight improvement, curative agent for sterility, used as a sedative and a pain killer. It is a rich source of vitamin B1 and B2.

Many higher plants are abundant sources of natural products used as pharmaceuticals, agrochemicals, flavour and fragrance ingredients, food additives and pesticides [1]. In the search for alternative to produce desirable medicinal compounds from plants various biotechnological approaches specifically Plant Tissue Culture are found to have huge potential as a supplement to conventional agriculture in the industrial and commercial production of bioactive plant secondary metabolites.[2]

Plants based secondary metabolites are biosynthetically compounds derived from primary metabolites by specific genetically controlled enzymatic ally catalysed reaction. The production of these compounds is often less than 1% of dry weight and depends greatly on physiological and developmental stages of the plant [3].

Plant tissue culture is a convenient, reproducible and efficient culture system for plant science and biotechnology research and development. It has shown great advantages as well as an economic alternative to the whole plant based system for producing bioactive products, which have been used produce valuable medicinal substances commercially [4,5]. However,

the low yield of secondary metabolites in plant cell culture has been a bottleneck for commercialization at industrial scale.

Consequently there have been numerous attempts to improve the productivity of secondary metabolites in plant cell culture such as medium optimization, cell line selection, cell immobilization, precursor addition, elicitation, and metabolic engineering [6,7]. Among these manipulation techniques, elicitation is a very attractive strategy for increasing the metabolite production in cell culture system, which can lead to increased yields and shortened culture times [8-11].

Elicitation is the induction of secondary metabolite production by either biotic or abiotic treatments. Nowadays, the use of pathogenic and non-pathogenic fungal preparations and chemicals as elicitors has become one of the most important and successful strategies to improve secondary metabolite production in plant cell culture [12].

Elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amount of elicitors [13].Elicitors can be classified on the basis of their nature like biotic and abiotic elicitors or on the basis of their origin like exogenous and endogenous elicitors. Biotic elicitors are derived from microorganisms or they are endogenous compounds of infected plants and latter types of elicitors are released after either by mechanical wounding or enzymatic hydrolysis of polymeric plant cell wall. [14]

Diosgenin is a precursor for the chemical synthesis of steroidal drugs & is tremendously important for pharmaceutical industry [15]. Considerable progress has been made on diosgenin bearing plants and in steroid industry. [16] Tal et al. reported the use of cell cultures of *Dioscorea deltoidea* for production of diosgenin. They found that carbon and nitrogen level greatly influence diosgenin accumulation in one cell line. [17].The work on the production and biochemistry of steroids has been reviewed by many workers [18-20].

Steroidal studies have been reviewed extensively using tissue culture [21-22]. Sapogenins are steroids attached with sugars are widely used in field of medicine s they are main precursor of steroidal hormones [23]. Diosgenin an important sapogenin is principally obtained from *Dioscorea*s pecies. [24-25]. It has also been described from few *Solanum* species. [26-29].

Therefore, it becomes imperative to study how we can use *Lycium barbarum* species for production of diosgenin a pharmaceutically significant secondary metabolite using PTC. Present investigation was conducted to study and optimize callus culture conditions and growth prospects of leaf explants of *Lycium barbarum*. Secondly the established culture will

be subjected to varying concentrations of biotic and abiotic agents to elicit the production of diosgenin. The diosgenin produced will be quantified using Thin Layer Chromatography.

2. Material And Methods

2.1 To establish Callus Culture of *Lycium barbarum* and Plot Growth Indices

Present Investigation was conducted at M.N Institute of Applied Sciences (MNIAS), Bikaner, Rajasthan which is located at 28° 01′ 00″N latitude,73◦18′43″Elongitude and at an altitude of 242mabove sea level. Shoots of *Lycium barbarum* were collected from NRCC, Jhorbead, Bikaner. All the chemicals used in this investigation were of analytical grade and were obtained from Department of PTC &Biotech Laboratory.

2.1.1ExplantsPreparation: Leaves of *Lycium barbarum* were washed thoroughly with 5% solution of liquid detergent Triton X followed by rinsing with sterile distilled water. After washing, leaves were surface sterilised with 0.1 % $HgCl_2$ for 2-3 minutes and then rinsed thoroughly with sterile distilled water for 3-4 times.

2.1.2 Media Preparation: Stock Solutions of MS Media [30] were used. Auxin & Cytokinin solutions were dissolved in few drops of 1N NaOH /Ethanol and 1N HCl respectively. The final volume was made using distilled water and was filter sterilised. pH of the media was adjusted to 5.8 prior to addition of agar at 0.8% concentration. Media was sterilized using autoclave.

2.1.3 Inoculation: Inoculation of explants on nutrient media was carried out aseptically under laminar air flow cabinet with a thoroughly cleaned working bench using cotton swab dipped in alcohol. Sterilized Explants (leaf) of *Lycium barbarum* were transferred to culture media supplemented with various concentrations of Auxin & Cytokinin.

2.1.4 Incubation Conditions: The cultures were maintained under aseptic uniform condition of temperature 26±2 deg C &55% relative humidity and diffused light of 300Lux.

2.1.5 Growth Rate Studies: Leaf Explantsof *Lycium barbarum* took 15 days for callus initiation. Initiation started from margin and extended to central part. The tissues were harvested at regular at their transfer ages of 2,4,6,8 and 10 weeks and growth Indices were calculated on dry weight basis.

$$\text{Growth Index} = \frac{\text{Final dry weight of tissue} - \text{Initial dry weigh}}{\text{Initial Dry weight}}$$

2.2 To study effect of Various Elicitors on Diosgenin Production in Calli of *Lycium barbarum*

2.2.1 Elicitor Preparation: In this investigation both Biotic and Abiotic elicitors were used to study the effect on Diosgenin production.

Biotic Elicitor: It was commercially available Yeast Extract Powder in local market.5 g of dried powder was dissolved in 25 ml of distilled water and equal amount of 80% ethanol was added and allowed to settle at 60 Deg C for 4 days. The supernatant was discarded and gummy precipitate was again washed and dissolved in 25 ml of distilled water. Same procedure was repeated for 02 times and final precipitate was used as biotic elicitor. Growing Tissues were transferred to sterilised MS media supplemented with various concentrations (2.5 ml, 5ml & 7.5 ml/100 ml media) of yeast elicitor.

Abiotic Elicitor: Salicylic acid and Cobalt Chloride ($CoCl_2$) were used as abiotic elicitors. Various optimized concentrations of these two elicitors were added in media preparations**.** Growing tissues were transferred to autoclaved MS Media supplemented with various optimized concentrations (2.5 mg, 12.5 mg & 37.5 mg / 100 ml media) of both Salicylic Acid and $CoCl_2$ respectively.

2.2.2 Incubation Conditions: The transferred tissues were maintained under aseptic uniform condition of temperature 26±2 deg C & 55% relative humidity and diffused light of 300Lux. The tissues subjected to various concentrations of elicitors were harvested regularly at 24, 48 and 72 hours of incubation to quantify Diosgenin in the culture following the procedure of Sanchez et al. [31].

2.2.3 Extraction Procedure: The harvested sample was dried and hydrolysed with 30% hydrochloric acid and was washed with distil water till pH reached 7.0. Test sample were re-dried and Soxhlet extraction was done in benzene separately [32]. The benzene extract was dried and residual crude extract was taken in chloroform for further quantification of diosgenin.

2.2.4 Quantitative Analysis: The quantification of diosgenin produced due to elicitation by various biotic and abiotic elicitors was done using Thin layer chromatography (TLC). The crude extracts along with reference Diosgenin was dissolved in benzene and were applied separately on silica gel 'G' coated and activated glass plates of thickness 0.3-0.4mm. The glass plates were developed in an organic solvent mixture of Hexane: Acetone -80:20 following procedure of Fazli et al. [33]. The glass plates were prepared in replica. One of the

developed glass plates was dried and visualised under UV light revealed fluorescent spots in each of test sample. These spots were sprayed with 50% sulphuric acid and subsequent heating at 100deg C for 10 minutes showed one spot coinciding with that of reference compound Diosgenin (RF-0.43). The spot was separately marked and collected along with silica gel from unsprayed plate and eluted with methanol and dried.

2.2.5 Preparative Thin Layer Chromatography: Each of the extract along with standard reference diosgenin was applied separately on 0.3-0.4 mm silica gel 'G' coated and activated glass plates. These plates were developed in organic solvent mixture of hexane: acetone (80:20). The developed plates were air dried and visualised under UV light. The fluorescent spot (RF-0.43) corresponding to reference standard sample of diosgenin in test sample was separately marked , collected along with silica gel from unsprayed plates, eluted with methanol and dried.

2.2.5 Quantitative Estimation: Reference Standard Stock solution of Diosgenin was separately prepared ranging from 10 µg to 120 µg in chloroform using the same protocol as for sample crude extracts. The 4 ml of methanol dried and eluted reference & test samples of diosgenin were taken and allowed to dry on water bath. To each resulting residue 4 ml of methanolic H_2SO_4 80% was added and kept for 02 hours. Absorbance from each known sample was measured on spectrophotometer set at 405 nm and a regression curve was prepared on basis of Beer's law.

3. Results And Discussion

3.1 Callus Culture of *Lycium barbarum* and Growth Indices :

The best growth response for callus culture was observed on MS media fed with 1.5 mg/l of 2, 4 D, 0.5mg/l of kinetin and 1mg/l IAA. The callus culture was creamy and compact in texture. The growth of callus was observed up to a period of 10 weeks. The maximum growth index (8.09) was observed in 08 weeks old culture. The tissue at 10 week old culture showed a decline in growth index (5.08) due to accumulation of waste products. Minimum growth index (4.50) was observed in a culture of 02 week age.

Thus, 08 week culture stage was taken for further analysis and diosgenin elicitation and quantification procedures.

Table 1: Table indicating growth indices of Callus Culture of *Lycium barbarum*

S .No.	Age of Cultures (in weeks)	Growth Indices (GI)
1	02	4.50
2	04	5.30
3	06	6.60
4	08	8.09
5	10	5.80

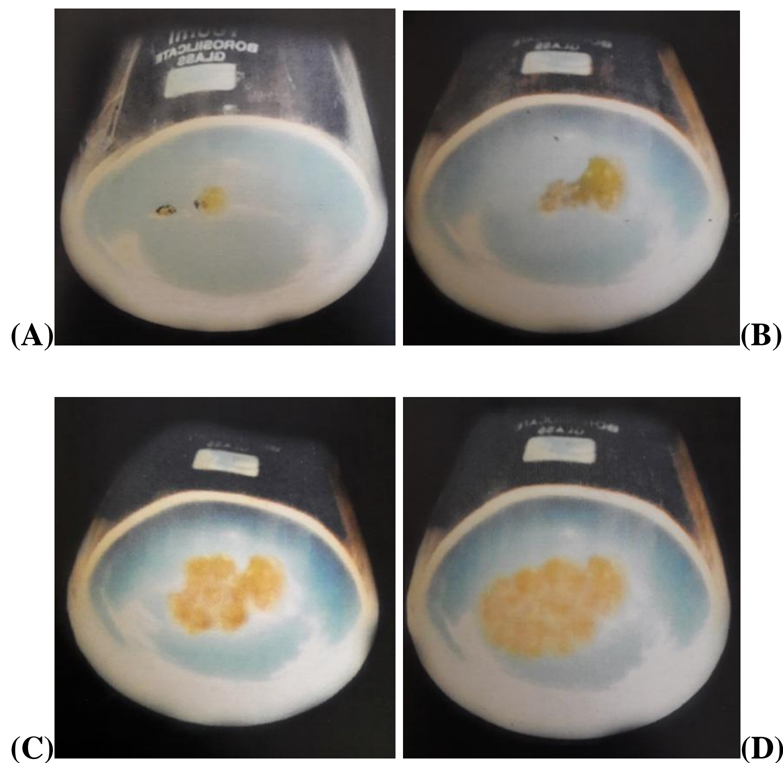

Figure 01: Depicting callus cultures of *Lycium barbarum* at
(A) 02 Weeks
(B) 04 Weeks
(C) 06 weeks
(D) 08 Weeks

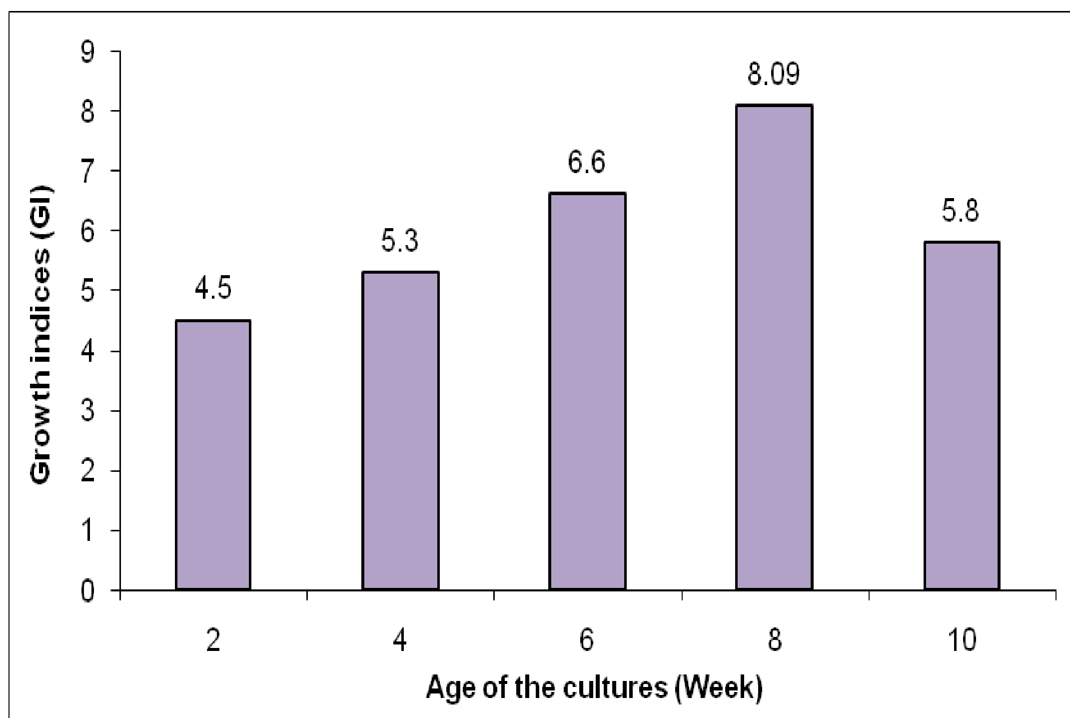

Figure 02: Graphical presentation of Growth Indices in callus culture.

In the present study it was observed that tissues of *Lycium barbarum* in callus culture became brown after a period of 08 weeks and showed a decline in GI mainly due to accumulation of secondary metabolites, waste products and depletion of nutrients.

3.2 **Effect of Elicitors on production of Diosgenin in tissue culture of *Lycium barbarum***

The elicitation of diosgenin in culture of *Lycium barbarum* revealed that with increasing amount of elicitor in the culture amount of diosgenin also increases. With yeast elicitor (7.5 ml/100 ml) maximum diosgenin content of (19.67 mg/gm) of callus was observed in 48 hours old culture.

With Salicylic acid as an abiotic elicitor maximum content of diosgenin (15.68 mg/gm) of callus was observed at a concentration of 37.5 mg/100 ml media in 48 hours old culture.

With $CoCL_2$ as an abiotic elicitor maximum content of diosgenin (10.56mg/gm) of callus was observed at a concentration of 37.5 mg/100 ml of media in 48 hours old culture.

These results reveal that $CoCl_2$ is the weakest elicitor among salicylic acid and yeast elicitor but it was still producing more diosgenin than the control sample (5.69 mg/gm) and MS media supplemented without hormone (6.70mg/gm.)

Table 2: Effect of Elicitor with variable concentration of elicitors

S No	Medium Formulation with various elicitor concentration	Diosgenin Content (mg/gm) dry weight			
		24 hours	48 hours	72 hours	Control Sample
1	MS Media with hormones(1.5 mg/l 2,4-D +0.5mg/l Kinetin+ 1 mg/l IAA)	-	-	-	5.69
2	MS Media without hormone	-	-	-	6.70
Abiotic Elicitors					
3	MS Media +2.5 mg/100 ml $CoCl_2$	7.25	7.55	6.89	-
4	MS Media +12.5 mg/100 ml $CoCl_2$	8.55	9.87	7.05	-
5	MS Media +37.5 mg/100 ml $CoCl_2$	9.10	10.56	7.65	-
6	MS Media +2.5 mg/100 ml Salicylic Acid	7.95	8.25	6.95	-
7	MS Media +12.5 mg/100 ml Salicylic Acid	8.99	12.85	7.25	-
8	MS Media +37.5 mg/100 ml Salicylic Acid	9.59	15.68	7.9	-
Biotic elicitors					
9	MS Media +2.5 ml/100ml Yeast elicitor	8.99	16.65	8.25	-
10	MS Media +5ml/100 ml Yeast elicitor	13.55	17.55	9.56	-
11	MS Media +7.5 ml/100ml Yeast elicitor	10.90	19.67	8.75	-

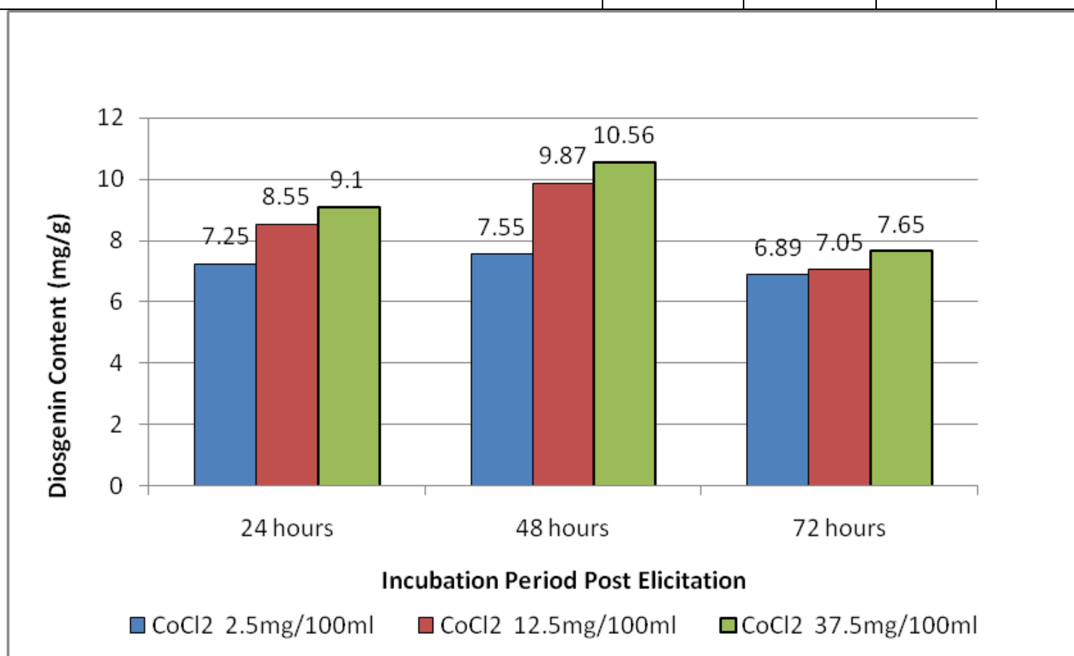

Figure 03: Graphical presentation of Disogenin production with Cobalt chloride as an elicitor.

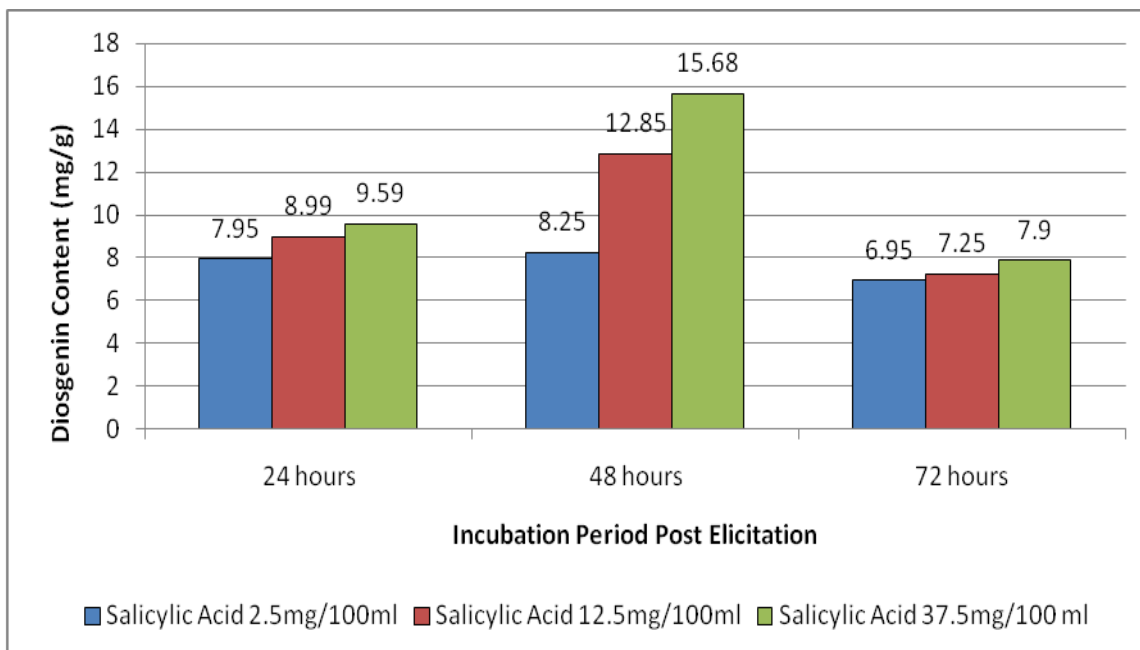

Figure 04: Graphical presentation of Disogenin production with Salicylic Acid as an elicitor.

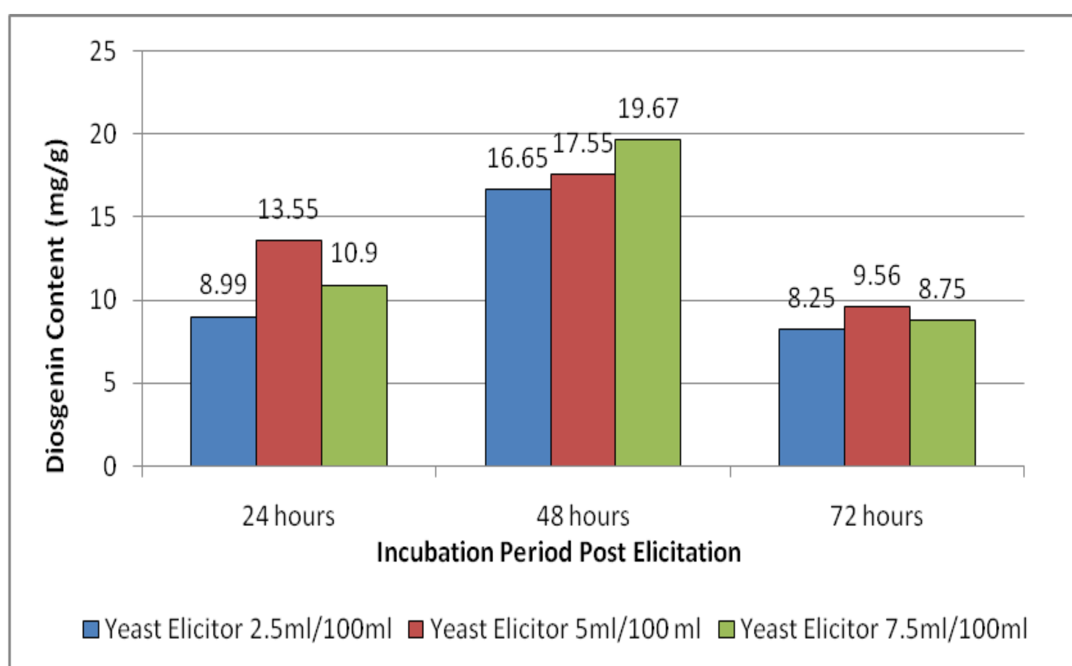

Figure 05: Graphical presentation of Disogenin production with Yeast Extract as an elicitor.

Most potent elicitation was done when yeast elicitor at a concentration of 7 ml/100 ml media was added in media and incubated for 48 hours .In this condition diosgenin produced if 2.45 fold the control one without any elicitor. This gives a good scope for commercial and industrial production of diosgenin using PTC techniques.

4. Conclusion

Elicitors play a significant role in increased production of diosgenin in callus culture of *Lycium barbarum* with varying concentrations of biotic and abiotic elicitors. The dried samples after elicitation were extracted with benzene and diosgenin content was studied in 24, 48 and 72 hours of elicitation.

The results revealed that increasing concentration of elicitor in media resulted in increased amount of diosgenin content in callus culture of *Lycium barbarum*. Increased amount of diosgenin content (19.67 mg/gm) was found in 48 hours old culture fed with yeast elicitor 7.5 ml/100 ml of culture media. Among biotic elicitors maximum amount of diosgenin content (15.68 mg/gm) was found with 37.5 mg/100 ml of media.

$CoCl2$ was weakest elicitor with 10.56 mg/gm in 48 hour old culture but it was still more than the control (5.69mg/gm) and MS Media without hormone (6.70 mg/gm.)

Thus present investigation revealed that elicitor strongly enhances the production of secondary metabolites. Elicitation in growth phase arrests the cell growth and changes in physiological states triggers overproduction of secondary metabolites.

Biotic elicitors are more potent than abiotic elicitors.

Thus it is evident with the results that as we increase the concentration of elicitor in medium plant is stressed to produce more diosgenin which is a very active pharmaceutical compound. Thus tissue culture techniques can be very well utilized for overproduction of diosgenin for human welfare.

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