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Efficient elicitor and precursor induced resveratrol production in suspension cultures of *Arachies hypogea*

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Abstract
Plant secondary compounds eliciting pharmacological or toxicological effects have long been processed for varied objective and they symbolize the perpetual compassion of nature by every means and are expressed in diverse human traditions. Plant cell cultures bequeath an alternative means for producing secondary metabolites. The current research is an effort to study the influence of elicitor salicylic acid and precursor phenylalanine and malonyl co A on the production of resveratrol, the most highly active phytochemical present in *A. hypogea***.** Suspension cultures were established on Murashige and Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D- 13.57 µM) and 6-benzylaminopurine (BAP- 0.88µM). Elicitor and precursor at different concentrations were supplemented to the medium and resveratrol produced was quantified by high performance liquid chromatography (HPLC). Several-fold increase in resveratrol concentration compared to the control was observed in elicited cultures. Precursor feeding (127.86±7.62) was found to be superior that elicited higher concentration of resveratrol, while noteworthy elicitation compared to precursor was observed in salicylic acid (74.45±5.62) on resveratrol production in the cell suspension culture of *A. hypogea*. Loss of cell viability was observed at high concentrations in both elicitor-precursor treated cultures. The results imply the production of resveratrol in elicitor-precursor treated suspension cultures are several times higher than in peanut plant and conceivably provide a new outlook for the further development.

Keywords: resveratrol, elicitor, precursor, suspension culture, *Arachies hypogea*

Introduction
Natural products extracted from conventional medicinal plants comprise potent therapeutic properties and ever since ancient times researchers have exploited a large numbers of such plants with restorative properties to treat various diseases and the need for novel and constructive compounds to provide assistance and relief in all aspects of the human condition is yet growing. Demand for understanding the approach and rationale used to isolate such bioactive compounds is of current interest. Resveratrol is one such comprehensively deliberated bioactive compound that has acknowledged much attention over decade due to its benefits to human health (Jocelyn and anna, 2014) [10]. These are naturally occurring phytoalexins produced in a selected number of plant species. The molecule has been endorsed as ‘‘the French Paradox’’, as low incidence of heart disease is observed flanked by a populace with a relatively lofty saturated fat diet and moderate wine consumption as significant levels of resveratrol is found in red wine [Frankel *et al*. 1993; Siemann and Creasy, 1992) [7, 19]. Over the years, various health benefits *viz.,* cardiovascular disease, various cancers, atherosclerosis and ageing have been linked with resveratrol (Baur and Sinclair, 2006; Roupe *et al*. 2006) [3, 18]. It has also shown to inhibit initiation, promotion, and progression of cancer (Jang *et al.* 1997) [12]. It is produced as a defense response to biotic and abiotic stresses. The molecule is synthesized by plants from the condensation of one molecule of *ρ*-coumaroyl CoA and three molecules of malonyl CoA by the action of the enzyme, stilbene synthase (Soleas *et al.,* 1997) [20]. The *ρ*-coumaroyl CoA is derived from phenylalanine, an amino acid synthesized in plants from sugars via the shikimate pathway, while malonyl CoA is derived from the elongation of acetyl CoA. Phenylalanine is converted to cinnamic acid by deamination, catalyzed by enzyme phenylalanine ammonia lyase. Cinnamic acid is then enzymatically hydroxylated to *ρ*-coumaric acid by cinnamate-4-hydroxylase (Jocelyn and anna, 2014) [10]. Peanut, *Arachis hypogaea,* is an important oil seed crop native to South America.

Besides income for the farmers, it provides high quality nutrition. They are opulent in bioactive compounds with a range of health benefits, such as stilbenes, flavonoids, phenolic acids, and phytosterols (Pennington, 2002) [17]. Hypocotyls and germinating seeds of groundnut have been found to contain resveratrol (Arora and Strange, 1991) [2]. The rapid advances made over the last two decades in biomedical research; there has been an unprecedented interest in unraveling the magical properties of this molecule and prompted scientists to think about the potentialities of investigation into cell cultures as a substitute for commercial utilization and also to be made cost effective. In this backdrop, the present investigation was carried to verify the impact of elicitor, salicylic acid and precursor phenylalanine and malonyl co-A on resveratrol production in suspension cultures of *A. hypogea*.

Materials and Methods

Callus Induction and Suspension Cell Cultures

The seeds of *A. hypogea* were procured from the krishi vignana Kendra, Bagalkot, Karnataka and were surfaces sterilized with 1% mercuric chloride for 5 minutes, followed by washing with sterile distilled water for 5–6 times to remove traces of surface-sterilant and were germinated on basal MS (Murashige and Skoog 1962) [15] medium. The germinated plants were served as an explant source. Callus was induced from *in vitro* leaf tissue explants cultured on MS medium supplemented with sucrose 3% (w/v), 2, 4-Dichlorophenoxyacetic acid (2,4-D) (13.57 µM) and 6-benzylaminopurine (BAP) (0.88µM) (w/v) and solidified with 0.8% agar-agar; pH was adjusted to 5.8 prior to autoclaving at 121◦C for 20 minutes. The culture tubes were incubated for 16 h photoperiod provided by cool white fluorescent lamps (25µmolm−2 s−1) at 25◦C and sub cultured at every second week. Callus (2 g cell fresh weight (FW)/100 ml medium) was transferred into 20 ml MS liquid medium in Erlenmeyer flasks, supplemented with 2, 4-D (13.57 µM), BAP (0.88µM) and 3% sucrose for proliferation. Suspension cultures were established by shaking on a rotary shaker (REMI, India) at 100 rpm at 22◦C, under a16 h photoperiod provided by cool white fluorescent lamps(25µmolm−2 s−1).

Preparation and application of precursor and elicitors to cell cultures

Precursor, phenylalanine and Malonyl CoA (50, 100, 150, 200 and 250 µM, each) and elicitor salysilic acid (50, 100, 150, 200 and 250 µM) were dissolved in sterile distilled water and was diluted with sterile double distilled water to get the final concentrations. PH was adjusted to 5.8 and solution was filter-sterilized before use. Treatments of cell suspensions with precursor and elicitor were performed 7 days after subculture. At the same time, control cell suspensions in MS liquid medium were supplemented with no additive (elicitor or precursor).

Measurement of *A. Hypogea* cell growth

The cell growth was supervised at every third day (up to15 days) by determining the fresh weight. Briefly the cells were accumulated by vacuum filtration, weighed for growth and analyzed on the same day. The fresh weight of the cells was recorded with the help of a physical balance. Alternatively cell growth was measured by sedimented cell volume (SCV) measurement. Cell viability was determined by vital staining with methylene blue stain.

Extraction and quantification of resveratrol

The cultures were separated by vacuum filtration and were washed with sterile distilled water to expel any adhering medium to the cell surface to record the fresh weight. Approximately 1 g of cell mass was taken and crushed using pestle and mortar by adding 10 ml methanol. The extract was centrifuged for about 05 min at 5000 rpm. The resulting supernatant was collected and was used for the estimation. HPLC quantification of resveratrol was carried out as described by Dong *et al.* (2001) [5] with appropriate modifications. Resveratrol content in the extract was estimated by HPLC analysis using Agilent, model 1260, QUAT pump with diode array detector and manual sampler injector using C-18 column. For determination of resveratrol from the samples obtained in this experiment, we used trifluro acetic acid : acetonitrile: water (0.2:20:80, v/v) as solvent A, and 100% acetonitrile as solvent B, at a flow rate of 1.0mL^{-min} with the following gradient :0–30% B linear (0–14 min), 30–100% B linear (14–18min), 100% B (18–23 min).This was followed by a 15min equilibrium period with initial conditions prior to injection of the next sample. Samples were filtered (0.45 mm, Millipore) and 20 µL was directly injected. Chromatograms were monitored at 305nm using the UV detector. Chromatographic peaks were identified through comparison with retention times of resveratrol (sigma aldrich), standards. Quantitative determination of resveratrol was performed using an external standard based on the area of peak under the optimal HPLC analytical conditions.

Statistical analysis

All experiments were conducted in replications. The data generated was subjected to statistical analysis using Microsoft Excel programme (MS Office, 2003) and represented as mean ± SE.

Results and Discussion

Durable modes of obtaining the secondary metabolites from plants are extraction from natural sources, partial synthesis from structurally similar compounds, total synthesis and through plant biotechnology. Conventional methods has a number of limitations such as plants are often not readily available because of geographical or governmental restrictions, requires huge area of land, which otherwise can be utilized for primary food crop. The process is labor intensive and time consuming, the quality may be affected by unforeseen environmental conditions and demands and supplies are difficult to manage. Total or partial synthesis of secondary metabolites proved to be extremely difficult and very expensive (Namdeo *et al.* 2007) [16].This has prompted industries, as well as scientists to deem the possibilities of investigation into cell cultures as an alternative supply for the production of plant pharmaceuticals (Vanisree and Tsay 2004) [24]. The aim of many industries are to establish plant cell culture techniques to the stage, where they yield secondary products more cheaply than extracting either the whole plant grown under natural conditions or synthesizing the product. *Arachis hypogaea* referred to as "*Poor man's almond*" employed in human nutrition contains resveratrol in considerable amounts. Over the past two decades, copious health benefits impacting cardiovascular disease, various cancers, atherosclerosis and aging have been linked with resveratrol. Attempt to increase resveratrol in other plant cell cultures by elicitation have been documented by several other reports (Jung *et al.* 2004; Annalisa *et al*. 2005; Keskin and Kunter 2010) [9, 1, 13]. Accumulation of resveratrol and RS

gene expression in response to stresses and hormones in peanut plant tissues in a glasshouse and in the experimental field has been reported by Ill-Min Chung *et al.* 2003. Production and secretion of resveratrol in hairy root cultures of peanut have been reported by Fabricio *et al*. 2007 [6] and Jong Se Kim *et al*. 2008 [11].

Effect of elicitor and precursor on the accumulation of resveratrol

Effect of elicitor and precursor on the accumulation of resveratrol are presented in table-1 and table-2. Contemplate to control, both the elicitor and precursor increased the accumulation of resveratrol in the cell suspension cultures of *A. hypogea*. Figure 1 shows the HPLC chromatograms of the standard resveratrol. Peaks were identified as resveratrol with retention time of 21.12 min by comparison with the external resveratrol standard. A several-fold increase in resveratrol was obtained in salicylic acid elicited cell cultures during day 3 to day 9. Further, from day 9, accretion of resveratrol was observed to decrease till day 15. An increase in 12.38-fold (74.45±5.62 mg^{-g}) of resveratrol was observed in 250 µM salicylic acid elicited cells on day 9 (Fig. 2).

The precursors for resveratrol are *p*-coumaroyl-CoA and malonyl CoA. The former is synthesized from phenylalanine by shikimate pathway. Later by the condensation of *p*-coumaroyl-CoA and malonyl CoA resveratrol is synthesized (George *et al.,* 2000) [8]. During the present study phenylalanine and malonyl CoA were used as precursor molecule for the production of resveratrol. Precursor effect on the influence of resveratrol production was rather offbeat to that of salicylic acid in the present study. It increased the resveratrol production from day 3 to day 12 at all the concentrations studied (Fig.3). The highest concentration of resveratrol was obtained (127.86±7.62mg g^{-1}) on day 12 at 250 µM l^{-1} with a 21.30 fold increase. Further wherein there was a drastic downturn in the accumulation of resveratrol till day 15, but the total concentration did not fall below the levels of elicitor treated cultures. The concentration of resveratrol on day 15 at 250 µM l^{-1} was found to be 38.54±7.51. In the present investigation, precursor proved to be very efficient compared to elicitor in increasing the resveratrol production. Dwindle in the resveratrol production was observed with the prolonged treatment of elicitor and precursor. This could be because of loss of cell viability (Stefania *et al.* 2002; Walker *et al.* 2002; Sonja *et al.* 2007) [22, 23, 21] in cultures as confirmed by staining of the cells for viability**.**

Table 1: Effect of Salicylic acid on the production of resveratrol[α] in suspension cultures of *A. hypogea*.

Salicylic acid (concentration in μM^{-lt})						
Days	**C**	**50**	**100**	**150**	**200**	**250**
03	2.05±0.02	16.53±0.35	23.67±0.12	35.45±0.31	42.78±0.14	54.18±0.23
06	2.74±0.09	25.76±0.42	32.56±0.32	45..93±3.02	56.67±4.12	62.61±4.16
09	5.26±0.21	31.52±0.12	42.79±1.42	50.01±4.32	62.35±5.43	74.45±5.62
12	6.01±0.14	28.73±0.21	34.78±0.52	38.84±0.15	43.87±0.28	46.41±0.14
15	4.03±0.01	18.96±0.23	21.07±0.12	24.04±0.46	28.04±0.46	32.46±0.46

*Data are expressed as an average of at least three separate experiments.

Table 2: Effect of Precursor on the production of resveratrol* in suspension cultures of *A. hypogea*.

Precursor(concentration in μM^{-lt})						
Days	**C**	**50**	**100**	**150**	**200**	**250**
03	2.05±0.02	8.52±0.23	10.52±0.41	13.75±0.82	18.42±1.32	22.42±1.32
06	2.74±0.09	16.67±0.21	19.53±0.32	32.79±0.65	34.80±5.37	34.98±5.23
09	5.26±0.21	33.46±0.12	48.51±0.78	96.92±3.57	98.52±6.13	112.42±6.12
12	6.01±0.14	46.32±0.42	56.23±3.65	106.86±5.23	107.91±7.02	127.86±7.62
15	4.03±0.01	36.47±0.72	42.63±2.41	36.09±6.51	36.72±7.06	38.54±7.51

*Data are expressed as an average of at least three separate experiments.

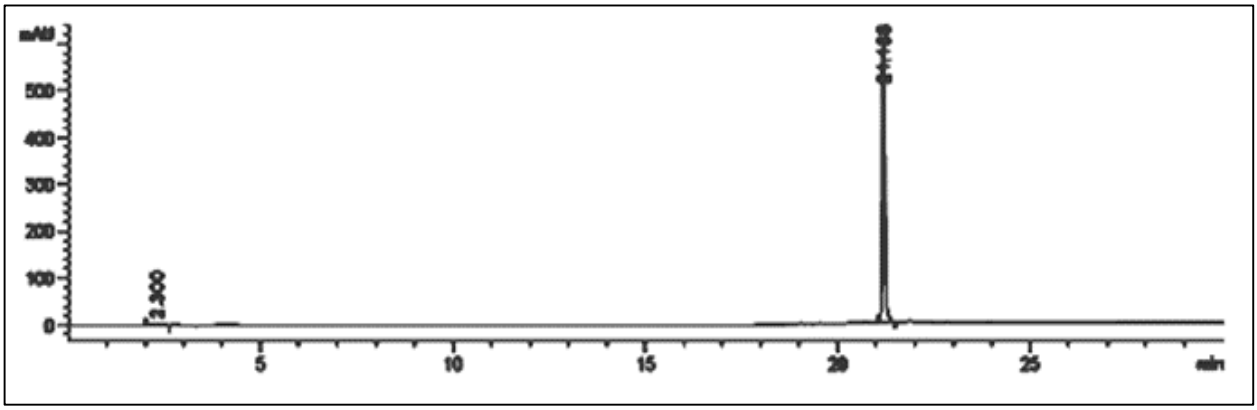

Figure 1: HPLC chromatogram of the standard resveratrol

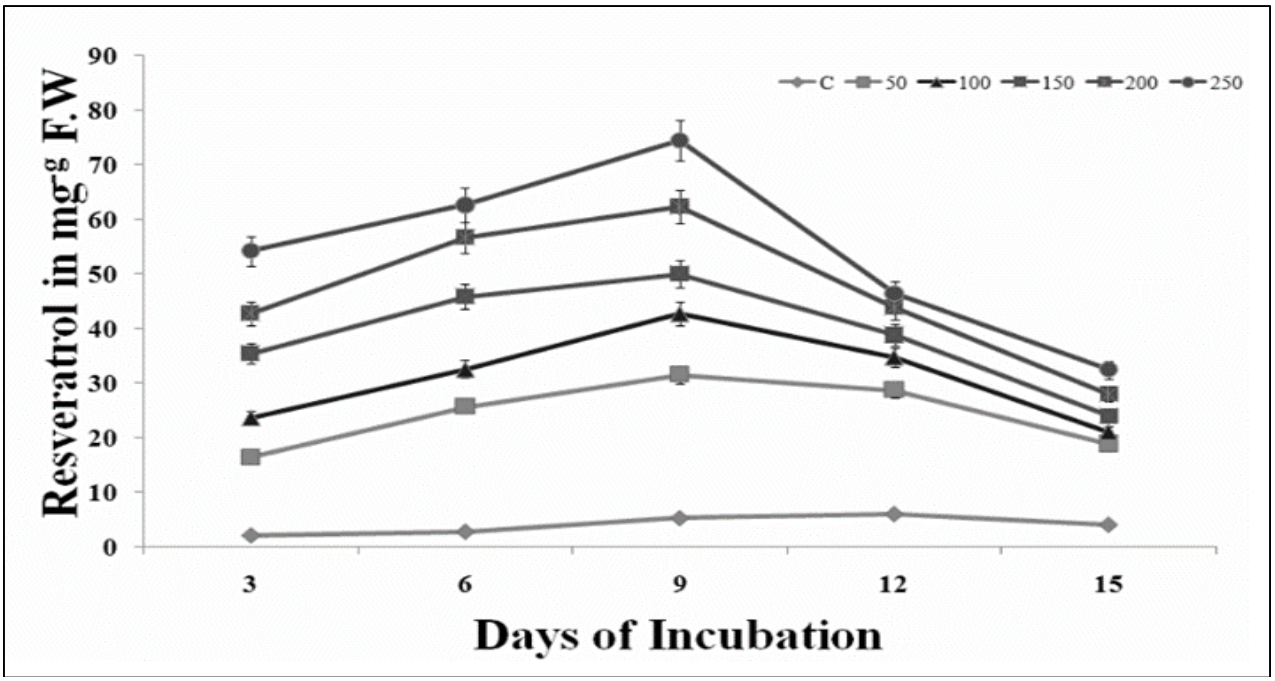

Fig 2: Effect of elicitor on the accumulation of resveratrol

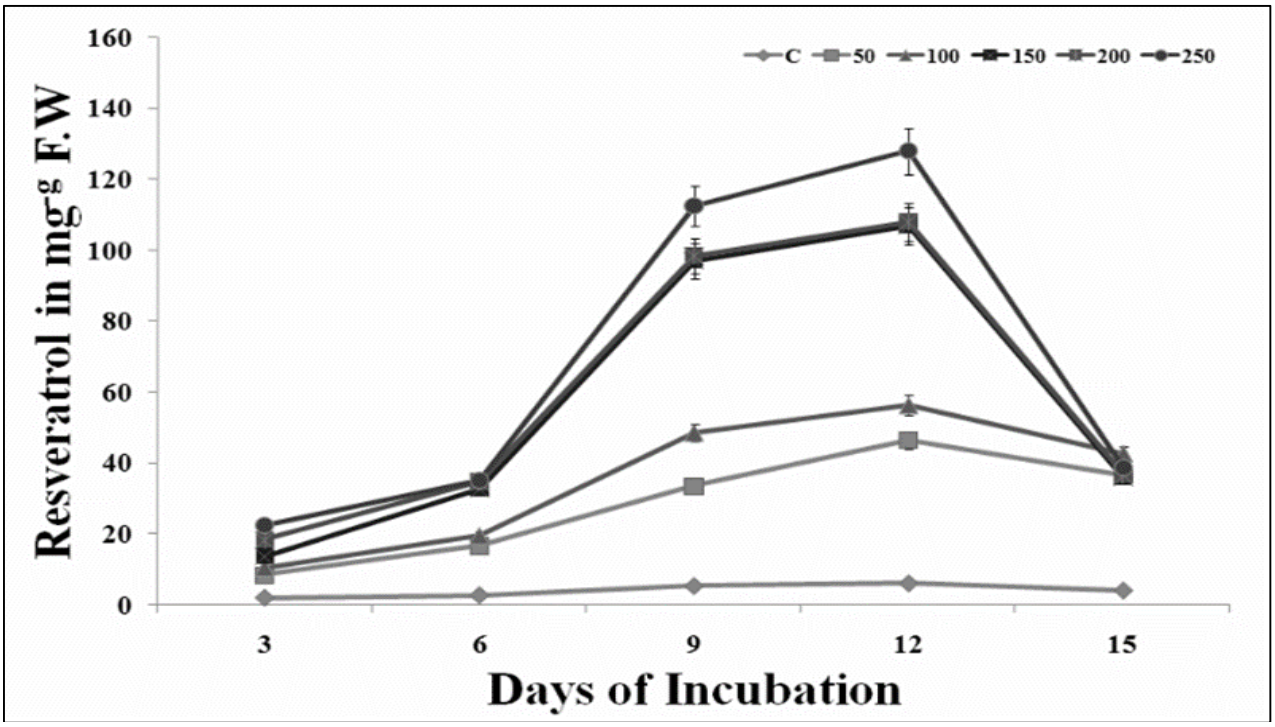

Fig 3: Effect of precursor on the accumulation of resveratrol

Effect of elicitor and precursor on cell growth

The elicitor salicylic acid in the current study at all the concentrations asserted a significant raise in cell growth in the beginning. Although there was substantial increase in cell growth observed in the beginning i.e. from day 0 to day 3 and further up to day 6, additional elicitation till day 9 decreased the cell growth and resulted in moderate browning of the cells. A decrease in growth from day 9 to day 15 was observed at all concentrations (Fig. 4). However, the results are not the same with the precursor fed cell cultures, where in there was gradual increase in the cell growth from day 3 to day 12 further treatment decreased the cell growth till day 15 (Fig. 5). Elicitation of plant cell cultures may lead to a defense response by switching away from primary metabolism to secondary metabolite production resulting in the cessation of growth temporarily or permanently (Leon *et al.* 2001) [14] and the results of the present study are in accord with the remark that there was cessation in the cell growth after day 9 in the cultures treated with salicylic acid and after day 12 in the cultures treated with precursor to day 15.

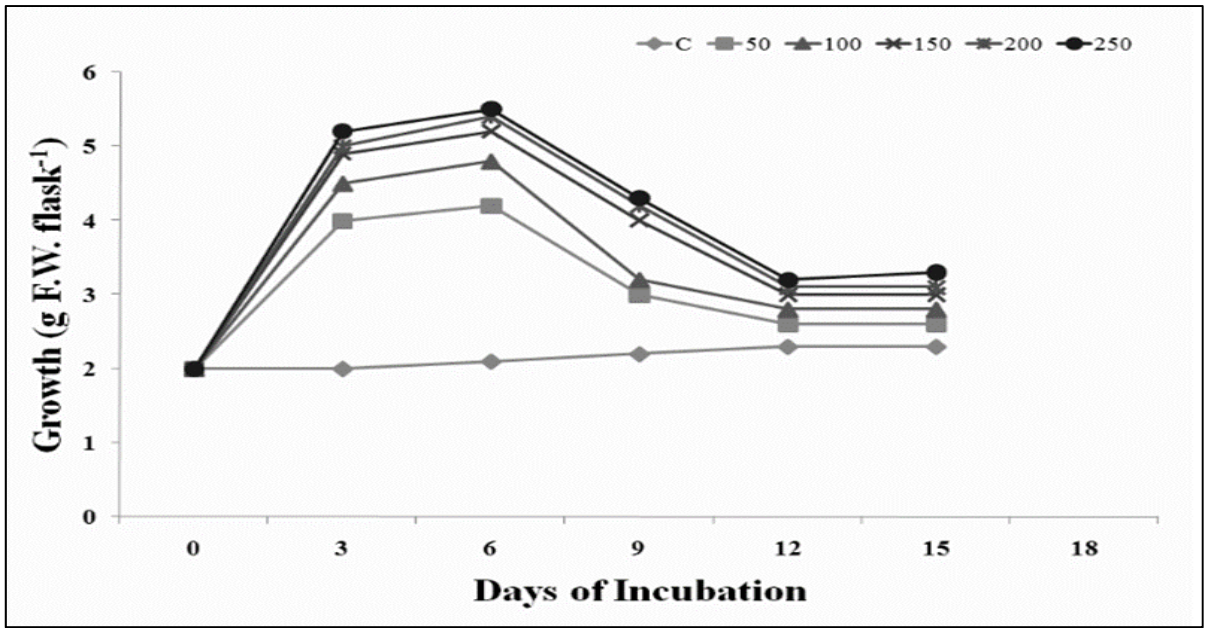

Fig 4: Effect of Salicylic acid on cell growth

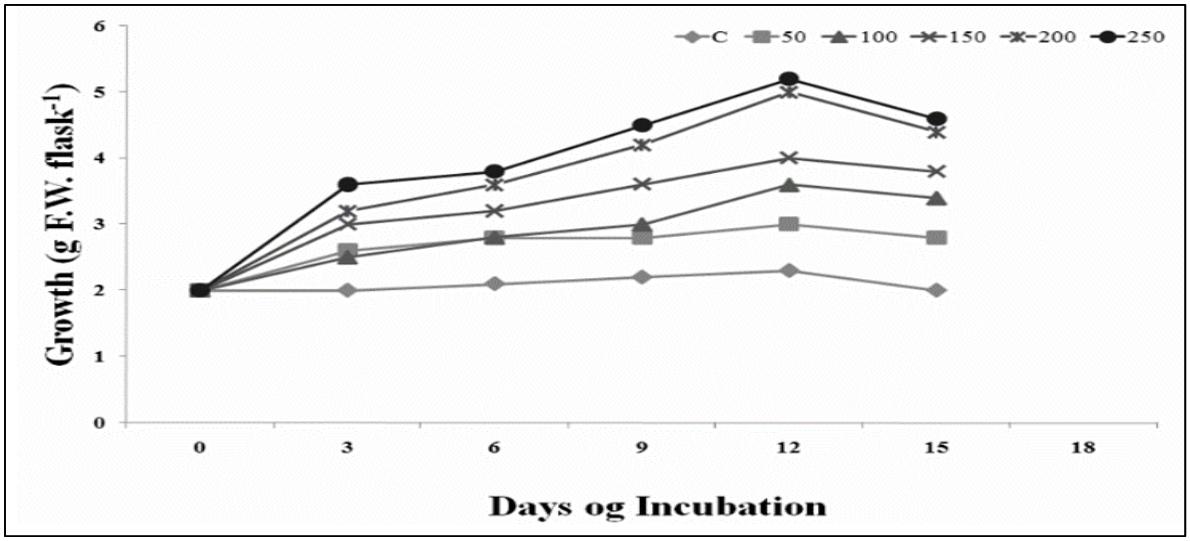

Fig 5: Effect of Precursor on cell growth

Effect of elicitor and precursor on cell viability

The results of the present study show that long-term treatment with elicitor and precursor caused cell browning and had a significant effect on cell viability. From day 3 to day 6, elicitation by salicylic acid, no significant variations in cell viability (80–90%) was observed; thereafter it was approximately 75%, which further progressively decreased by day 15. Decrease in resveratrol accumulation in the elicitor-treated cell cultures was noticed after day 9. In variance to elicitor, the precursor fed cell cultures the similar impact was till day 12 ie, (80–90%), after which the cell viability decreased gradually till day 15. Yet the production of resveratrol in both elicitor and precursor treated cultures did not fall below the control levels (6.01±0.14).

Conclusion

In the present research, it was very clear that precursor supply has been appraised to be the best option for resveratrol production that yielded the utmost quantity of resveratrol then the elicitor. Resveratrol yield was found to be reliant on concentration of both precursor and elicitor. The low yield might be attributed to the loss of cell viability in presence of higher concentrations and also even at low concentrations because of exposure for the longer durations (15 days).

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