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Differential effects of elicitor and precursor feeding on the enzyme tyrosine hydroxylase and their influence on the production of L- Dopa in the cell suspension cultures of *Mucuna prurita* H.

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Abstract
L-dopa (3, 4-dihydroxy L-phenylalanine) is a neurotransmitter precursor being used for symptomatic relief of Parkinson's disease. It is one of the most highly active allele chemicals present in *Mucuna* species. L-Dopa is produced via the oxidation of tyrosine by the copper containing enzyme tyrosine hydroxylase. The effect of elicitors and precursor molecules on the activity of tyrosine hydroxylase and their effect on the production of L-Dopa in cell suspension cultures of *Mucuna prurita* Hook were studied. Callus cultures were initiated on MS medium supplemented with BAP and IAA. The callus was transferred to MS liquid medium supplemented with BAP, IAA and elicitors methyl jasmonate, chitin, pectin, yeast extract and also a precursor L-tyrosine at different concentrations. Compared to the control, several fold increase in the activity of tyrosine hydroxylase was observed. With the progressive increase in the activity of tyrosine hydroxylase also, several fold increase in L-Dopa production was observed in the elicited and precursor fed cell suspension cultures. Compared to elicitor treatments, precursor feeding had more impact on L-Dopa production and on the activity of tyrosine hydroxylase.

Keywords: *Mucuna prurita*, L-Dopa, Tyrosine hydroxylase, elicitor, precursor, callus, suspension culture

Introduction
Plant cell cultures not only have a higher rate of metabolism than differentiated plants, but also have the biosynthetic cycles compressed into shorter time periods (Zenk 1991) [36]. Plant cell and organ cultures grown *in vitro* usually exhibit changes in physiological and bio- chemical responses upon exposure to biotic and abiotic elicitors (Sircar and Mitra 2008) [28]. In plant cell culture, the addition of precursors and elicitors plays an important role in enhancing the secondary metabolism of cells (Dornenburg and Knorr 1995) [7]. Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plant cells to ensure their survival, persistence and competitiveness (Moumita *et al*. 2009) [14]. Elicitation of *in vitro* cultures is a useful approach to enhance and extend production of desirable products (Oksman-Caldentey and Inze´ 2004) [19] within a shorter production times (Discosmo and Misawa 1985) [6]. Both plant defense mechanism and metabolite production are interrelated via secondary metabolism (Sahai and Shuler 1984) [26]. Exogenous supply of a biosynthetic precursor to culture medium may also increase the yield of the desired product. The concept is based on the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases (Moreno *et al*. 1993; Whitmer *et al*. 1998; Silvestrini *et al.* 2002) [13, 34, 27].
The genus *Mucuna* is a well known medicinal plant, but the studies of its pharmacological properties and the corresponding compounds still continues. The importance of *Mucuna* as a medicinal plant is mainly due to the presence of L- Dopa. L-dopa (3, 4-dihydroxy L-phenylalanine) is a neurotransmitter precursor being used for symptomatic relief of Parkinson's disease. It is produced by the oxidation of tyrosine by the enzyme tyrosine hydroxylase. Once formed L-dopa can be converted into several neurologically important catecholamines such as neurotransmitter dopamine and the important hormones adrenaline and noradrenaline (Riley 1997) [24]. Few studies have been carried out on the accumulation of L-Dopa in the cell cultures of *Mucuna*, in *Mucuna pruriens* (Brain 1976; Wichers *et al*. 1993) [4, 35], *Mucuna hassjoo*, *M. Pruriens*, and *M. deeringiana* (Teramoto and Komamine 1988) [31].

This study investigates the effects of elicitors and precursor feeding on the activity of the biosynthetic enzyme in the production of L- Dopa, tyrosine hydroxylase and also production of L- Dopa in cell suspension cultures of *Mucuna prurita*, the synonymous member of *Mucuna pruriens*.

Materials and Methods

Callus induction and suspension cell cultures from M. prurita

The seeds of *Mucuna prurita* were collected from wild environment from the forests of Western Ghats, Shimoga District, Karnataka India. The seeds were surface sterilized with 1% mercuric chloride for 5 min, followed by washing with sterile distilled water for 5-6 times to remove traces of surface sterilant and germinated on basel MS (Murashige & Skoog 1962) [15] medium. The plant was identified by senior taxonomist Prof S B Kamalakar, Department of Botany, Sahyadri Science College, Shivamogga. The plant such grown were used as explants source.

Callus was induced from *in vitro* leaf tissue explants cultured on MS medium supplemented with sucrose 3% (w/v), IAA (11.41 µM) and BAP (0.88 µM) (w/v) and solidified with 0.8% agar-agar, pH was adjusted to 5.8 prior to autoclaving at 121 ºC for 20 min. The culture tubes were incubated at 16 h photoperiod provided by cool white fluorescent lamps (25 µmol m^{-2} s^{-1}) at 25 ºC and sub-cultured every 2nd week. Callus (2 g cell fresh weight (FW)/100 ml medium) was transferred into 20 ml MS liquid medium in Erlenmeyer flasks, supplemented with IAA (11.41 µM) and BAP (0.88 µM) and 3% sucrose for proliferation. Suspensions were established by shaking the cultures on a rotary shaker (REMI, India) at 100 rpm at 22 ºC, under a 16 h photoperiod provided by cool white fluorescent lamps (25 µmol m^{-2} s^{-1}).

Preparation and treatment of elicitors and precursor to cell cultures

Methyl jasmonate (MJ) (50, 100, 150, 200 and 250 µM), pectin and Yeast extract (YE) (50, 100, 150, 200 and 250 mg l^{-1}) was dissolved in sterile distilled water, chitin (50, 100, 150, 200 and 250 mg l^{-1}) was solubilized in 1 or 2 drops of 50 % H_2SO_4, L-tyrosine (50, 100, 150, 200 and 250 mM) was prepared by dissolving initially with 1 or 2 drops of 1 N NaOH and later both chitin and tyrosine 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.

Measurement of cell growth

The cell growth was monitored at every 3rd day (up to 15 days) by determining the fresh weight. Briefly the cells were harvested on every 3rd day to collect cells by vacuum filtration, weighted for growth analysis 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. All data are expressed as an average of three separate experiments.

Extraction and quantification of L-Dopa

The suspended cells were separated from the medium by vacuum filtration. The separated cells were washed with sterile distilled water to remove any adhering medium to the cell surface to record the fresh weight. Extraction of L- Dopa was done as described by Myhrman (2002) [16] with appropriate modifications. Approximately 1 g of cells was taken and crushed using pestle and mortar by adding 10 ml of distilled water. The extract was boiled for 10 min and cooled and centrifuged for about 10 min at 5000 rpm. The supernatant was collected, boiled and cooled. Again the supernatant was centrifuged and the resulting supernatant was collected and was used for the estimation.

L-Dopa content in the extract was estimated by HPLC analysis using Waters, model 2487, pump 1515 with 2487 dual absorbance UV detection and 2414 RI detectors, manual sampler injector using C-18 column and a guard pre-column was packed with material, as in the main column. Isocratic elution was carried out using water : methanol : phsosphoric acid [975.5:19.5:1 (v/v)] as described by Perumal Siddhuraju and Klaus Becker (2001) [21]. Separation was performed at room temperature (25 ºC) and after the injection of 20 µl, the column was operated with the flow rate of 1.2 ml^{-1}. Absorbance was monitored at 282 nm and peak heights and areas were determined. Sample was eluted between 3rd and 4th min. The amount of L-Dopa present in the tissue extracts was calculated with the help of standard curve for L- Dopa (Sigma, St. Louis, MO., USA). Amount of L- Dopa present in tissue extract was expressed as mg g^{-1} tissue dry weight. All the samples were passed through a glass-filter (0.20 µm pore size, Millipore, USA) and analyzed by HPLC for quantitative determination of L-Dopa.

Tyrosine hydroxylase activity (Tyrosinase)

Tyrosine hydroxylase activity was measured spectrophotometrically as described by Tigist *et al.* (2001) [32] using L-tyrosine as the substrate at 20 ºC with modifications, in presence of 5 mM L-ascorbic acid to inhibit the polyphenol oxidase activity. The reaction mixture contained 2.95 ml of 10 mM L-tyrosine solution in 0.05 M Tris-HCl buffer (pH 8.5) and 0.05 ml of the enzyme extract. To distinguish between a phenoloxidase and the possibility of peroxidase activity responsible for hydroxylation of L-tyrosine, the tyrosine hydroxylase activity was performed in the presence of 6500 U catalase (Sigma, Sto. Louis, MO., USA). Absorbance was recorded at 475 nm and the enzyme activities were calculated using the standard curve for L-dopa (Sigma, St. Louis, MO., USA). One unit of tyrosine hydroxylase is that amount which catalyzes the formation of 1 µmol of product per min under the assay conditions. Specific activity is expressed as unit's mg^{-1} protein.

Statistical analysis

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

Results and Discussion

Influence of elicitors and precursor on cell growth

The elicitation with all the elicitors used in the present study decreased the cell growth. In MJ, pectin, and YE there was slight increase in the cell growth from day '3' to day '6' and further moderate browning of the cells as well as decrease in growth was observed at day '9' at all the concentrations of these elicitors, except for chitin. The similar effect was observed from the day '6' itself (Figure 1-4). Usually, when elicitors are added to the *in vitro* cultures of plants they cease to grow temporarily or permanently, which may also lead to a defense response by switching to primary metabolism (Leon *et al*. 2001). Similar to this in our study also there was

cessation in the cell growth after day '9' till day '15'. The result was not the same with the precursor, L- tyrosine molecule. There was gradual increase in the cell growth from day '3' to day '15' (Figure 5).

Influence of elicitors and precursor on cell viability

The results from the present study showed that long term treatment with elicitors caused brown color and had significant effect on cell viability. Cell viability was determined by vital staining with methylene blue stain as described by Ravishankar *et al.* (1998) [23]. From day '3' to day '6' for all the elicitors except for chitin (day '6'), we did not observe significant variations in cell viability (80–90%). After which, the viability was around 75% which further progressively decreased on day '15'. However, cell viability was not much lower in YE treated cells than compared to MJ, Chitin and Pectin. Fresh weight of elicited cells decreased during the time of culture. This was not the case in tyrosine treated cell cultures; from day '3' to day '15' we did not observe any loss of cell viability.

Efficacy of elicitors and precursor on the enzyme tyrosine hydroxylase (TH) and accumulation of L- Dopa

Elicitor activity was assessed according to the TH activity and accumulation of L-Dopa in the cell suspension cultures of *M. prurita* from day '3' to day '15'. Increased levels of several folds in TH activity and L-Dopa content during day '3' to day '9', except chitin (day '6') was observed. However after this, decrease levels were observed till day '15' at all the concentrations. In MJ treated cells the TH activity and concentration of L-Dopa rose significantly but more notable results was observed in cell cultures elicited with pectin and YE. L-Dopa is produced via the oxidation of tyrosine by the copper containing enzyme tyrosine hydroxylase in the presence of molecular O_2 (Pattison *et al*. 2002) [20]. Hence in the present study L-tyrosine, supplementation in the medium was used as precursor to study its influence on the production of L-Dopa.

Effect of MJ on TH and accumulation of L- Dopa

MJ was found to be an important elicitor to increase the TH activity and L- Dopa production in cell cultures effectively. The result of the enzyme activity after the addition of MJ into the culture is shown in Figure 1 and contents of L- Dopa in Table 1. The enzyme activity of TH began to increase rapidly on day '3' and reached a maximum on day '9', then there after the activity started to decrease to near the level before the elicitation on day '15' (Figure 1) at all the concentration of this elicitor. The highest activity at 200 µM MJ on day '9' was 840.52±1.86. The contents of L- Dopa changed similarly after the addition of MJ, a gradual increase in the content from day '3' to day '9' after which there was decrease in its concentration. A 13.5 fold (31.9±0.14 mg g^{-1}) increase in L-dopa concentration was obtained in 200 µM MJ elicited cells on day '9'.

Table 1: Effect of Methyl jasmonate (µmole l^{-1}) on the production of L- Dopa[α] in suspension cultures of *M. prurita*

Days	Control	Concentration of Methyl jasmonate in µmole l^{-1}			
		50	100	150	200
03	1.92±0.04	2.62±0.02	3.01±0.03	4.03±0.02	21.01±0.16
06	2.18±0.11	3.03±0.02	3.03±0.02	4.02±0.96	25.92±0.23
09	2.32±0.12	5.93±0.42	7.89±0.07	12.12±0.02	31.9±0.14
12	2.35±0.10	3.05±0.02	3.02±0.02	7.94±0.07	8.93±0.13
15	2.36±0.12	2.62±0.03	2.62±0.02	2.62±0.01	2.62±0.03

[α] Data are expressed as an average of at least three separate experiments.

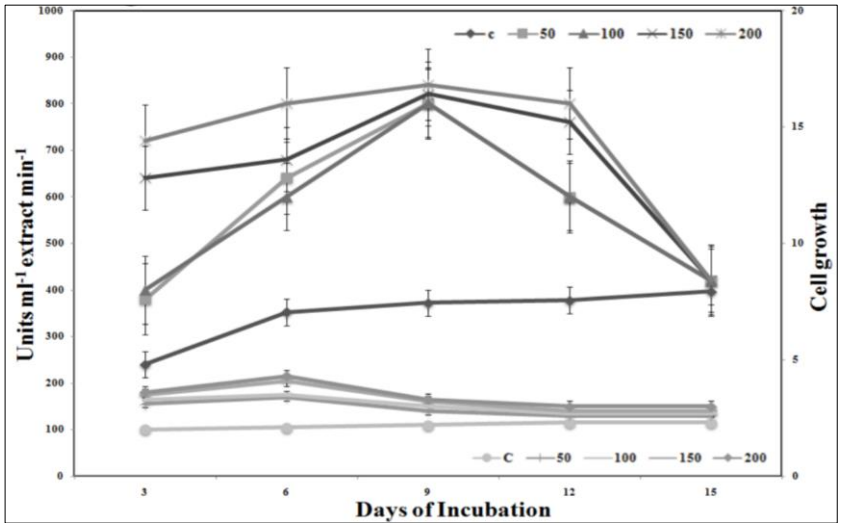

Fig 1: Effect of methyl jasmonate on the enzyme tyrosine hydroxylase and cell growth in *M. prurita*

Effect of chitin on TH and accumulation of L- Dopa

Chitin was the second elicitor in our study. The result of the enzyme activity after its addition is shown in Figure 2 and contents of L- Dopa in Table 2. After the addition of chitin the activity of TH increased but the extent increase was not as much as the other three elicitors, and also here the maximum activity of the enzyme was on day '6' (840.92±3.02) after which the activity started to decrease till day '15'(Figure 2) at all the concentrations. Chitin did not have much impact on the L-Dopa accumulation. Nevertheless', L-Dopa accumulation increased marginally under the influence of this elicitor (Table 2) with a fold increase of 5.34. The highest concentration of L-Dopa was obtained on day '6' (12.62±0.12) in the cultures treated with 200 mg l^{-1}.

Table 2: Effect of Chitin (mg l^{-1}) on the production of L- Dopa$^{\alpha}$ in suspension cultures of *M. prurita*

Days	Control	Concentration of Chitin in mg l^{-1}			
		50	100	150	200
03	1.92±0.04	4.33±0.01	5.98±0.03	9.63±0.10	10.02±0.09
06	2.18±0.11	7.84±0.07	9.13±0.04	10.02±0.09	12.62±0.01
09	2.32±0.12	3.13±0.02	3.43±0.02	6.42±0.02	7.94±0.17
12	2.35±0.10	3.21±0.01	3.42±0.01	4.56±0.02	5.09±0.12
15	2.36±0.12	2.54±0.06	2.41±0.03	2.41±0.03	2.45±0.32

$^{\alpha}$ Data are expressed as an average of at least three separate experiments.

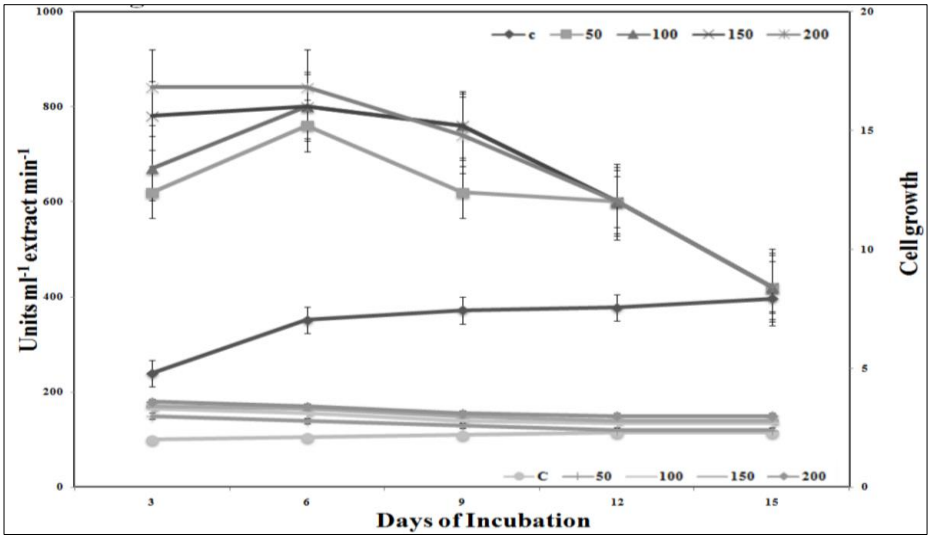

Fig 2: Effect of chitin on the enzyme tyrosine hydroxylase and cell growth in *M. prurita*

Effect of pectin on TH and accumulation of L- Dopa

The effect of pectin elicitation on the enzyme TH and on the L- Dopa content is shown in the Figure 3 and Table 3 respectively. After the addition of pectin the enzyme activity and the L- Dopa concentration increased as found in MJ till day '9' (1120.74±5.36), after which decreased activity of TH and also L- Dopa accumulation was observed till day '15'. But the extent increase was not as much as the MJ elicited cells. The pectin treated cultures on day '9' induced the accumulation of 40.43±0.12 mg g^{-1} of L-Dopa with 17.3 fold increase at 200 mg l^{-1} (Table 3).

Table 3: Effect of Pectin (mg l^{-1}) on the production of L- Dopa$^{\alpha}$ in suspension cultures of *M. prurita*

Days	Control	Concentration of Pectin in mg l^{-1}			
		50	100	150	200
03	1.92±0.04	10.52±0.03	14.53±0.12	19.87±0.13	21.19±0.14
06	2.18±0.11	14.93±0.32	21.29±0.15	24.16±0.23	34.35±0.41
09	2.32±0.12	16.43±0.25	23.57±0.42	31.74±0.42	40.43±0.12
12	2.35±0.10	2.74±0.13	2.36±0.02	3.21±0.17	3.21±0.13
15	2.36±0.12	2.54±0.60	2.54±0.06	2.54±0.06	2.54±0.06

$^{\alpha}$ Data are expressed as an average of at least three separate experiments.

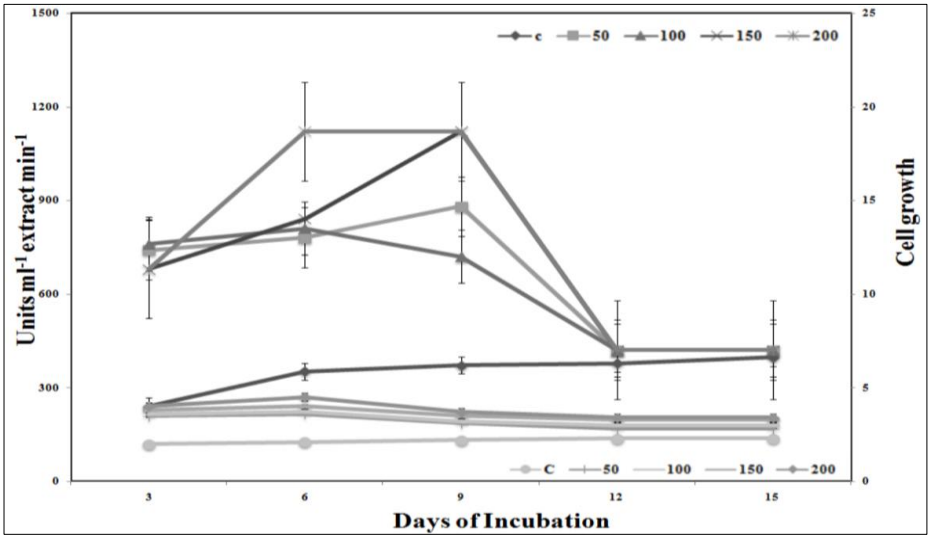

Fig 3: Effect of pectin on the enzyme tyrosine hydroxylase and cell growth in *M. prurita*

Effect of YE on TH and accumulation of L- Dopa

YE was added as an elicitor to improve L- Dopa production. The enzyme activity and L- Dopa content change was similar to that of MJ and pectin treated cell cultures i.e. increase in the activity and Dopa content from day '3' today '9'(1520.79±6.14) after which there was decrease in both till day '15' at all the concentrations studied (Figure 4, Table 4). However, the extent decrease was observed to be not as that of with the other elicitor, as the lowest concentration of L-Dopa obtained with highest concentration of YE treatment is 9.073±0.16 mg g^{-1}. The highest concentration of L-Dopa was obtained (60.45 ±5.13 mg g^{-1}) on day '9' in 200 mg l^{-1}. In the present study YE was found to be better elicitor compared to the other three elicitors with a 25.6 fold increase in L- Dopa.

Table 4: Effect of Yeast extract (mg l^{-1}) on the production of L- Dopa$^{\alpha}$ in suspension cultures of *M. prurita*

Days	Control	Concentration of Yeast extract in mg l^{-1}			
		50	100	150	200
03	1.92±0.04	14.73±0.12	20.58±0.32	32.72±0.53	40.69±1.32
06	2.18±0.11	23.37±0.23	30.76±0.12	42.58±2.34	54.52±4.21
09	2.32±0.12	29.65±0.18	35.45±0.31	50.61±4.12	60.45±5.13
12	2.35±0.10	12.52±0.21	16.72±0.41	23.52±0.21	30.57±0.21
15	2.36±0.12	7.46±0.18	8.52±0.37	8.76±0.25	9.73±0.16

$^{\alpha}$ Data are expressed as an average of at least three separate experiments.

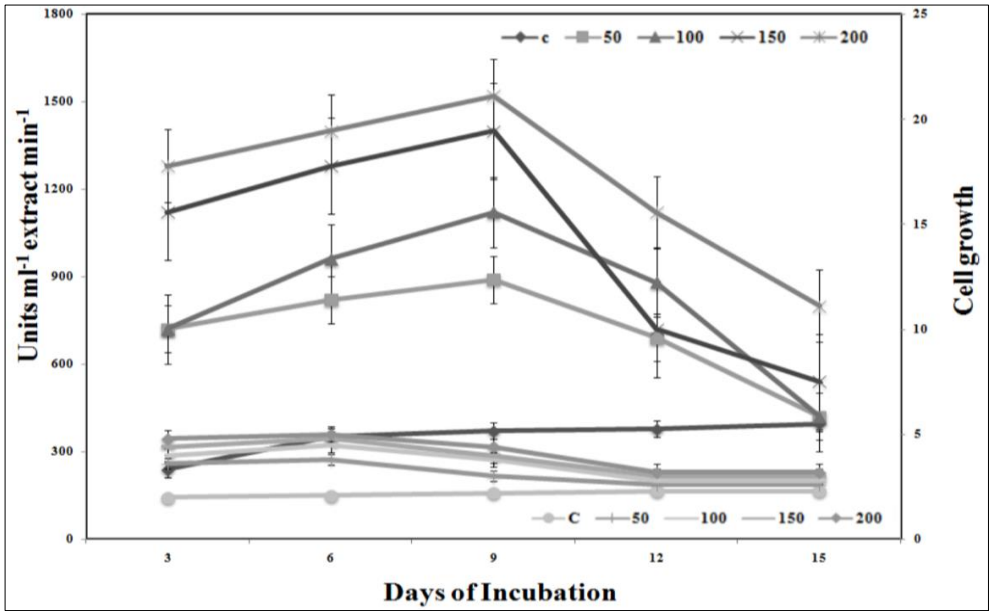

Fig 4: Effect of yeast extract on the enzyme tyrosine hydroxylase and cell growth in *M. prurita*

Effect of precursor on TH and accumulation of L- Dopa

The influence of precursor was rather different than that of the four elicitors used in the present study on the activity of TH and on the accumulation of L-Dopa (Figure 5, Table 5). There was constitutive increase in the TH activity and L-Dopa production from day '3' to day '15' at all the concentrations of tyrosine used. The highest concentration of L-Dopa was obtained (90.54±7.05 mg g^{-1}) with an increase of 36.36 fold on day '15' in 200 mg l^{-1} L-tyrosine treated cultures. There was no decrease in the TH activity (1590.54±7.34) and L-Dopa content throughout our study even after day '9' as was in the case of elicitors till day '15'. No loss in cell growth and cell viability was observed. Similar observations were also been reported by Whitmer *et al*. (1998) [34] and Ashish and Dixit (2008) [3].

Table 5: Effect of Tyrosine (mM l^{-1}) on the production of L- Dopa$^{\alpha}$ in suspension cultures of *M. prurita*

Days	Control	Concentration of L- Tyrosine in mM l^{-1}			
		50	100	150	200
03	1.92±0.04	10.52±0.23	11.52±0.41	24.86±0.02	35.46±0.42
06	2.18±0.11	15.67±0.21	20.53±0.32	32.79±0.65	45.23±3.65
09	2.32±0.12	18.92±0.04	24.32±0.12	42.92±3.57	62.86±5.23
12	2.35±0.10	24.32±0.12	42.63±2.32	54.52±4.21	85.78±7.06
15	2.36±0.12	30.47±0.72	45.26±3.45	62.96±5.43	90.54±7.05

$^{\alpha}$ Data are expressed as an average of at least three separate experiments.

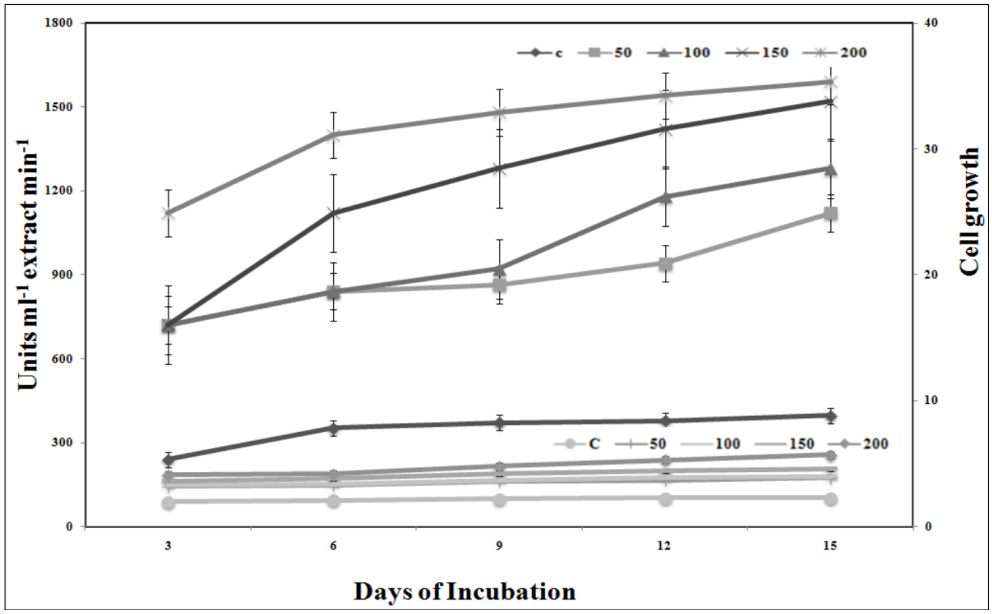

Fig 5: Effect of tyrosine on the enzyme tyrosine hydroxylase and cell growth in *M. prurita*

Accumulation of various classes of secondary metabolite molecules in cell cultures of legumes by YE and MJ have been described (Suzuki *et al*. 2002, 2005; Achnine *et al*. 2005; Broeckling *et al*. 2005; Farag *et al*. 2007; Naoumkina *et al*. 2007) [29, 30, 1, 5, 9, 17]. Pectin has been reported to be as an effective elicitor that has increased significantly menthol in *Mentha piperita* cell cultures (Amrita and Sharmila 2008) [2], similarly chitin has also been used as a stress molecule in the study of its effect on *Citrus aurantium* (Maria *et al*. 2007) [12]. Even though accumulation of L-Dopa was found to increase in the initial treatment with elicitors, long term treatment decreased the accumulation of L-Dopa and also loss in cell viability in the cell cultures. This may be due to cell dediferentiation process that often leads to a lower productivity (Verpoorte *et al*. 2002) [33]. Similar results have also been reported by Moreno *et al*. (1993) [13], Negeral and Javelle (1995) [18] and Rijhwani and Shanks (1988) [25]. Attempt to increase the production of desired molecules by supplementing precursors in the medium have been effectively studied in case of *Solanum lyratum* and *Cistanche salsa* (Lee *et al*. 2007; Liu *et al*. 2007) [10-11]. Production of anthocyanin and phenylethanoid glycosides was enhanced by feeding L-phenyl alanine and related precursors to the cell cultures of strawberry and *Cistanche deserticola* respectively (Edahiro *et al*. 2005; Quyang *et al*. 2005) [8, 22].

In plant cell cultures which produce the desired compound(s), a number of approaches have been adopted to increase yield, selection of high-producing lines, culture medium composition, and treatment with elicitors. Another important aspect concerns the fact that secondary metabolism represents one facet of plant differentiation; consequently, morphologically undifferentiated cultures of many species fail to produce secondary metabolites. Results of the present work indicate that, treatments with elicitors for long period caused brown coloration of cell cultures and decrease in cell growth, had slight effect on cell viability and even on the TH activity and the production of L-Dopa. Of the precursor tyrosine and all the four elicitors studied, tyrosine has a greater impact on the TH activity and L- Dopa production. Hence the results suggest that precursor feeding have more advantage compared to elicitors and could be applied successfully to large-scale production of L-Dopa.

As to date no reports are available on the enhancement of L-Dopa by elicitors and precursor feeding in the genus *Mucuna prurita*. This study represents the first successful cell culture based approach for the production of L-Dopa.

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