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# Production of hyoscyamine and scopolamine using stress in *Datura metel* L.

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## ABSTRACT

Highest biomass in terms of Fresh Weight (FW) and Dry Weight (DW) obtained on 2, 4-D (1 mg/l) for all studied medium, and for higher alkaloids production i.e. for hyoscyamine and scopolamine we found that 2, 4-D, BAP (0.5, 0.5 mg/l) was the best medium. So this medium was used for manipulation of alkaloids using different stress producing agents (elicitors) and therefore it was used to carry out further experiment. Highest hyoscyamine and scopolamine content was obtained at 0.25mM treatment of Salicylic acid (SA) which was 0.254 mg/g DW and 8.588 mg/g DW respectively, at 36 h. High level of hyoscyamine (0.224 mg/g DW) and scopolamine (8.490 mg/g DW) was obtained at 200 mg/l of YE treatment at 36 h. Highest hyoscyamine and scopolamine content was obtained at pH 5.5 at 12 h and it was 0.217 mg/g DW, 8.333 mg/g DW respectively. 

## KEYWORDS

*Datura metel* L.;
Cell suspension culture;
Salicylic acid;
Yeast extract;
pH.

## INTRODUCTION

The genus *Datura* produces great range of tropane alkaloids, and two of them are hyoscyamine and scopolamine which are important for pharmaceutical industries[1]. World consumption of scopolamine is several folds higher than that of hyoscyamine mainly due to the fact that scopolamine is used as starting material for semi-synthesis of several important drugs[2]. Tropane alkaloids have significant medicinal importance as they are compounds with a variety of pharmacological effects on some human organs such as eyes, nerve system, heart, blood circulation and respiration[3]. Tropane alkaloids inhibit the muscarinergic acetylcholine receptors and show parasympatholytic properties. As such they are used in medicine to treat spasms, to sedate patients and for dilation (mydriasis) of pupils. Furthermore tropane alkaloids affect neuronal activities and are known as hallucinogens[4]. The Tropane alkaloids hyoscyamine[5] and scopolamine are used in medicine for its anticholinergic activity[6]. Scopolamine is the most valuable tropane alkaloid, preferred for its higher physiological activities and fewer side effects.

Plants Cell cultures have been developed as a promising alternative for production of secondary metabolites which is difficult to obtain using other methods such as chemical synthesis or plant extraction[7]. Cell suspension of *D. innoxia* Mill.[8] and *D. stramonium*[3] were investigated for growth and production of tropane alkaloids. Some aspects for manipulation of secondary metabolites were studied by various workers. One of the aspect widely used is making of elicitors, abiotic stress and other approaches[7].

The present investigation aimed to use different stress

producing agent i.e. Elicitors for manipulation of tropane alkaloids in cell suspension cultures of *D. metel* L.

## MATERIALS AND METHODS

### Collection and sterilization of explants

Plant materials for the tissue culture experiments were collected from in and around University of Pune area. For sterilization leaf explants were kept under running water for 30 min. with 2-3 drops of Tween-20 followed by a wash of 70% alcohol (v/v) for 10 sec. Then explants were sterilized with 0.1% $HgCl_2$ (w/v) (Sigma) for 3 min. The explants were then washed three times with sterile distilled water to remove the traces of $HgCl_2$.

### Inoculation of leaf explants

The leaf disc were cut in size of 5 mm and were used as explants for inoculation with abaxial surface in contact with MS medium supplemented with different concentration of 2, 4-D. The different concentrations of 2, 4-D (0.5, 1, 1.5, 2, 4, and 6 mg /l) were used for raising the callus. The MS basal medium was used as control.

### Cell suspension cultures

For cell suspension culture initiation, friable calli obtained on MS + 1mg/l 2, 4-D was used as inoculums. For optimization of biomass cell suspension cultures were raised on different growth regulators of different concentrations such as IAA (0.5, 2 mg/l), NAA (0.5, 2 mg/l), BAP (0.5, 2 mg/l), and 2, 4-D. (0.5, 1, and 2 mg/l). Approximately 2-3 g of callus was used as inoculum in 40 ml medium in 250 ml flask which was kept on shaker at 125 rpm. All the experiments had 3 replicates and each experiment was repeated twice.

### Alkaloid production

The alkaloid content was checked for every combination of PGR. The cell suspension cultures were harvested after every seven days for checking the alkaloid content up to three weeks. The combinations of PGR which showed highest alkaloid content were used for the enhancement process by using elicitors.

### Enhancement of alkaloids

Three elicitors were used for enhancing alkaloids.

### Salicylic acid (SA)

Stock of 10 mM of SA was prepared and the pH was adjusted to 5.8 before autoclaving. Different concentrations of SA (0.1, 0.25, 0.50 mM and 0 mM used as control) were used in this experiment.

### Yeast extract (YE)

Stock of 1.0 g per 10 ml of YE was prepared and the pH was adjusted to 5.8 before autoclaving. Different concentrations of YE (100, 200, 400 mg/l and cultures without YE used as control) were used in this experiment.

### pH variation

Different ranges of pH were used (4.5, 5.5, 6.5 and control (5.8)).

Fourteen day old cultures were used for this experiment. Cells from cell suspension cultures which were treated with SA, and YE were harvested at different time durations i.e. at 24, 36, and 48 h. along with control and for pH treatment cells were harvested at 6, 12, and 36 h. along with control. Fresh weight of cell suspension culture was taken and cells were dried at 50°C to obtain dry weight.

### Extraction of alkaloids

Berkov[9] method was used for extraction of alkaloids with some modifications. Fresh weight of cells from cell suspension culture was taken and dried at 50°C to take its dry weight. Dried sample was powdered and to this sample10 ml of 3% $H_2SO_4$ was added. Extract was sonicated for 10 min at 33 KHz. Extract was heated at 40-45°C for 60 min. Extract was filtered using Whatman Filter Paper No. 1 and filtrate was made alkaline using 20% $NH_4OH$ and pH was adjusted between 9 - 10. To this filtrate dichloromethane was added in separating funnel and mixed well. Organic phase obtained in from separating funnel was collected and kept on water bath at 60°C until dry residue was obtained. Obtained residue was dissolved in methanol and mixed well using vortex mixer. After mixing it was kept overnight before using for further analysis.

### Qualitative methods for Identification of Tropane alkaloids.

Thin layer chromatography (TLC) was used for qualitative identification using solvent system Chloroform

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: Methanol (8:2). The TLC plate was removed from TLC chamber after the solvent had traveled up to 10 cm and sprayed with Dragendroff locating reagents. Orange color represented presence of alkaloids and background changed to cream color. This was compared with Rf of Standard Hyoscyamine and Scopolamine.

**Quantitative method for estimation of tropane alkaloids.**

Quantification of Hyoscyamine and Scopolamine content was done by spectrophotometric analysis. The cell suspensions grown in different media were used to harvest cells and the biomass was assessed in terms of Fresh weight (FW) and Dry weight (DW). The alkaloid extraction was done as described previously. Std. Hyoscyamine and Scopolamine (Sigma) were used at different concentrations at 545nm and 264nm and quantification of experimental samples was carried out using std. graph.

## RESULTS AND DISCUSSION

**Biomass and metabolites production in cell cultures**

For the growth analysis of *D. metel* L., cell suspension cultures different parameters were used such as cell count (number of cells/40ml), Packed Cell Volume (%), Fresh Weight (g), Dry Weight (g). On the basis of production of alkaloids i.e. hyoscyamine and scopolamine we found that the medium C (2, 4-D, BAP 0.5mg, 0.5mg) was the best for enhancement of alkaloids and therefore it was used to carry out further experiments.

**Manipulation of secondary metabolites production using different elicitor treatments-Effect of salicylic acid (SA)**

For SA treatment highest biomass was obtained in terms of FW at 0.50mM treatment which was 8.033 at 24 h, while lowest biomass found for 0.1mM treatment of SA (2.707 g) (TABLE-1). Highest Dry weight was found in control at 24 h. (0.098 g), while lowest Dry weight was obtained in treatment with 0.25mM SA (0.051 g) at 36 h (TABLE-1). For alkaloid production highest hyoscyamine and scopolamine content was obtained at 0.25mM treatment of SA which was 0.254 mg/g DW and 8.588 mg/g DW respectively, at 36 h (Figure 1 and 2).

Balwin[10] reported that methyl jasmonate and salicylic acid are involved in signal transduction and induce the transcription of biosynthetic enzymes involved in the formation of defense compounds in plants and Jaber-Vazdekis et al[11] reported that use of different concentration of SA and analysis time did not affect culture growth compared to the control in *Atropa baetica*, although at 48 h there was a statistical difference between 1.0 and 0.1mM treatments. On the contrary in our experiment we have observed that SA treatment was statistically significant as compared to control, (Figure 1 and 2). This is probably because we have used different concentrations of SA (0.1, 0.25, 0.50 mM) and different durations of cell harvesting (24, 36, and 48 h). In our experiment we have observed that the treatments of SA are statistically significant as compared to control. According to Pitta – Alvarez et al[12] relationship between total scopolamine and hyoscyamine did not vary for SA treated cultures of *Brugmansia candida* compared to control for the different durations of the treatment. He used 0.01, 0.10, and 1.0 mM concentrations of SA and duration of study after treatment was 24, 48, and 72 h. In our study we have used 0.1, 0.25, and 0.50 mM concentrations of SA and harvesting of cells was done at 24, 36, and 48 h (Figure 1 and 2). The difference in our results as compared to Pitta –Alvarez[12]

**TABLE 1 : Effect of SA treatment on growth of *D. metel* L. Cell cultures (FW and DW).**

| No. | SA (mM) | Fresh weight (g) | | | Dry weight (g) | | |
|---|---|---|---|---|---|---|---|
| | | 24 hrs | 36 hrs | 48 hrs | 24 hrs | 36 hrs | 48 hrs |
| 1. | 0.1 | 2.717 ± 0.7 | 3.145 ± 0.9 | 3.066 ± 0.4 | 0.064 ± 0.004 | 0.055 ± 0.002 | 0.070 ± 0.001 |
| 2. | 0.25 | 3.848 ± 1.1 | 6.131 ± 2.1 | 5.642 ± 2.3 | 0.062 ± 0.002 | 0.051 ± 0.002 | 0.074 ± 0.009 |
| 3. | 0.50 | 8.033 ± 0.6 | 7.103 ± 2.0 | 5.938 ± 2.4 | 0.065 ± 0.004 | 0.060 ± 0.003 | 0.076 ± 0.002 |
| 4. | Control | 3.924 ± 0.6 | 4.718 ± 1.6 | 2.996 ± 0.9 | 0.098 ± 0.003 | 0.095 ± 0.010 | 0.095± 0.006 |

*** All results are mean of 3 replicates ± S.D.**

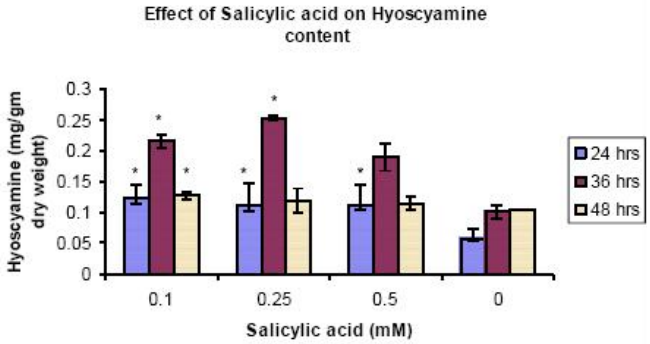


**Figure 1 : Effect of SA on Hyoscyamine Content (mg /g Dry weight) in cell culture of *D. metel* L.**

***Indicates significant increase in Alkaloid production (Hyoscyamine and Scopolamine) as compared to the control on applying T-test with a p value < 0.05.**

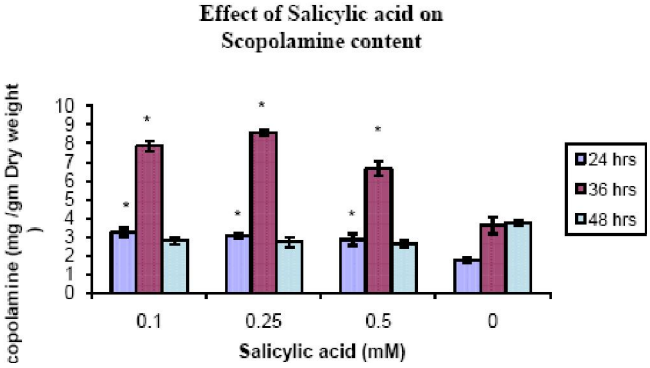


**Figure 2 : Effect of SA on Scopolamine Content (mg/g Dry weight) in cell culture of *D. metel* L.**

***Indicates significant increase in Alkaloid production (Hyoscyamine and Scopolamine) as compared to the control on applying T-test with a p value < 0.05.**

might be due to use of different concentration of SA, and harvesting time. Pitta – Alvarez[12] have also reported that SA probably induces the biosynthetic rout during the first 48 h of treatment, but without altering the alkaloid profile, which has clearly favored scopolamine production in this period of growth. Our results showed agreement with this analysis of Pitta – Alvarez[12]. SA is also reported as on an alternative potent stimulator of secondary metabolites production[13]. The role of SA as part of the signaling cascade leading to systematic acquired resistance has been intensely studied[14]. Kang et al[15] reported that exogenous SA did not have positive effect on hyoscyamine. Treatment with 2.0 mM SA slightly increased hyoscyamine at 0.5 h after elicitation but it was still lower than hyoscyamine production by the control. In comparison, our results showed that the enhanced hyoscyamine production is statistically significant as compared to control at different concentrations of SA (0.1, 0.25, 0.50 mM) and at different duration (24, 36, and 48 h). This could be attributed to the different concentrations of SA and different time of cell harvesting.

SA is shown to increase the expression level of enzyme H6H[15]. H6H is an important enzyme which converts hyoscyamine to scopolamine[16]. In our experiment we also found high concentrations of scopolamine and it may be because of effect of SA on the level of enzyme. Also we have used *Datura metel* L. which already has higher concentration of scopolamine as compared to other scopolamine producing plants[6].

In our experiments at higher concentration of SA (0.50mM) cell growth and the production of alkaloids was reduced. Similar observations have been reported in *Vicia faba* L.[17] and *Atropa belladonna*[18]. It is reported that due to release of alkaloids into medium cell growth probably got hampered because of poisoning effect of alkaloids. This may be one of the reasons for less growth in our cultures.

## Effect of yeast extract (YE)

In YE treatments highest biomass in terms of fresh weight was obtained at 100 mg/l concentration which was 5.236g at 36 h. treatment duration while lowest fresh weight was obtained at 24 hrs and it was 3.376 g in 400 mg/l YE treatment. Highest dry weight (0.067 g) was obtained in 100 mg/l at 48 h. while lowest dry weight was obtained at 200 mg/l of YE concentration at 36 h (TABLE 2). For alkaloid production high level of hyoscyamine (0.224 mg/g DW) and scopolamine (8.490 mg/g DW) was obtained at 200 mg/l of YE treatment at 36 h. (Figure 3 and 4).

Enhanced accumulation of secondary metabolites by YE has received wide acceptance[19, 20]. In our study the effect of YE on the tropane alkaloid production in cell suspension cultures of *D. metel* L. was investigated (TABLE 2, Figure 3 and 4). Maximum tropane alkaloid was obtained at 200 mg/l YE treatment and at 36 h duration for elicitation. To the best of our knowledge in the literature, this is first report on effect of YE on the tropane alkaloid production in the cell suspension cultures of *D. metel* L. Treatment of suspension cultures of *D. metel* L. with a crude yeast extract improved

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TABLE 2 : Effect of YE treatment on growth of *D. metel* L. Cell cultures (FW and DW).

| No. | YE (mg/l) | Fresh weight (g) | | | Dry weight (g) | | |
|---|---|---|---|---|---|---|---|
| | | 24 hrs | 36 hrs | 48 hrs | 24 hrs | 36 hrs | 48 hrs |
| 1. | 100 | 4.229 ± 0.7 | 5.236 ± 0.5 | 4.434 ± 0.9 | 0.059± 0.002 | 0.063 ±0.004 | 0.067 ±0.002 |
| 2. | 200 | 4.090 ± 0.8 | 5.006 ± 0.5 | 5.603± 0.6 | 0.055± 0.002 | 0.053 ±0.003 | 0.056± 0.006 |
| 3. | 400 | 3.376 ± 0.2 | 5.120 ± 0.9 | 5.043 ± 0.1 | 0.065 ±0.002 | 0.057 ±0.005 | 0.062± 0.002 |
| 4. | Control | 3.786 ± 0.2 | 5.536 ± 0.1 | 5.400 ± 0.1 | 0.072 ±0.002 | 0.063 ±0.002 | 0.061 ±0.005 |

*** All results are mean of 3 replicates ± S.D.**

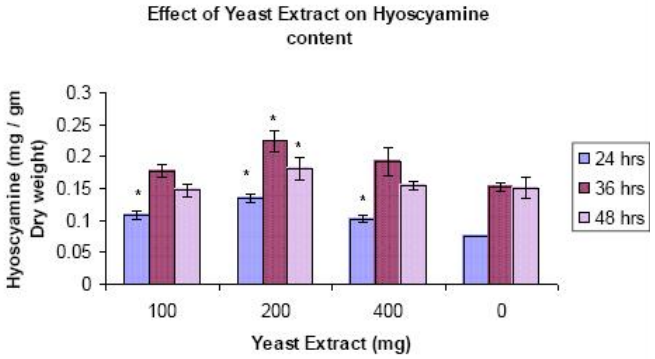


**Figure 3 : Effect of YE on hyoscyamine content in cell culture of *D. metel* L.**

***Indicates significant increase in Alkaloid production (Hyoscyamine and scopolamine) as compared to the control on applying T-test with a p value < 0.05.**

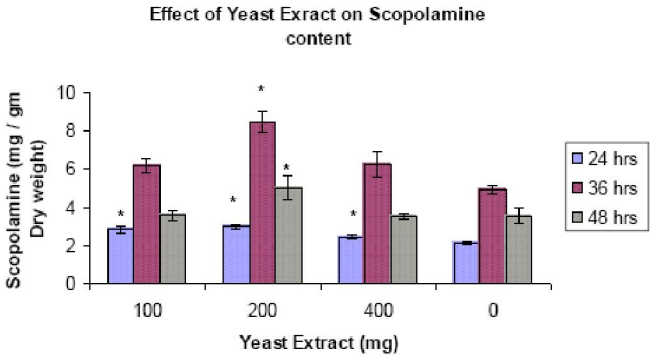


**Figure 4 : Effect of YE on Scopolamine Content in cell culture of *D. metel* L.**

***Indicates significant increase in Alkaloid production (Hyoscyamine and Scopolamine) as compared to the control on applying T-test with a p value < 0.05.**

production of hyoscyamine 1.46% folds higher and scopolamine approximately 1.17% folds higher than that of the control. In *Taxas* (treatment duration used was 5, 10, 15, days)[21, 22] and in *Panax ginseng* cells treated with 10 g/l concentration of YE showed hypersensitive response, cells turned brown and cell growth was arrested[23]. In our study we found that higher level of alkaloid was produced at high concentration of YE (200 mg/l) and no hypersensitive effect was found on cell growth. It was found that elicitation time, elicitor concentrations are probably the important parameters for tropane alkaloid production. Maximum tropane alkaloid production was reported at 200 mg/l concentration of YE (TABLE 2, Figure 3 and 4). As compared to other concentrations of YE, alkaloid content was less at 100mg/l YE and the probable reason is the stress produced by the elicitor was probably insufficient to enhance alkaloids (TABLE 2, Figure 3 and 4). At 400 mg/l concentration of YE tropane alkaloid production was low, probably due to inhibition at higher concentration of YE. Our observation supports the view of Avancini et al[24] that elicitation time and concentrations are the important parameters in enhancement of alkaloids.

YE have been reported to effectively bind to receptors on the plant cell, induce the synthesis of PAL and enhance secondary metabolites accumulation in plant cell cultures[25]. Cramer et al[26] concluded that elicitor induced the *de novo* synthesis of mRNA's encoding CHS, PAL, and CHI as part of an extensive shift in the pattern of mRNA synthesis. They have suggested that a specific change in gene expression occurs at an early stage in the plant defense mechanism which could lead to elucidation of signal response coupling mechanism in plant pathogen interaction and the development strategies to manipulate and enhance the response of plant cells to biological stress[26]. The effect of YE is also due to its role in increase in phenylalanine ammonia lyase activity which is a key enzyme of phenylpropanoid pathway that catalyses L-phenylalanine deamination and trans cynamic acid production which links primary metabolism to the secondary one, and formation of vast secondary metabolites with phnylpropanoid skeleton[27].

Although the physiological mechanism for this phenomenon is not well understood, it may be associated with the shifting of metabolic flux toward different secondary metabolites which can be induced different secondary metabolites which can be induced by elicita-

**TABLE 3 : Effect of pH treatments on growth in cell culture of *D. metel* L (FW and DW).**

| No. | pH | Fresh weight (g) | | | Dry weight (g) | | |
|---|---|---|---|---|---|---|---|
| | | 6 hrs | 12 hrs | 18 hrs | 6 hrs | 12 hrs | 18 hrs |
| 1. | 4.5 | 4.266 ± 0.9 | 2.533 ± 0.8 | 4.063 ± 1.4 | 0.062 ± 0.001 | 0.067 ± 0.002 | 0.064 ± 0.003 |
| 2. | 5.5 | 4.561 ± 1.2 | 2.520 ± 0.9 | 5.333 ± 1.3 | 0.056 ± 0.002 | 0.054 ± 0.003 | 0.058 ± 0.005 |
| 3. | 6.5 | 3.498 ±1.5 | 5.208 ± 0.9 | 3.920 ± 1.5 | 0.065 ± 0.002 | 0.055 ± .0009 | 0.066 ± 0.002 |
| 4. | 5.8 (control) | 3.083 ± 1.0 | 3.413 ± 0.2 | 3.573 ± 0.9 | 0.073 ± 0.003 | 0.064 ± 0.004 | 0.062 ± 0.004 |

***All results are mean of 3 replicates ± S.D.**

tion[7]. On the basis of these speculations, we believe that YE probably caused a complex stress response in cultures leading to accumulation of tropane alkaloids because of activation of metabolic pathways as suggested by earlier workers.

5.5 and it was 5.333 g (18 h) and 2.520 g (12 h) respectively. Highest dry weight (0.067 g) was obtained with pH 4.5 at 6 h, while lowest dry weight (0.054 g) was obtained at pH 5.5 at 12 h (TABLE-3). Highest hyoscyamine and scopolamine content was obtained at pH 5.5 (12 h) and it was 0.217 mg/g DW, 8.333 mg/g DW respectively (Figure 5 and 6).

Pitta- Alvarez and Giulietti[28] have reported that a decrease in pH did not affect growth significantly in hairy root cultures of *Brugmansia candida* during the initial 48 h, but after 72 h, and at low pH levels, growth was inhibited and furthermore some moderate browning of the roots was observed. In our experiment we have used different range of pH as pulse treatment and treatment duration used was short (6, 12, 18 h) for cell harvesting. In our results decrease in pH (i.e. at pH 4.5) affected cell growth (DW) during first 6 h. Control (pH 5.8) and pH 6.5 treatments showed difference in results as compared to the reports of Pitta- Alvarez and Giulietti[28] and this might be because the system we have used is cell suspension cultures (Unorganized system) and Pitta- Alvarez and Giulietti[28] (1999) used hairy root cultures for study (Organized system).

For production of alkaloids it was observed that pH 5.5 is best. pH 6.5 treatment also produced higher amount of alkaloids than control (pH 5.8) and pH 4.5 (Figure 5 and 6). So we can conclude that optimum range of pH for production of alkaloids is probably between 5.5 to 6.5.

The extracellular pH may have a role in cell signaling, and it potentially could affect the regulation of secondary metabolism. The pH is known to affect the uptake rate of carbon and nitrogen nutrients[29]. The difference in nutrient level would then affect the energy status of the cell. Additionally, the maintenance of pH gradient between the intracellular and extracellular media could affect the cellular energy status. Furthermore, a controlled external pH may lead to an alternation of cytoplasmic and vacuolar pH. Any of these factors could lead to altered gene expression or enzyme activities. Furthermore by controlling pH, the accumulation pattern and amounts of secondary metabolites can be con-

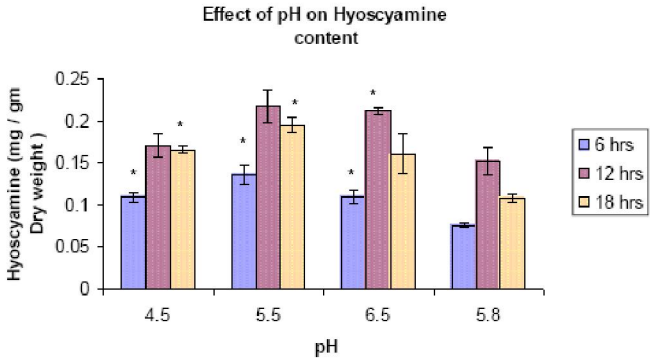


**Figure 5 : Effect of pH on Hyoscyamine Content in cell culture of *D. metel* L.**

***Indicates significant increase in Alkaloid production (Hyoscyamine and Scopolamine) as compared to the control on applying T-test with a p value < 0.05.**

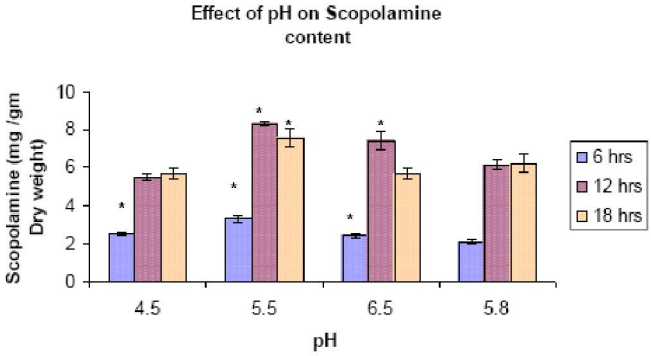


**Figure 6 : Effect of pH on Scopolamine Content in cell culture of *D. metel* L.**

***Indicates significant increase in Alkaloid production (Hyoscyamine and Scopolamine) as compared to the control on applying T-test with a p value < 0.05.**

trolled[29]. So in our experiment we could conclude that probably the effect of different range of pH may alter cell growth and secondary metabolites production by producing stress in cultures leading to enhanced production of alkaloids.

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