Influence of biotic and abiotic elicitors on accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L.

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Leaf-derived root cultures of *Datura metel* L., established on B5 medium containing 1.2 µM IAA, were employed to study the influence of biotic (*Aspergillus niger, Alternaria* sp., *Fusarium moniliforme* and yeast extract) and abiotic (salicylic acid, AlCl$_3$, CaCl$_2$, NaCl and Na$_2$SO$_4$) elicitors on the growth and production of hyoscyamine and scopolamine, the medicinally important tropane alkaloids. The hyoscyamine and scopolamine contents in control root cultures were 1.39 mg/g dw and 0.069 mg/g dw, respectively. The highest hyoscyamine (4.35 mg/g dw) and scopolamine (0.28 mg/g dw) accumulation was obtained in cultures treated with 500 µM salicylic acid, followed by treatment with 0.75 g L$^{-1}$ yeast extract (3.17 mg/g dw hyoscyamine & 0.16 mg/g dw scopolamine) and 250 µM AlCl$_3$ (2.49 mg/g dw hyoscyamine and 0.11 mg/g dw scopolamine).

**Keywords:** *Datura metel*, elicitors, hyoscyamine, scopolamine, tropane alkaloids

**Introduction**

*Datura metel* L. (Solanaceae), a small branched perennial herb with purple coloured flowers, is distributed in the tropical and sub-tropical regions of the world. This medicinal plant has been traditionally used as intoxicant, emetic, digestive and healing since ancient times$^{1,2}$. The main active constituents of the plant are the medicinally important tropane alkaloids—hyoscyamine and scopolamine. Being anticholinergic agents, these are used in medicine as antispasmodics, preoperative medication, analgesics, narcotics, sedatives and in treatment of asthma, Parkinson’s disease and motion sickness$^3$. Due to complex chemical structures, they require production from natural sources$^4$ and are mainly obtained from plant species like *Atropa beladona, Hyoscyamus muticus* and *Datura* species. Among the 85 different genotypes of Solanaceous plants investigated for tropane alkaloid content, *D. metel* showed very high content of hyoscyamine$^5$ and scopolamine$^6$.

Propagation of *D. metel* through seed is unreliable due to poor germination. On the other hand, harvesting the plant on mass scale from natural habitat leads to depletion of natural population. Therefore, there is urgent need to develop a plantation method either by developing the method for breaking the seed dormancy and improvement of seed germination or by micropropagation$^2$. The production of important alkaloids through different biotechnological means is an interesting alternative, since it would guarantee a stable and uniform year-round supply, independent of seasonal variations of field-grown plants$^7$. At the same time, the indiscriminate collection of plant from natural habitats can be prevented. Despite this, the production of different secondary metabolites through *in vitro* cell culture has met with limited success due to low yields.

The use of elicitors is one of the effective strategies employed to increase the production of important alkaloids in cell and organ culture$^3,7,8$. Although *D. metel* is one of the major sources of the hyoscyamine and scopolamine, only few reports are available on *in vitro* propagation$^2$ and production of alkaloids through hairy root culture$^9$. Therefore, the main objective of the work reported here was to improve the production of tropane alkaloid through the application of biotic and abiotic elicitors in root cultures of *D. metel*.

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Material and Methods

Plant Material and Culture Condition
The healthy young leaves of purple variety of *Datura metel* were obtained from the plants growing naturally on the banks of the river Mutha, Pune, India. Leaf explants were washed under running tap water and surface sterilized by shaking for 5 min in 0.1% mercuric chloride solution (w/v). The leaves were then washed several times in sterile distilled water and used as explants. Segments of 10-12 mm^2 leaf explant were placed on B5^10 basal medium supplemented with vitamins, 2% (w/v) sucrose, 0.8% agar and 0.5 to 22 µM IAA and NAA individually. The pH of the medium was adjusted to 5.8 before autoclaving. The medium was autoclaved at 1.1 kg cm^-2 pressure and 121°C for 15 min. Cultures were incubated at 25 ± 2°C under 8 h light/16 h dark photoperiod (50 µmol m^-2 s^-1).

Root Culture
The roots induced from the leaf explant on B5 medium containing 1.2 µM IAA and 2% (w/v) sucrose after 4 wk of culture showed extensive proliferation. The selected induced roots were further proliferated on the same medium for further experiments. For proliferation, an inoculum of 500±2.5 mg fresh roots was subcultured at 4 wk interval in 250 mL conical flask containing 40 mL of liquid B5 medium supplemented with 1.2 µM IAA and 2% (w/v) sucrose. The cultures were placed on rotary shaker at 80 rpm.

Fungal Elicitor Cultures
Fungal strains of *Aspergillus niger*, *Alternaria* sp. and *Fusarium monoliforme* were obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India. The fungal cultures were established in liquid potato dextrose medium. The fungal mycelia were separated by filtration and washed four times with sterile distilled water. The mycelia were homogenized and the homogenate was used as elicitor.

Effect of Biotic and Abiotic Elicitors on Growth and Tropane Alkaloid Production
The roots were cultured for 6 wks on parental medium containing different concentrations of fungal extracts (0, 0.1, 0.25, 0.5, 0.75 & g L^-1); Yeast extract (0, 0.1, 0.25, 0.5, 0.75 &1 g L^-1); sodium chloride (NaCl; 0.17, 2, 43.1, 86.2, 129.3 & 172.4 mM); sodium sulphate (Na_2SO_4; 0, 7.0, 17.6, 35.2, 52.8 & 70.4 mM); calcium chloride (CaCl_2; 0.0, 1, 10, 15, 30 & 40 mM); salicylic acid (SA; 0, 25, 250 & 500 µM) and aluminium chloride (AlCl_3; 25, 250 & 500 µM) added individually on the day of inoculation (0 d). Since the growth and tropane alkaloid content of root reached maximum on the 5th wk of culture, the root growth (fresh wt, dry wt & growth index) and hyoscyamine and scopolamine content were measured after 5 wks of culture. The growth index was calculated as:-

\[
\text{Growth index} = \frac{(\text{Final weight} - \text{Initial weight})}{\text{Initial weight}}
\]

Extraction and Estimation of Hyoscyamine and Scopolamine Content
Oven dried samples were powdered in mortar and extraction was carried out following the method described by Feldman and Bruce^11_. 1 g sample was macerated for 12 h in a mixture of 0.75 mL ammonium hydroxide (28%), 1 mL 95% ethyl alcohol and 2 mL ethyl ether. The tissues were dried and extracted with methanol. The extract was evaporated under infrared heat and the residue was redissolved in methanol. The methanolic extract was centrifuged and the solution was used for the analysis of alkaloids.

Tropane alkaloids were analyzed by HPTLC (CAMAG TLC Scanner 3). Alkaloids were examined using cellulose and silica gel 60 F254 TLC precoated plates (Merck). The developing solvent was toluene:ethylacetate:diethylamine (7:2:1). Standard hyoscyamine and scopolamine (Sigma, USA) and samples were loaded on TLC plates. The product spots were detected by spraying with modified Dragendorff's reagent^12,13_. The plates were air dried in the dark and Dragendorff-positive zones were marked. Hyoscyamine and scopolamine content was calculated by area of standard and area of sample observed at 290 and 230 nm respectively^11-13_.

Statistical Analysis
All experiments were repeated at least thrice with minimum of 21 replicates per treatment. Significance of treatment effects was determined by using analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test^14_ (p ≤ 0.05) to determine significant differences among treatment means.
Results and Discussion

Time Course Growth and Alkaloid Accumulation

Fig. 1 illustrates the time course growth and alkaloid accumulation in root cultures of *D. metel*. The lag phase was about 6 d and the linear growth phase was between 7th to 27th d, followed by the stationary phase. The figure shows that growth of the culture was associated with tropane alkaloid accumulation. The maximum biomass (0.27 g) was obtained at the end of linear growth phase at 35th d, which was also the peak time of hyoscyamine accumulation (1.13 mg/g dw) in the root culture. The stationary phase was between 28th to 35th d, after which the biomass showed a steady decline. Similar pattern of growth and alkaloid production was observed in cultured tissues and organs of other tropane alkaloid producing plants.8,15

Effect of Biotic Elicitors on Growth and Alkaloid Production

The results show that the incorporation of increasing concentration of homogenate of biotic elicitors, *A. niger*, *Alternaria* sp. and *F. monoliforme*, in the medium resulted in higher hyoscyamine and scopolamine accumulation with the reduced growth of roots. The growth index declined gradually with the increasing concentration of the fungal homogenate in the medium. However, among the same concentrations of elicitor homogenates, no significant difference was observed for the growth index. Root cultures treated with 1.0 g L⁻¹ of *A. niger* homogenate resulted in higher hyoscyamine (1.77 mg/g dw) and scopolamine (0.087 mg/g dw) production than that of the *Alternaria* sp. and *F. monoliforme* (Table 1). Further increase in the concentration of elicitor homogenates (1.5 g L⁻¹) inhibited the growth of roots as well as accumulation of tropane alkaloids. Similar results were observed in other *Datura* species.8 and other tropane alkaloid producing plants.16 The results indicate that the *A. niger* homogenate is favourable for promoting the accumulation of tropane alkaloids in *D. metel*.

Addition of increasing concentration of yeast extract (YE) (0.1-0.5 g L⁻¹) in the medium resulted in an increased growth index as well as tropane alkaloid accumulation (Fig. 2). The inclusion of YE at the concentration of 0.75 g L⁻¹ was inhibitory for the growth, but promoted the accumulation of roots. The values represent the mean ± SE of three independent experiments each performed on twenty one replicates. Values followed by similar letters do not differ significantly at p ≤ 0.05 as per DMRT.

Table 1—Influence of biotic elicitors on the alkaloid content in the callus and root culture of *D. metel* L

<table>
<thead>
<tr>
<th>Elicitors (g L⁻¹)</th>
<th>Growth index</th>
<th>Hyoscyamine (mg/g dw)</th>
<th>Scopolamine (mg/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>5.79</td>
<td>1.39±0.004</td>
<td>0.069±0.001</td>
</tr>
<tr>
<td>0.1</td>
<td>6.32</td>
<td>1.46±0.003</td>
<td>0.077±0.0004</td>
</tr>
<tr>
<td>0.5</td>
<td>5.61</td>
<td>1.75±0.005</td>
<td>0.082±0.0005</td>
</tr>
<tr>
<td>1.0</td>
<td>5.07</td>
<td>1.77±0.005</td>
<td>0.087±0.001</td>
</tr>
<tr>
<td>1.5</td>
<td>4.34</td>
<td>1.63±0.004</td>
<td>0.081±0.001</td>
</tr>
<tr>
<td><em>Alternaria sps.</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>5.79</td>
<td>1.39±0.004</td>
<td>0.069±0.001</td>
</tr>
<tr>
<td>0.1</td>
<td>5.96</td>
<td>1.43±0.006</td>
<td>0.074±0.00006</td>
</tr>
<tr>
<td>0.5</td>
<td>5.43</td>
<td>1.59±0.004</td>
<td>0.079±0.0008</td>
</tr>
<tr>
<td>1.0</td>
<td>4.89</td>
<td>1.64±0.003</td>
<td>0.084±0.001</td>
</tr>
<tr>
<td>1.5</td>
<td>4.24</td>
<td>1.52±0.004</td>
<td>0.078±0.001</td>
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<tr>
<td><em>Fusarium monoliforme</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>5.79</td>
<td>1.39±0.004</td>
<td>0.069±0.001</td>
</tr>
<tr>
<td>0.1</td>
<td>6.14</td>
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<td>0.072±0.014</td>
</tr>
<tr>
<td>0.5</td>
<td>5.61</td>
<td>1.63±0.005</td>
<td>0.075±0.0007</td>
</tr>
<tr>
<td>1.0</td>
<td>5.25</td>
<td>1.66±0.004</td>
<td>0.081±0.0006</td>
</tr>
<tr>
<td>1.5</td>
<td>4.92</td>
<td>1.55±0.006</td>
<td>0.072±0.0006</td>
</tr>
</tbody>
</table>

The values represent the mean ± SE of three independent experiments each performed on twenty one replicates. Values followed by similar letters do not differ significantly at p ≤ 0.05 as per DMRT.
tropane alkaloid. However, incorporation of very high concentration of YE (1.0 g L\(^{-1}\)) resulted in the decline of growth as well as alkaloid content. Among all the biotic elicitors, the highest level of hyoscyamine and scopolamine was obtained at 0.75 g L\(^{-1}\) YE; representing about 2.2 times more than the control. In a previous study, *D. stramonium* culture when exposed to YE resulted in only slight increase in hyoscyamine content but 2.5 times increase in scopolamine\(^{17}\). Yeast extract is composed of variety of compounds, apart from amino acids, vitamins and minerals\(^{18}\), and it is also possible that elicitation effects might be due to the contents of cations like Zn, Ca and Co in the yeast extract.

**Effect of Abiotic Elicitors on Growth and Alkaloid Production**

Various concentrations of NaCl and Na\(_2\)SO\(_4\) were tried to stimulate the potential of root culture for accumulation of tropane alkaloids (Fig. 3). It was observed that increasing concentration of NaCl suppressed the growth of roots. However, there was increase in the accumulation of both hyoscyamine and scopolamine upto 129.3 mM NaCl, where the increase was about 1.5- and 1.3-times more, respectively in comparison to control. Addition of 172.4 mM NaCl was harmful for the growth as evident from a sharp decline in growth index. The addition of NaCl in the medium may result in an increase in the endogenous level of methyl jasmonate, which can stimulate the activity of enzymes involved in the biosynthesis of tropane alkaloids and consequently their increased accumulation\(^{19}\). Compared to NaCl, the presence of Na\(_2\)SO\(_4\) in the medium was less inhibitory for the growth and less stimulatory for accumulation of alkaloids (Fig. 3).

The results depicted in Fig. 4 show that inclusion of 1-15 mM concentration of CaCl\(_2\) in the medium promoted the growth and tropane alkaloid accumulation in the root. The inclusion of higher level (15 mM) of CaCl\(_2\) in the medium resulted in increased accumulation of hyoscyamine (1.83 mg/g dw) and scopolamine (0.087 mg/g dw) content, which was about 2- to 3-times more than the control. The concentrations of calcium ions in the culture medium regulate the putrescine methyl transferase (PMT enzyme) activity and consequently the capacity for the synthesis of tropane alkaloids\(^{20}\). In the present investigation, increasing the CaCl\(_2\) concentration upto 30 mM in the medium promoted the growth and proliferation of root but it was inhibitory for alkaloid accumulation. High level of Ca\(^{2+}\) in the medium activates peroxidases, which are involved in the degradation of secondary products\(^{21}\).

![Fig. 3—Effect of salt stress on growth and tropane alkaloid accumulation in root cultures of *D. metel* L.](image)

![Fig. 4—Influence of CaCl\(_2\) on growth and tropane alkaloid accumulation in root cultures of *D. metel* L.](image)
Increased contents of hyoscyamine and scopolamine were also obtained in hairy root cultures of *Brugmansia candida* after treatment with AlCl$_3$. The reason for this might be that most of the genes up-regulated by aluminium shared homologies with those related to pathogenesis, suggesting aluminium may act as an elicitor. In the present investigation, there was increase in the hyoscyamine and scopolamine content in root culture in the medium containing AlCl$_3$ (25-250 μM; Fig. 5). These results indicate that aluminium chloride can stimulate the production of tropane alkaloids.

Addition of salicylic acid (SA) slightly reduced the growth index as compared to control, but promoted the accumulation of tropane alkaloid in the root culture. The highest hyoscyamine and scopolamine accumulation was detected at 500 μM SA (Fig. 5), representing about 3.5- and 4-times more than control, respectively. The positive responses of the root culture to elicitation are possibly associated with the fact that SA is one of the key endogenous signals involved in the activation of numerous plant defense responses and its ability to produce pathogenesis-related proteins in plants, even in the absence of pathogenic organism.

The results of the present investigation clearly indicate that incorporation of yeast extract and salicylic acid in the nutrient medium may result in the elicitation of hyoscyamine and scopolamine contents in the *in vitro* root cultures of *D. metel* to the levels comparable to the parent plant (3.28 mg/g dw hyoscyamine & 1.45 mg/g dw scopolamine). In conclusion, the protocol presented here can be used for the production of these medicinally important tropane alkaloids by the pharmaceutical industry, subject to economic considerations.

**Acknowledgement**

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**References**