Some Accounts of Nicotine Biosynthesis in Tobacco Callus Tissues by the Use of Effective and Ineffective Strains

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Nicotine biosynthesis in tobacco callus tissues was investigated by comparing high nicotine producing strain OMT-53 with scarcely producing strain NT-5. NT-5 strain which had been subcultured for about 5 years with a medium containing 1.0 ppm 2,4-D, produced very little amount of nicotine even after more than 47 subcultures on OT-23 medium containing 0.15 ppm α-NAA. On the other hand, OMT-53 strain maintained a constant high level nicotine producing (ca. 2% or more on D.W. basis) capacity over 50 successive cultures on the same medium. The growth response of NT-5 callus tissues to α-NAA concentration was quite different from that of OMT-53. Nicotine production assumed more distinctive patterns with these two strains. NT-5 strain never produced nicotine at any concentrations of α-NAA examined, whilst OMT-53 strain produced abundant amount of nicotine within a narrow range of α-NAA concentration having the optimal value of 0.15 ppm. Nicotine supplemented to culture medium could not be decomposed either with NT-5 or with OMT-53 callus tissues. The nicotine production by OMT-53 was not affected at moderate level (1 mM) of supplemented nicotine, but at a very high level (5 mM) of the supplemented nicotine, the nicotine production seemed to have been affected considerably. Some precursors supplemented to NT-5 callus cultures did not stimulate the nicotine biosynthesis at all, even though slightly positive effect was recorded with OMT-53 callus cultures. NT-5 callus tissues had much smaller pool size of total free amino acids than OMT-53. The ratio of each amino acid in total free amino acids was not so different between both callus tissues. On the basis of these results, some discussions were made on nicotine biosynthesis in tobacco tissue cultures.

Biosynthetic pathway of microbial products has often been effectively studied by the use of a mutant which lacks a part of metabolic chain furnished by an ordinary microbial strain. In the study of biosynthesis of plant materials, similar method has been employed with plant tissue cultures of tobacco and carrot.

Nicotine production in tobacco tissue cultures is generally poor, and many trials have been made to increase the nicotine production. It is expected that in future entire regulation of nicotine production in tobacco callus tissues will find its way when each step of the nicotine biosynthetic pathway will be disclosed. To elucidate limiting steps in the process of biosynthesis in tobacco callus tissues, comparative studies with active and inactive strains would be useful.

In the previous papers, a tobacco callus strain, OMT-53 was shown to have high nicotine producing capacity. On the other hand, a tobacco callus strain, NT-5 which produces very little nicotine had been obtained in the course of the research. These two strains, OMT-53 and NT-5 were considered to afford valuable informations on regulatory mechanism of nicotine biosynthesis in tobacco tissue cultures.

The present investigation deals with the comparison of metabolic responses of these active and inactive strains OMT-53 and NT-5.

MATERIALS AND METHODS

Callus tissues. OMT-53 callus strain was derived from a segment of sterile tobacco seedling (Nicotiana tabacum var. Bright Yellow) on a medium containing 0.15 ppm α-NAA and successively cultured on OT-23.
medium\(^4\) which also contains the same quantity of \(\alpha\)-NAA. This callus strain produces abundant amount of nicotine on the same medium.\(^4,5\) A callus strain, NT-5 was derived from the same variety of tobacco seedlings and subcultured for about 5 years on a basal medium\(^6\) containing 1 ppm 2,4-D. This strain has never produced detectable amount of nicotine in successive cultures.

**Culture method.** Callus tissues were cultured with OT-23 medium (40 ml/flask) by the same method as described in a previous paper,\(^5\) otherwise indicated in the text.

**Analysis.** Determination and identification of nicotine in the culture were made by the same way as reported previously.\(^5\) Amino acid analysis was made as follows: Each sample of fresh callus tissues was ground in a mortar with 70\% alcohol and sea sand, then centrifuged. The sediment was extracted again with 70\% alcohol and centrifuged. The extracts were combined and evaporated in vacuo to dryness. The residue was dissolved in dil. HCl (pH 2.2). The solution was used for amino acid analysis with JLC-5AH Amino Acid Analyzer (Japan Electron Optics Laboratory Co., Ltd.).

**RESULTS AND DISCUSSION**

**Nicotine producing activities of NT-5 and OMT-53 callus tissues**

NT-5 callus tissues subcultured for about 5 years with a medium containing 0.1 ppm \(\alpha\)-NAA and successively cultured in the dark at 25\(\degree\)C. Nicotine content of callus tissues of each passage was determined after each incubation period as indicated.

As shown in Table I, unexpectedly, nicotine was not detected at any degree in the callus tissues over the followed 10 successive cultures. After 15th passage, callus tissues became to produce very little amount of nicotine, and the maximal content of nicotine did not exceed 0.06\% on D.W. basis over more than 47 subcultures. Furuya \textit{et al.}\(^1\) reported that the transfer of tobacco callus tissues from 1 ppm 2,4-D containing medium to 1 ppm \(\beta\)-IAA containing medium resulted in almost complete recovery of nicotine biosynthetic activities after 3 successive cultures.

Therefore, it can be suggested that NT-5 callus tissues have a quite stable inert nature in producing nicotine. On the other hand, OMT-53 strain maintained a constant high level nicotine producing (ca. 2\% or more on D.W. basis) capacity over 50 successive cultures.\(^4,5\)

The inert nature of NT-5 strain in producing nicotine may have been built through the two mechanisms. One is a chromosomal mutation which is commonly induced by 2,4-D and its analogues.\(^12\) The other is the selective reproduction of cells which adapt to a medium containing 2,4-D through many successive cultures.

The very minute recovery in nicotine producing activity after more than 10 successive cultures on OT-23 medium definitely indicates that the inert nature of NT-5 strain had not been built merely through selective reproduction, because the long successive culture in OT-23 medium would be enough to develop sufficiently potential activity in the tissue under the concept of selective regeneration.

<table>
<thead>
<tr>
<th>Passage on OT-23 medium</th>
<th>1</th>
<th>5</th>
<th>8</th>
<th>10</th>
<th>15</th>
<th>47</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture period (days)</td>
<td>21</td>
<td>17</td>
<td>15</td>
<td>23</td>
<td>21</td>
<td>42</td>
</tr>
<tr>
<td>Growth D.W. (mg)</td>
<td>405</td>
<td>429</td>
<td>563</td>
<td>547</td>
<td>540</td>
<td>614</td>
</tr>
<tr>
<td>Nicotine content % on D.W. basis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
<td>0.06</td>
</tr>
</tbody>
</table>
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though it is very small, of nicotine producing activity in NT-5 callus cultures after more than 15 successive cultures makes it difficult to consider that all of the inert nature of NT-5 callus tissue has been due to the chromosomal mutation.

Therefore, it would be reasonable to assume that the nature of NT-5 callus tissues is the reflection of a complex entity resulted from the two mechanisms mentioned above. In this sense, the pathway of nicotine biosynthesis in tobacco callus tissues, in general, is considered to be under a regulation defined by the mechanisms of chromosomal mutation and selective reproduction as well as of metabolic adaptation to environmental conditions.

Under these considerations, further experiments were made with NT-5 callus tissues which were previously subcultured 10 times successively on OT-23 medium.

Response of callus tissues to α-NAA concentration

In the previous papers, nicotine biosynthesis in tobacco callus tissues was shown to vary intensively by the level and kind of plant growth regulators in the culture medium. In addition, it was suggested that different callus strain from the same plant may not be the same in metabolic responses to the same condition with plant growth regulators. The response of NT-5 and OMT-53 callus tissues to plant growth regulators in nicotine biosynthesis could be different at varied concentrations of α-NAA. Therefore, NT-5 and OMT-53 strains were cultured at various levels of α-NAA and nicotine content was examined.

As shown in Table II, NT-5 callus tissues grew very well over a wide range of α-NAA concentrations from 0.15 ppm to 2.0 ppm. On the other hand, the growth of OMT-53 was favoured within a narrower range (0.15 to 0.5 ppm) of α-NAA. Nicotine production assumed more distinctive patterns with these two callus strains. NT-5 strain never produced nicotine at any concentrations of α-NAA employed in this experiment, whilst OMT-53 strain produced abundant amount of nicotine within a narrow range of α-NAA concentration having the optimal value of 0.15 ppm.

Takahashi and Yamada suggested that auxin might directly or indirectly act on gene expression in the biosynthesis of nicotine in tobacco callus tissues. The present results with NT-5 strain may not be explained by this mechanism.

The inert nature of NT-5 strain in producing nicotine over a wide range of α-NAA concentration in the medium would again confirm the assumption that the strain NT-5 had received high degree of chromosomal mutation and reproductive selection during the previous successive passages cultured with 2,4-D containing medium.

Decomposition of nicotine by callus tissues

NT-5 callus tissues were shown to produce very little amount of nicotine after more than 15 successive cultures on OT-23 medium. It was feared that nearly nil accumulation of nicotine in NT-5 callus cultures over a wide range of α-NAA concentrations and also in OMT-53 callus cultures especially at high level of α-NAA might have been due to the possible decomposition of nicotine in the cultures.

To examine the possibility if nicotine is decomposed in these callus cultures, the callus

<table>
<thead>
<tr>
<th>α-NAA conc. (ppm)</th>
<th>NT-5 Growth</th>
<th>Nicotine content % on D.W.basis</th>
<th>OMT-53 Growth</th>
<th>Nicotine content % on D.W.basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21</td>
<td>0</td>
<td>56</td>
<td>0.08</td>
</tr>
<tr>
<td>0.01</td>
<td>64</td>
<td>0</td>
<td>92</td>
<td>0.68</td>
</tr>
<tr>
<td>0.05</td>
<td>511</td>
<td>0</td>
<td>272</td>
<td>0.74</td>
</tr>
<tr>
<td>0.15</td>
<td>513</td>
<td>0</td>
<td>447</td>
<td>1.49</td>
</tr>
<tr>
<td>0.5</td>
<td>590</td>
<td>0</td>
<td>401</td>
<td>0.12</td>
</tr>
<tr>
<td>1.0</td>
<td>477</td>
<td>0</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>193</td>
<td>0</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

—: not determined.
TABLE III. DECOMPOSITION OF NICOTINE ADDED TO A CULTURE MEDIUM BY NT-5 AND OMT-53

<table>
<thead>
<tr>
<th>Callus strain</th>
<th>Nicotine added to culture medium (mg/flask)</th>
<th>Growth F.W. (g)</th>
<th>Nicotine (mg/flask)</th>
<th>Difference $^a$</th>
<th>Nicotine production (g nicotine/100g tissue F.W.)</th>
<th>Recovery ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT-5</td>
<td>0.0</td>
<td>23.2</td>
<td>0</td>
<td>0.5</td>
<td>0.3</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>24.0</td>
<td>0.3</td>
<td>2.8</td>
<td>0</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>24.8</td>
<td>0.4</td>
<td>5.1</td>
<td>0</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>23.7</td>
<td>3.3</td>
<td>26.0</td>
<td>0</td>
<td>0.87</td>
</tr>
<tr>
<td>OMT-53</td>
<td>1.0</td>
<td>9.7</td>
<td>2.6</td>
<td>12.1</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>6.0</td>
<td>5.5</td>
<td>17.0</td>
<td>0.142</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Difference = total nicotine − (added nicotine × recovery ratio).

As shown in Table III, NT-5 callus tissues grew very well even though considerable concentration of nicotine was maintained in the medium during the culture period. More striking thing is that considerable amount of nicotine was absorbed into the callus tissues. Recovery ratio of nicotine in these cultures ranges from 0.83 to 0.93 having an average of 0.87. Considering these recovery ratio over wide range of nicotine concentrations, the nicotine decomposition by NT-5 callus tissues could be neglected.

The nicotine production in cultures with OMT-53 has not been affected at a moderate level (1 mM) of supplemented nicotine. But at a very high level (5 mM) of the supplemented nicotine, the nicotine production seems to have been affected considerably. If the latter is the case, nicotine decomposition in OMT-53 callus tissues could also be neglected. On the contrary, assuming that nicotine production in OMT-53 tissues was not affected at the very high level (5 mM) of supplemented nicotine, considerable degree of nicotine decomposition should be concluded. The decisive evidence could be obtained by the use of labelled nicotine for supplementation. This should await further experiments.

Mizusaki et al. reported that supplemented nicotine in culture solution strongly repressed the synthesis of enzymes involved in nicotine biosynthesis in tobacco intact plants. In the present experiment, nicotine concentration in callus tissues of OMT-53 was very much elevated (2.46 mg/g F.W.) in the culture supplemented with large amount of nicotine, and considerable amount of nicotine was excreted to the medium in a control flask having no nicotine supplementation. Considering these results, depression of nicotine production in OMT-53 in the culture supplemented with nicotine would be the most reasonable mechanism in explaining the data in Table III.

Effect of some precursors on nicotine biosynthesis

Effect of precursors, especially of putrescine and nicotinic acid, on nicotine production in OMT-53 was previously examined and gave unfavourable results. This might be due to the toxic effect on growth of these precursors even at $10^{-3}$ M concentration. Less amount of precursors are not enough to give a significant value to the amount of nicotine produced by the control callus culture during the incubation period. In a preliminary experiment, NT-5 callus tissue was known to grow favourably even at higher concentrations than $10^{-3}$ M of putrescine. Therefore, NT-5 callus tissues were cultured on OT-23 medium supplemented...
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with putrescine and nicotinic acid at concentrations of $10^{-3}$ M or $10^{-4}$ M separately or in combination. The nicotine content was determined after 4 weeks' incubation.

The nicotine biosynthesis in callus cultures was not stimulated even by supplementing putrescine and/or nicotinic acid at $10^{-3}$ M or $10^{-4}$ M concentration, separately or in combination, although the growth of the callus tissues was not inhibited at all in these culture conditions.

Mizusaki et al.\textsuperscript{14-16) reported that when $^{14}$C-labelled ornithine or putrescine were supplied to tobacco cell suspension cultures, considerable amounts of radioactivity were incorporated into p-coumaroyl, caffeoyl and feruloyl putrescine, but not into nicotine moiety. Therefore, they suggested that nicotine biosynthesis in the tobacco cell suspension culture was being blocked at or after the step of methylation of putrescine.

Considering these results, the pathway of nicotine biosynthesis in NT-5 strain would presumably be completely blocked after the formation of putrescine and nicotinic acid. On the other hand, OMT-53 strain would be slightly restricted in nicotine production by the shortage of precursors, because the supplementation of ornithine at $2 \times 10^{-4}$ M concentration resulted in a slight enhancement of nicotine biosynthesis in the tissue. However, some restriction of nicotine biosynthesis in the course from putrescine and nicotinic acid to nicotine could not be excluded even in callus strain OMT-53.

\textbf{Pool size and composition of free amino acids}

Amino acids constitute general precursors for alkaloid biosynthesis in intact plants, even though they are not necessarily immediate constituting materials. Ornithine is known to contribute in synthesizing pyrrolidine ring of nicotine molecules in intact tobacco plants. Informations on pool size and composition of free amino acids in tobacco callus tissues were thought to afford supplemental valuable materials to estimate the capacity of nicotine biosynthesis in the tissues. Therefore, NT-5 and OMT-53 strains were cultured on OT-23 medium for 3 weeks, and the pool size and composition of free amino acids were examined.

As shown in Table IV, NT-5 callus tissues had much smaller pool of total free amino acids than OMT-53. Steward \textit{et al.}\textsuperscript{17) reported that rapidly proliferating tissues had a much lower content of free amino acids and amides than non-growing tissues in potato and carrot tissues. This relation well coincides with the present results, because the growth rate of NT-5 callus tissues much exceeds that of OMT-53 tissues as shown in Tables II and III. Free amino acids would presumably be consumed rapidly in fast growing tissues for synthesizing cell protein. The shortage of free amino acids would inevitably constitute

\begin{table}[h]
\centering
\caption{Free Amino Acids and Nicotine in Tobacco Callus Tissues}
\begin{tabular}{lcc}
\hline
\textbf{Amino acid} & \textbf{NT-5} & \textbf{OMT-53} \\
\hline
Aspartic acid & 207.1$^a$ & 876.9$^a$ \\
Threonine & 20.2 & 45.3 \\
Serine & 18.8 & 40.2 \\
Glutamic acid & 12.9 & 35.4 \\
Proline & 19.3 & 32.4 \\
Glycine & 7.5 & 13.3 \\
Alanine & 3.1 & 3.0 \\
Cysteine & 3.8 & 3.0 \\
Valine & 9.9 & 13.5 \\
Leucine & 2.4 & 3.9 \\
Tyrosine & 2.1 & 3.5 \\
Phenylalanine & 4.6 & 7.6 \\
Lysine & 5.3 & 22.9 \\
Histidine & 3.9 & 5.7 \\
Total & 320.9 & 1106.6 \\
\hline
\textbf{Nicotine production} & & \\
\text{(mg nicotine/100 g tissue F.W.)} & 0.0 & 75.8 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} This fraction probably contains also asparagine and glutamine in high proportion.
unfavourable conditions in synthesizing unavailable useless secondary metabolites. This might be one aspect of inert nature of synthesizing nicotine in fast growing callus tissue NT-5.

The ratio of each amino acid in total free amino acids was not so different between NT-5 and OMT-53 tissues. The result reasonably coincides with data presented by Koiwai et al.\textsuperscript{18} Biosynthesis of nicotine in tobacco callus tissues, therefore, does not seem to be affected very much from the concentration of defined free amino acids in the tissues.

Taking account all of these things, the most susceptible place of restriction in nicotine biosynthesis in tobacco callus tissues seems to lie in the path after putrescine and nicotinic acid biosynthesis.

REFERENCES