Physiology and Biochemistry of the Timothy Leaf Spot Fungus, 
*Cladosporium phlei* de Vries

II. Utilization of inorganic nitrogen

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**Introduction**

When the timothy leaf spot fungus, *Cladosporium phlei*, was cultured in the medium containing ammonium nitrate as a sole nitrogen source, the pH of the medium was shown to lower with the culture age. In this study, an attempt was made to elucidate this phenomenon from the aspect of selective utilization of ammonium and nitrate ions by the fungus. This paper describes the time course changes of ammonium and nitrate ion concentrations, and of the pH in the culture media.

**Materials and Methods**

*Organism and its culture*

The timothy leaf spot fungus, *Cladosporium phlei* de Vries, was used throughout this work. The preliminary culture was carried out as described previously. Composition of the basal medium was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>0.2 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>1.2 g</td>
</tr>
<tr>
<td>FeSO₄·4H₂O</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>0.2 mg</td>
</tr>
</tbody>
</table>

in 1 l of deionized water. The final concentrations of thiamine and pyridoxine were 0.1 and 0.5 ppm, respectively. After adjusted to pH 6.0 with 1 N HCl, the medium was sterilized at 120°C for 20 min. From the preliminary culture, a loopful of spores of the fungus were transferred into 25 ml of test medium and cultured at 20°C in the dark.

*Determination of the fungal growth*

The fungus was collected by filtration through gauze and washed three times with deionized water, followed by sucking the water thoroughly with filter paper. The fresh weight of the fungus was measured with a chemical balance.

*Measurement of pH and glucose concentration*

The fungus was removed from the culture medium. Measurement of the pH in the medium was conducted with pH meter (Toa Denpa, Model HM5A). After the pigment...
excreted by the fungus was removed from the medium with ether, glucose amount was determined by the method of Somogyi Nelson. Determination of ammonium, nitrate and nitrite ions
The concentrations of ammonium, nitrate and nitrite ions were determined using indophenol, brucine and sulfanylic acid-α-naphthylamine.

Results
The selective utilization of ammonium and nitrate ions was examined using the medium supplemented with thiamine and pyridoxine. As shown in Fig. 1, the pH of the medium continued to lower during the first 17 days and thereafter rose. The results presented in Fig. 2 indicate that the fungus utilized preferentially ammonium ion at the early stage of culture and successively nitrate ion at the later stage after ammonium ion excretion by the fungus was removed from the medium with ether, glucose amount was determined by the method of Somogyi Nelson.

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Fig. 1. Time course change of pH in the medium supplemented with thiamine and pyridoxine.

Fig. 2. Time course changes of NH₄⁺, NO₃⁻ and NO₂⁻ concentrations in the medium supplemented with thiamine and pyridoxine.

Fig. 3. Time course changes of glucose concentration and fresh weight of the fungus in the medium supplemented with thiamine and pyridoxine.

Fig. 4. Time course change of pH in the medium excluding thiamine and pyridoxine.
were fairly consumed. The appearance of nitrite ion in the medium was first observed in the lapse of 5 days culture. This suggests that though a small quantity, nitrate ion could be utilized from the comparatively early stage even in the presence of ammonium ion. As shown in Fig. 3, nitrate ion were also utilized after the maximal growth was attained. A similar experiment was conducted using the medium excluding any vitamin (Figs. 4-6). Though the growth was considerably slow (Fig. 6), essentially the same result was obtained. Considering all the results together, an explanation can be made that the lowering and rising of pH at the early and latter stages are due to preferential utilization of ammonium ion prior to active utilization of nitrate ion.

Discussion

In this study, utilization of inorganic nitrogen by C. phlei was examined using two glucose-NH$_4$NO$_3$ media including or excluding thiamine and pyridoxine. TANAKA et al. reported that the blast disease fungus, Piricularia oryzae, utilized ammonium ion in the early stage of culture with decreasing pH, using ammonium nitrate as a nitrogen source. Also in our experiments, C. phlei was shown to utilize preferentially ammonium ion at the early stage and then actively nitrate ion after ammonium ion were fairly consumed in both media (Figs. 2 and 5). However, it is not yet clear whether the biosynthesis of nitrate reducing system was repressed or the activity of the system itself was inhibited by the presence of ammonium ion.

The lowering and rising of pH at the early and later stages, respectively, were observed in both media (Figs. 1 and 4). From the results obtained in this study, an explanation was proposed that selective utilization of ammonium and nitrate ions was responsible for the variation of pH in the course of culture. Viewing the reports that
many acidic and basic substances were produced by molds\(^1\), there is a possibility that the variation of pH depends also on the products formed by this fungus. This problem remains to be further investigated. Besides it will be an interesting subject to solve how ammonium and nitrate ions, together with the other nitrogen sources, in timothy leaves are practically utilized by C. phlei.

**Summary**

Utilization of inorganic nitrogen for the timothy leaf spot fungus, *Cladosporium phlei* de Vries, was examined in this study. When the fungus was cultured in glucose-ammonium nitrate medium supplemented with thiamine and pyridoxine, the pH in the medium lowered gradually during the first 17 days and thereafter rose. A similar experiment was conducted using the medium excluding any vitamin and essentially the same result was obtained. The lowering and rising of pH at the early and later stages were explained in terms of preferential utilization of ammonium ion prior to active utilization of nitrate ion.

**Acknowledgements**

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**References**


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チモシー斑点病菌 Cladosporium phlei de Vries の生理学的ならびに生化学的研究

第2報 無機窒素の利用性について

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要 旨

チモシー斑点病菌 Cladosporium phlei de Vries によるアンモニア態窒素および硝酸態窒素の利用性を調べた。本実験はビタミンB1とB6を添加したブドウ糖・硝酸アンモニウム培地で増殖させると、培地のpHが17日目まで低下し続けその後上昇することが認められた。ビタミンを含まない培地を用いて同様の実験を行なったところ、本菌の増殖速度はかなり低下したが、pHの変動はビタミンB1とB6を添加した培地の場合とほぼ同じ傾向をたどった。本実験において得られた結果から、本菌の増殖過程におけるpHの変動は最初にアンモニア態窒素が優先的に利用され、その後硝酸態窒素が利用されることに帰因することが明らかとなった。

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