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Nitrogen Requirements of Some Ascosporic Members of the Aspergillus nidulans Group.

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Out of several Aspergilli known the nitrogen metabolism of three of them viz., *Aspergillus fischeri* (Wenck, Peterson and Fred, 1935) *Aspergillus oryzae* (Tamiya and Usami, 1940) and *Aspergillus niger* (Steinberg, 1942) has been investigated. Of all nitrogen compounds, amino acids have a specific and important role in the metabolism of an organism. Further, it has been reported (Lilly and Barnett, 1951) that a mixture of amino acids may or may not be utilized better than a single amino acid because the effect of one amino acid on the utilization of another depends upon the organism employed. The present study deals with a comparative study of five ascosporic species of *Aspergillus nidulans* group with respect to their nitrogen requirements. It also includes estimation of the capacity of these organisms to utilize various amino acids when supplied singly and also in a mixture.

Materials and Methods

Pure cultures of the following species of *A. nidulans* group were selected for the present study: *A. nidulans* (Eidam) Wint., *A. rugulosus* Thom and Raper, *A. quadrilineatus* Thom and Raper, *A. variecolor* (Berk. and Br.) Thom and Raper and *A. violaceus* Fennell and Raper. The following basal medium was used: Sucrose 10 gms.; KH_2PO_4 1 gm., $MgSO_4$. $7H_2O$ 0.5 gm., KCl 0.5 gm. and distilled water 1000 ml. To this basal medium various nitrogen compounds were added singly so as to furnish 494 mgs. of nitrogen per litre except in case of peptone which was added in amounts equal to that of sodium nitrate. 20 ml. of each of the media was poured in a set of fifteen 150 ml. Pyrex Erlenmeyer flasks. The flasks were inoculated by seeding equal volumes of ascospore suspensions on the surface of the medium. They were then incubated at 25° C for 15 days. After the period of incubation the contents of each flask were filtered separately on a previously weighed filter paper. The filter papers containing the mycelium were then dried at 65° C for two days and the difference in dry weights was taken as the weight of the fungal mats. The data thus obtained were classified into good, moderate and poor on the basis of their statistical analysis, in which the standard error (S. E.) was calculated by the formula

$$\text{S. E.} = \sqrt{\frac{\text{Mean square of the error}}{\text{Number of the replicates}}}$$

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and the critical differenze (C. D.) was determined by the formula

$$\text{C. D.} = \text{S. E.} \times t \times \sqrt{2}$$

The degree of sporulation was based on critical visual obervation as practised by many workers.

In the second series of experiments the fungi were separately grown in flasks containing glutamic acid, aspartic acid, histidine, alanine and leucine and on the mixture of these amino acids. The method of inoculation, period of incubation and temperature were the same as in the previous experiment. The culture media of 1—15 days were chromatographically analysed by circular partition paper chromatography (Mehrotra & Agnihotri, 1961). The pH of all the media was adjusted at 6.5 before autoclaving. The chromatograms were run in n-butanol, acetic acid and water (4:1:5). They were then dried at room temperature and sprayed with 0.1 % ninhydrin in n-butanol. The Rf values of various bands were noted and they are given at appropriate places in the text.

Observations

The results are tabulated in Table 1. From a perusal of the table it would be apparent that in all cases the behaviour of the different species studied is different. These results are of special significance because the species taken were closely related in morphological characters.

Both the nitrate as well as the nitrite nitrogen were mostly excellent sources of nitrogen for the growth and sporulation of the present fungi. The final pH of the media in both the cases shifted towards the alkaline side. Ammonium nitrogen supported only poor growth but considerably lowered the pH of the medium.

While thiourea was a poor source for the present organisms, urea was found to be a good source for *A. quadrilineatus* and *A. variecolor* but for the rest of Aspergilli it was a moderate source. Peptone and acetamide, in general, proved to be good sources for the growth of all the species under investigation.

Of the 14 amino acids tested only serine proved to be equally good for all species of Aspergilli investigated. Arginine was good for the growth of *A. violaceus*, *A. quadrilineatus* and *A. variecolor* and moderate for *A. rugulosus* and *A. nidulans*. Histidine was poor for *A. rugulosus*, moderate for *A. nidulans* and for the rest of species it was a good source of nitrogen. Asparagine was utilised well by *A. rugulosus*, *A. quadrilineatus* and *A. variecolor* but was moderate and poor for *A. violaceus* and *A. nidulans* respectively.

Of the amino acids, belonging to dicarboxylic group, viz., glutamic acid and aspartic acid, the former was a poor source for *A. variecolor* but was a good source for the rest of the Aspergilli and the latter acid

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Table Showing the dry weight in mgs., sporulation and drift in pH by *A. nidulans*, *A. rugulosus*, *A. variecolor*, *A. violaceus*, and *A. quadrilineatus* on the 15th day.

Nitrogen Compounds	*A. nidulans* Dry wt.	Sporulation	pH	*A. rugulosus* Dry wt.	Sporulation	pH	*A. variecolor* Dry wt.	Sporulation	pH	*A. violaceus* Dry wt.	Sporulation	pH	*A. quadrilineatus* Dry wt.	Sporulation	pH
1. Sodium nitrate	92.0	++++	8.0	97.3	++++	8.2	110.0	++++	8.1	108.6	++++	8.4	86.6	++++	8.0
2. Sodium nitrite	83.0	++++	8.2	54.0	++++	9.1	102.0	+++	7.9	72.3	++++	8.2	65.3	++++	8.5
3. Ammonium chloride	42.0	+	4.0	24.0	+	4.3	39.0	+	4.0	36.0	+	4.0	30.0	+	4.0
4. Ammonium nitrate	33.0	+	5.2	20.3	+	5.2	37.0	+++	5.0	41.6	+	4.5	21.3	++	5.5
5. Urea	60.0	+++	6.7	63.6	+++	6.7	102.0	+++	7.6	62.0	++++	7.1	67.6	++++	7.0
6. Peptone	88.0	++++	7.0	93.0	++++	6.7	117.3	++++	7.0	93.3	++++	7.0	72.0	++++	7.3
7. Acet-amide	81.3	++++	7.0	69.0	+++	6.1	91.6	+++	7.9	90.0	++++	8.2	101.0	+++	6.4
8. Thio urea	33.0	+	6.1	18.3	+	5.5	16.0	+	5.5	22.3	+	5.8	16.0	+	5.5
9. Dl-Leucine	48.6	++++	7.3	48.6	++++	7.9	74.3	+++	7.6	71.0	++++	7.0	55.3	+++	7.0
10. Dl-Iso-leucine	54.6	++++	7.0	77.3	++++	7.3	80.6	++++	7.0	63.3	++	7.0	91.6	+++	7.0
11. L- Aspartic acid	62.0	++++	9.4	68.0	++++	8.8	61.0	++	8.5	71.0	++++	8.3	66.6	+++	8.5
12. L- Glutamic acid	71.3	++++	8.5	74.0	+++	8.5	67.6	++++	8.8	74.0	++++	8.5	71.0	++	9.4
13, Glycine	60.0	++++	7.0	76.3	++++	6.7	76.3	+++	7.0	67.0	+++	7.0	58.3	+++	6.7
14. Dl-Serine	83.6	++++	6.8	109.6	++++	6.1	95.0	+++	7.3	73.3	+++	7.2	85.0	+++	7.3
15. L- Arginine	65.0	++++	6.7	59.6	++++	7.0	107.3	++++	7.0	82.0	++++	7.0	97.3	++++	7.0
16. L- Histidine	57.6	++++	6.8	52.3	++++	6.7	77.6	+++	7.0	68.6	+++	6.7	70.6	+++	6.7
17. L- Cystin	73.6	+++	4.3	67.3	++	4.6	60.6	+	4.6	54.3	++	4.1	76.3	+++	4.0
18. L- Methionine	51.6	+	4.6	51.6	+	5.4	72.6	+	6.7	60.0	+	5.5	45.0	+	4.0
19. L- Asparagine	52.0	+++	5.8	71.3	++++	6.1	79.0	++++	6.1	65.0	++++	6.0	70.3	++	6.1
20. L- Phenylalanine	62.6	+	4.0	75.3	+	4.3	66.3	+	4.0	51.0	+	4.0	66.3	+	4.0
21. Dl-Alanine	66.0	+++	7.0	79.0	++++	7.3	81.3	+++	7.0	75.3	++++	6.9	65.6	++++	6.7
22. Dl-Valine	62.3	+++	6.7	71.6	+++	6.1	59.0	+++	6.7	51.0	+++	6.5	65.3	+++	6.1
23. Hydroxylamine	62.3	+++	4.3	55,0	++	3.7	78.0	+	4.5	66.3	++	4.8	46.0	++	4.8
24. Control	0.0		0.0	0,0			0.0			0.0			0.0		
General mean	60.4			61.5			73.02			63.09			62.1		

Degree of Sporulation: ++++ excellent; +++ good; ++ fair; + poor.

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was good for *A. rugulosus*, *A. violaceus* and *A. quadrilineatus*, moderate for *A. nidulans* and poor for *A. variecolor*.

Of the sulphur containing amino acids methionine was a moderate source for *A. violaceus* and *A. variecolor*, but was a poor nitrogen source for the rest of the Aspergilli. Cystin was good for *A. rugulosus*, A. *nidulans* and *A. quadrilineatus*, moderate for *A. variecolor* and poor for *A. violaceus*.

All the six monoamino-monocarboxylic acids exhibited different behaviour with the different species under experiment. Glycine proved to be a good source for *A. rugulosus* and *A. violaceus* but for the rest it was a moderate source. Dl-valine supported good growth of *A. rugulosus*, moderate of *A. nidulans* and *A. quadrilineatus* and poor for *A. violaceus* and *A. variecolor*. Dl-alanine was good for all Aspergilli except *A. quadrilineatus* for which it was a moderate source. Leucine was good for *A. violaceus*, moderate for *A. variecolor* and poor for the rest of the species. Iso-leucine was good for *A. rugulosus*, *A. quadrilineatus* and *A. variecolor*, moderate for *A. violaceus* and poor for *A. nidulans*. Serine supported good growth of all the Aspergilli under investigation.

Utilization of individual amino acids: The results of chromatographic analysis of individual amino acids are depicted in Fig. 1 and the dry weights in Table 2. Leucine was totally consumed on the 8th day by *A. quadrilineatus*, on the 10th day by *A. nidulans* and *A. rugulosus*, on the 13th day by *A. variecolor*. However, *A. violaceus* could not consume it within the incubation period. Histidine was fully utilized on the 10th day by *A. violaceus* and *A. variecolor*, on the 12th day by *A. nidulans* and *A. rugulosus* but *A. quadrilineatus* could not finish it even within the incubation period. Aspartic acid was assimilated completely on the 8th day by *A. variecolor*, on the 12th day by *A. violaceus* and the rest of Aspergilli could nit consume it within the incubation period. Glutamic acid was fully consumed on the 8th day by all the species except *A. nidulans* which could do so on the 9th day. Alanine was consumed on the 6th day by *A. quadrilineatus*, on the 8th day by *A. nidulans*, on the 10th day by *A. rugulosus*, and on the 12th day by *A. violaceus* and *A. variecolor*.

Assimilation rate of amino acids in a mixture

The results of the chromatographic analysis are depicted in Fig. 2. Dry weights of the mycelium are given in Table 2 and changes in pH of the media are graphically represented in Fig. 3.

It has been found that histidine was completely consumed on the 3rd day by *A. rugulosus* and *A. variecolor*, on the 4th day by *A. violaceus* and *A. quadrilineatus* and on the 5th day by *A. nidulans*. Glutamic acid was assimilated completely by the 6th, 8th and 10th day by *A. rugulosus*, *A. quadrilineatus* and *A. nidulans* respectively while *A. violaceus* and *A. variecolor* could not finish it within the incubation period.

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Table 2.

Table showing the dry weight in mgs. on individual amino acids and on their mixture on 5th, 10th and 15th day by *A. nidulans, A. rugulosus, A. violaceus, A. variecolor* and *A. quadrilineatus* respectively.

Amino acids	Days of incubation	*A. nidulans*	A. *rugulosus*	*A. violaceus*	*A. variecolor*	*A. quadrilineatus*
1. Alanine (Rf. O. 53)	5	36.0	40.0	28.0	25.0	30.0
	10	60.0	68.0	56.0	49.0	48.0
	15	66.0	79.0	75.3	81.3	65.6
2. Histidine (Rf. O. 21)	5	20.0	24.0	36.0	28. 0	36.0
	10	34.0	40.0	48.0	45.0	68.0
	15	57.6	52.3	68.0	76.6	68.0
3. Leucine (Rf. 0.8)	5	18.0	20.0	37.0	33.0	22.0
	10	30.0	45.0	48.0	50.0	53.0
	15	48.6	48.6	71.0	74.3	55.3
4. Glutamic acid (Rf. O.47)	5	35.0	33.0	25.0	31.0	36.0
	10	62.0	72.0	48.0	52.0	56.0
	15	71.3	74.0	74.0	67.3	71.0
5. Aspartic acid (Rf. O.43)	5	21.0	33.0	35.0	28.0	25.0
	10	48.0	42.0	68.0	52.0	44.0
	15	62.0	68.0	71.0	61.0	66.3
6. Mixture of amino acids	5	40.0	53.0	57.0	44.0	58.0
	10	64.0	77.0	80.0	67.0	70.0
	15	84.0	80.0	92.0	69.0	98.0

Aspartic acid was used up on the 8th day by *A. violaceus, A. rugulosus* and *A. quadrilineatus* and on the 10th and 12th day by *A. nidulans* and *A. variecolor* respectively. Leucine was completely assimilated on the 6th day by *A. nidulans* and *A. rugulosus* while *A. quadrilineatus, A. violaceus* and *A. variecolor* could do so on the 4th, 5th and 8th day respectively. Alanine was consumed on the 4th, 5th, 6th, 8th and 12th day by *A. quadrilineatus, A. variecolor, A. nidulans, A. rugulosus* and *A. violaceus* respectively.

It was also observed that almost all the amino acids included in the mixture were completely finished within the incubation period. The same amino acids when supplied singly were not fully utilized even upto the 15th day. Further it was found that both good and poor amino acids were utilized simultaneously from the mixture from the very beginning. A simultaneous decrease in the intensity of bands of all the amino acids was observed from 2nd day to the end of the incubation period viz., 15th day.

All the Aspergilli shifted the pH of the medium towards the alkaline side.

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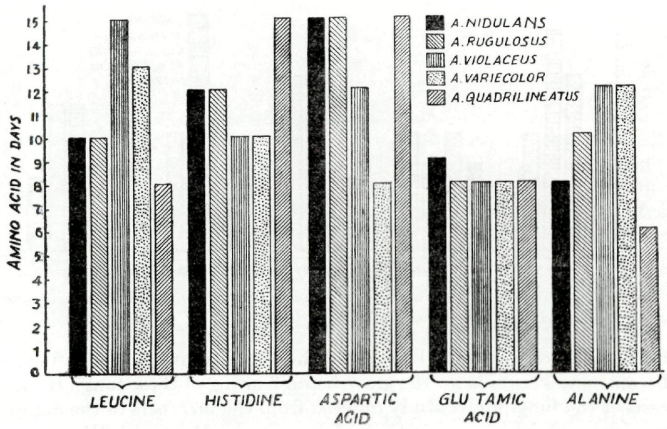

Fig. 1. Utilization of leucine, histidine, aspartic acid, glutamic acid and alanine by *A. nidulans*, *A. rugulosus*, *A. violaceus*, *A. variecolor* and *A. quadrilineatus*.

Discussion

From the results obtained in the present investigation it is evident that all the Aspergilli tested able to utilize nearly all forms of nitrogen.

It has been proved by many workers that nitrites are toxic for the growth of many fungi. The toxicity of nitrites on the acid pH values is due to the presence of free nitrous acid ions in the medium (Cochrane, 1950) which produce a destructive effect on the proteins and amino acids of fungal cells. (Lilly and Barnett, 1951). In the present investigation sodium nitrite permitted favourable growth of most of the Aspergilli and the same has been the finding of Sakaguchi and Wang (1936) for *Aspergillus oryzae* and *A. aureus*. Lemoigne et al. (1936) working with *Aspergillus niger* found that this organism was capable of reducing nitrite to hydroxylamine which the fungus could employ as a source of nitrogen for protein synthesis. Possibly this is also true in the case of the present species of Aspergilli.

The growth of Aspergilli in media containing nitrate nitrogen with the accompanying fall in the pH of the medium shows that these species are capable of reducing nitrate nitrogen to the reduction level of ammonia.

Ammonium nitrogen when supplied as such was poorly utilized by these moulds. This was due to the sharp fall in the pH level which resulted due to the more rapid utilization of anions of the ammonium nitrogen than cations. Even in media supplied with ammonium nitrate

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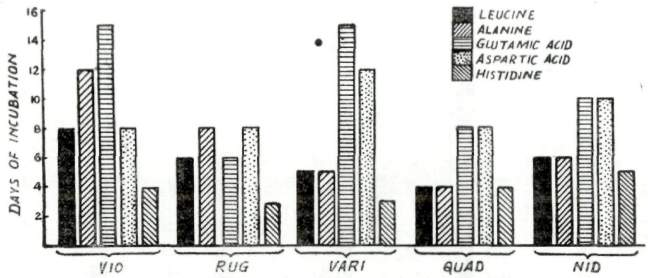

Fig. 2. Utilization of amino acids in a mixture by *A. violaceus, A. rugulosus, A. variecolor, A. quadrilineatus* and *A. nidulans.*

the pH was considerably lowered to the extent that the medium soon became unfavourable for the growth and fruiting of these fungi. In this respect the fungi under study differed from the members of the Saprolegniaceae (Bhargava, 1945) and from some Mucorales (Raizada, 1957) where ammonium nitrate has been found to be a good source of nitrogen.

Good growth of the present species in peptone was because it is made up of a number of nitrogenous compounds and water soluble vitamins (Stokes et al. 1944). Lilly and Barnett (1951) have reported that peptone is a useful source of nitrogen when it is desired to culture a large number of species on a single medium.

Urea has been found to be a favourable source of nitrogen for the growth of the present species because of the general occurrence of its hydrolysing enzyme urease in moulds.

Of the amino acids tried aspartic and glutamic acids were important. Both of them supported equal amount of growth of all the Aspergilli but glutamic acid favoured slightly better growth than aspartic acid because of the larger ratio of carbon with nitrogen and because of its utility in respiration.

Though no definite explanation for the better growth of these Aspergilli on a mixture of amino acids than on a single amino acid is at present available yet most probably it is due to the capacity of these organisms to produce more easily a number of enzymes in minute quantities to assimilate amino acids than a single enzyme in large quantity to assimilate a single amino acid.

There are two theories with regard to the utilization of amino acids (Lilly and Barnett, 1951). According to on the amino acids are deaminated before utilization and the other holds that the amino acids are utilized directly.

In the present investigation the fall in pH in early stages of assimilation of amino acids and rise in pH in later stages was due to

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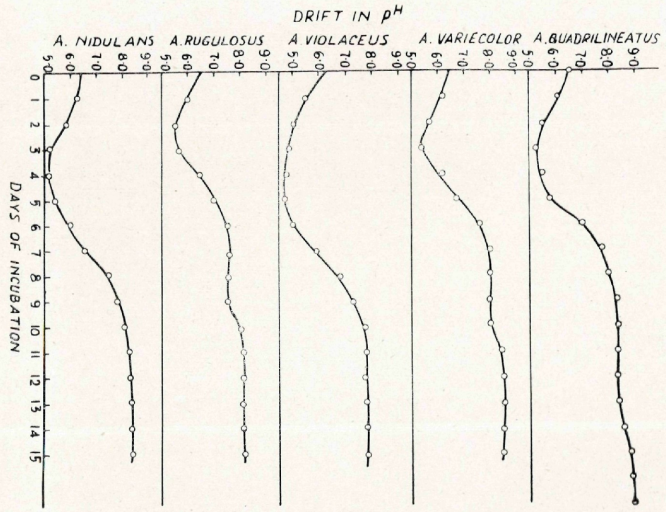

Fig. 3. Drift in pH during the utilization of amino acids in a mixture by *A. nidulans*, *A. rugulosus*, *A. violaceus*, *A. variecolor* and *A. quadrilineatus*.

the utilization and accumulation of ammonia from and in the medium respectively.

No exhaustive studies have been made in the past on the role of nitrogenous compounds on sporulation. Hawker (1950) is of the opinion that no generalization can be made regarding the fruiting in relation to nitrogen sources. The present results support her statement as no correlation between growth and sporulation was seen and practically all grades of sporulation were recorded with significantly good and poor growth.

Summary.

Nitrogen requirements of *Aspergillus nidulans* (Eidam) Wint,. *A. rugulosus* Thom and Raper, *A. violaceus* Fennell and Raper, *A. variecolor* (Berk. and Br.) Thom and Raper and *A. quadrilineatus* Thom and Raper were studied under controlled conditions. These organisms were able to utilize organic and anorganic forms of nitrogen. Of the six monoamino mono carboxylic acids, serine proved to be best for all *Aspergillus* spp. The sulphur containing amino acid i. e., methionine was a moderate source for *A. violaceus* and *A. variecolor* but was a poor source for rest of the Aspergilli tested. Cystin was good for A. *rugulosus*, *A. nidulans* and *A. quadrilineatus*, moderate for *A. variecolor*

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and poor for *A. violaceus*. Of the two dicarboxylic acids, glutamic acid was found to be better than aspartic acid for all the Aspergilli studied.

Chromatographic studies showed that there was no antagonistic effect of different amino acids and even the poor sources of amino acids did not adversely influence the growth when they were given in combination with good amino acids.

There was no correlation between growth and sporulation.

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