Fruit Body Formation of Red Bread Mold *Neurospora crassa* IV. Effect of Ammonium and Nitrate Ion in the Medium on Size of the Perithecium

by Taro ITO*

Received September 3, 1961

A study on the mechanism of the production of the perithecium in the fertilization stage of the sexual reproduction process, including protoperithecial formation, ascospore production and fruit body formation, should be generally completed after information upon their genetical and physiological natures has been obtained. In physiological study on the effect of concentration of KNO_3 on the formation of the female sexual organ and fruit body, Hirsch¹⁾ has suggested that the existence of the nitrate ion had no direct relation to the fertilization and the fruit body development, but showed an effective influence on the differentiation of the female sexual organ. Further he showed that the exhaustion of the nitrate ion in the culture medium was not necessary for the formation of a female sexual organ, but for the formation of perithecia.

In the sexual reproduction, the process of the formation of the fruit body following sexual organ formation seems to be one of morphogenesis. Therefore, in connection with the investigation on the morphogenesis of this fungus, an analysis of the external and internal factors which affect the reproductive growth phase, seems to be a subject of considerable importance. In previous papers^{2,3)}, the author has shown that the perithecia were produced in the culture medium containing both ammonium and nitrate salts in a particular ratio, and that perithecia without ascospore were formed on the single strain mycelium in the culture filtrate which was obtained from the culture medium containing these two nitrogen salts at the beginning of the fertilization. These investigations involve the examination on the factors controlling the cell formation (heterogenous development), in association with the expression of sexuality in morphogenesis. For the further understanding of the fruit body formation, it may be necessary to analyse the factors affecting perithecial enlargement (homogenous development). The present report deals mainly with the effects of concentration ratio of nitrate to ammonium ion on the development of perithecial size.

Materials and Methods

The strains used in this experiment are designated as 4A and 8a. They are macroconidial wild type strains which form fruit bodies, after 10 to 14 days, on synthetic media containing inorganic salts, inorganic nitrogen, suitable organic carbon and the vitamin biotin. The minimum medium used for this experiment is the crossing medium designated by Westergaard and Mitchell⁴⁾. The four kinds of minimal media were prepared for the test of perithecial formation. The medium used for the study on the effects of ammonium and nitrate ion in the culture mixture was the one supplemented with ammonium tartrate of 10 different concentrations from $0.5 \times 10^{-3}\text{M}$. to $0.5 \times 10^{-2}\text{M}$. and potassium nitrate (10^{-2}M .). In the other culture series, KNO_3

* Laboratory of Botany, Dairy Section, Obihiro Zootechnical College, Obihiro, Japan.

solutions were prepared at 10 different final concentrations from 10^{-3} M. to 10^{-2} M. with ammonium tartrate (10^{-2} M.). The effect of a sole nitrogen source was similarly tested by the independent application of one of these nitrogen sources in the concentration range from 10^{-3} M. to 2×10^{-2} M. The quantitative analysis for the residual ammonium- and nitrate-ion was carried out spectrophotometrically, and the hydrogen ion concentration in the liquid medium was also measured.

Experimental Results

In the first experiment, the influences of both nitrate and ammonium salts in the cross medium on protoperithecial and perithecial development were tested by measuring the diameter of perithecia formed on the slant medium in the test tube. The effect of CF on the formation of the protoperithecium and the perithecium was tested by the use of the crossing medium of Westergaard and Mitchell⁴). +CF was obtained from the liquid synthetic media, in which a single culture of A (+) type strain hyphae was made, and similarly -CF was prepared from the culture solution of a (-) type strain. Ten of both the protoperithecia and perithecia formed by 10 to 14 days incubation at 25° were picked up at random and their diameters were measured with the micrometer under microscope. The results are shown in Table 1-(I) & (II).

As seen in Table 1-(I), remarkable stimulation of the growth of perithecia was observed when both the ions were supplied at the ratios of 10:10, 5:10, 3:10 and 0:10. The effect of +CF upon the growth of perithecia was striking at the ionic ratios of 2:10 and 0:10, and that of -CF was at 10:10 and 7:10. Significant negative correlation was recognized between the concentration of ammonium ion and size of the perithecium. The correlation coefficient between the size of perithecia and the amount of ammonium or nitrate ion was higher in the ammonium minimum medium rather than in the nitrate minimum medium. It was also found that the perithecial growth

Table 1-(I). Size of perithecia formed on the culture media supplemented with the various amount of both ammonium and nitrate.

Conc. grade	0	1	2	3	4	5	6	7	8	9	10
Conc. ratio [NH ₄ ⁺]:[NO ₃ ⁻]	0:10	1:10	2:10	3:10	4:10	5:10	6:10	7:10	8:10	9:10	10:10
Range in size (μ)	583— 699.6	—	408.1— 583	466.6— 699.6	408.1— 524.7	408.1— 583	349.8— 583	349.8— 466.6	408.1— 583	349.8— 466.4	408.1— 583
Average size (μ)	641.3 ± 18.5		489.6 ± 19	606.2 ± 24	478.0 ± 4.6	512.8 ± 1.8	442.6 ± 19	419.4 ± 6.5	478.0 ± 19	396.2 ± 19	500 ± 28

Cor. co. (1)*: -0.8.

Cor co. (2)*: -0.5.

Conc. grade	11	12	13	14	15	16	17	18	19	20	21
Conc. ratio [NH ₄ ⁺]:[NO ₃ ⁻]	10:0	10:1	10:2	10:3	10:4	10:5	10:6	10:7	10:8	10:9	10:10
Range in size (μ)	—	—	—	349.8— 699.6	408.1— 583	408.1— 641.3	560.2— 594.6	349.8— 524.7	466.4— 699.6	408.1— 641.3	466.4— 583
Average size (μ)				489.6 ± 30	510.2 ± 21	501.2 ± 21	583.0 ± 0	478.0 ± 21	537.6 ± 25	501.2 ± 30	513.1 ± 21

Cor. co. (1)*: -0.01.

Cor. co. (2)*: 0.2.

Table 1-(II). Size of perithecia formed on the crossing medium supplemented with the CFs from the culture of either A (+) or a (-)

+CF	Conc. grade	0	1	2	3	4	5	6	7	8	9	10
	Range in size (μ)	466.4—641.3	233.2—524.7	466.4—699.6	256.4—583	466.4—699.6	466.4—699.6	408.1—641.3	466.4—699.6	349.8—583	408.1—583	408.1—583
	Average size (μ)	559.2 ± 16.2	384.6 ± 25.5	570.8 ± 23.2	489.6 ± 16.2	537.6 ± 25.5	536.0 ± 23.2	537.6 ± 23.2	489.6 ± 23.2	489.6 ± 23.2	489.6 ± 16	478.0 ± 23.2
Cor. co. (1)*: -0.1.						Cor. co. (2)*: 0.04.						
+CF	Conc. grade	11	12	13	14	15	16	17	18	19	20	21
	Range in size (μ)	174.9—233.2	291.5—524.7	233.2—408.1	233.2—466.4	—	174.9—349.8	326.0—466.4	349.8—641.3	349.8—524.7	233.2—466.4	—
	Average size (μ)	209.4 ± 2.5	361.4 ± 25	302.8 ± 5.8	326.0 ± 23	—	279.6 ± 23	421.0 ± 23	489.6 ± 30	421.0 ± 5.8	326.0 ± 28	—
Cor. co. (1)*: 0.1.						Cor. co. (2)*: 0.2.						
-CF	Conc. grade	0	1	2	3	4	5	6	7	8	9	10
	Range in size (μ)	349.8—583	—	291.5—466.4	349.8—583	349.8—524.7	349.8—524.7	408.1—583	524.7—757.9	291.5—583	349.8—524.7	408.1—583
	Average size (μ)	454.2 ± 27.8	—	333.0 ± 20.8	454.2 ± 25.5	442.6 ± 16.2	442.6 ± 16.2	501.2 ± 18.7	583 ± 18.7	419.4 ± 30.3	442.6 ± 16.2	489.6 ± 18.7
Cor. co. (1)*: 0.3.						Cor. co. (2)*: 0.6.						
-CF	Conc. grade	11	12	13	14	15	16	17	18	19	20	21
	Range in size (μ)	291.5—524.7	233.2—583	233.2—349.8	233.2—466.4	233.2—583	349.8—524.7	233.2—466.4	233.2—466.4	174.9—466.4	291.5—349.8	233.2—583
	Average size (μ)	419.4 ± 18	361.4 ± 30	291.5 ± 14	326.0 ± 26	421.0 ± 31	442.6 ± 17	373.0 ± 34	373.0 ± 29	337.6 ± 35	304.4 ± 15	361.4 ± 30
Cor. co. (1)*: 0.05.						Cor. co. (2)*: 0.1.						
Without any CFs**		Range in size (μ)		421.0—583								
		Average size (μ)		478.0 ± 14								

* The correlation coefficient between size and ammonium (1) or nitrate (2).

** The crossing medium without supplementation of the CFs.

decreased more strikingly with an increase in the amount of ammonium ion added than with that of nitrate ion. The positive correlation between the amount of ammonium ion and the size of perithecia formed was revealed by the application of -CF. In order to ascertain the difference of the effect of varied ionic ratios ($[\text{NH}_4^+]: [\text{NO}_3^-] = 10^{-3} \sim 9 \times 10^{-3}: 10^{-2}$ or $10^{-2}: 10^{-3} \sim 9 \times 10^{-3}$) between the two lots, the analysis of variance of the means was calculated. No highly significant difference was observed between the means of the two lots ($P > 0.05$).

The second experiment was carried out to see the effect of ammonium and nitrate minimum medium on the growth of perithecia and also to confirm the influence

of the high concentration of the sole nitrogen source of either ammonium or nitrate. The concentration range of the synthetic media was made up between 10^{-3} M. and 2×10^{-2} M. The results are shown in Table 2.

Table 2. Size of perithecia formed on the culture media supplemented with the sole nitrogen ion of either ammonium or nitrate.

Conc. grade	0	1	2	3	4	5	6	7	8	9	10
Conc. of $(\text{NH}_4)_2\text{C}_4\text{H}_4\text{O}_6$	0	$10^{-3} \times 0.5$	$\times 1$	$\times 1.5$	$\times 2$	$\times 2.5$	$\times 3$	$\times 3.5$	$\times 4$	$\times 4.5$	$\times 5$
Range in size (μ)	466.4–583	357.0–571.2	357.0–571.2	428.4–571.2	428.4–714	357.0–642.2	499.4–856.8	428.4–571.2	428.4–714	357.0–571.2	—
Average size (μ)	542.0 ± 28	485.2 ± 23	485.2 ± 23	485.2 ± 29	613.8 ± 30	499.4 ± 33	571.2 ± 39	499.4 ± 21.3	571.2 ± 25	527.8 ± 29	

Cor. co. (1): 0.4.

Conc. grade	11	12	13	14	15	16	17	18	19	20
Conc. of $(\text{NH}_4)_2\text{C}_4\text{H}_4\text{O}_6$	$\times 5.5$	$\times 6$	$\times 6.5$	$\times 7$	$\times 7.5$	$\times 8$	$\times 8.5$	$\times 9$	$\times 9.5$	$\times 10$
Range in size (μ)	428.4–571.2	285.6–428.4	213.8–428.4	285.6–499.4	—	213.8–356.6	—	213.8–356.6	—	285.6–356.6
Average size (μ)	542.0 ± 28	328.2 ± 4.3	314.1 ± 21	385.0 ± 25.5		285.6 ± 18		285.6 ± 3		314.1 ± 3

Cor. co. (1): -0.3.

Conc. grade	0	1	2	3	4	5	6	7	8	9	10
Conc. of KNO_3	0	$10^{-3} \times 1$	$\times 2$	$\times 3$	$\times 4$	$\times 5$	$\times 6$	$\times 7$	$\times 8$	$\times 9$	$\times 10$
Range in size (μ)	—	—	285.6–856.8	—	571.2–714	357.0–642.2	214.2–499.4	214.2–571.2	357.0–642.2	—	—
Average size (μ)			456.9 ± 42		585.4 ± 22.5	499.4 ± 24	357.0 ± 20	385.0 ± 35	471.0 ± 34		

Cor. co. (2): 0.3.

Conc. grade	11	12	13	14	15	16	17	18	19	20
Conc. of KNO_3	$10^{-2} \times 1.1$	$\times 1.2$	$\times 1.3$	$\times 1.4$	$\times 1.5$	$\times 1.6$	$\times 1.7$	$\times 1.8$	$\times 1.9$	$\times 2$
Range in size (μ)	349.8–524.7	—	408.1–524.7	408.1–524.7	291.5–583	291.5–466.4	291.5–466.4	291.5–583	233.2–583	291.5–524.7
Average size (μ)	542.0 ± 24		501.2 ± 19	478.0 ± 15	489.6 ± 36	421.0 ± 29	421.0 ± 26	542 ± 25	408.1 ± 18	419.4 ± 23

Cor. co. (2): -0.8.

As seen in Table 2, the mean obtained in the first experiment was higher than that in the second experiment. A significant increase in size of the perithecium was observed at the three concentration grades 4, 8 and 11.

In the third experiment the residual nitrogen ion in the ammonium minimum medium in culture of either A (+) or a (-) strain was quantitatively measured by the spectrophotometrical method to find out the effect of residual ammonium and nitrate ion in the CFs (Fig. 1).

Fig. 1. The residual amount of NH_4^+ and NO_3^- in the CF. The signs, and , show the residual concentration of NH_4^+ and NO_3^- , and the initial concentration of both ions.

The residual amount of nitrate in the +CF decreased less than 10 per cent of the initial one at the concentration grades from 1 to 4 and from 8 to 10, while it increased at the grade 7. The residual amount of ammonium decreased at the grades from 1 to 5, 7 and 9, while it increased at the grades 6, 8 and 10. In the -CF the residual amount of nitrate decreased at the all concentration grades from 1 to 10 and that of ammonium increased at the concentration grades from 6 to 10. Both the ions were exhausted when supplied at the lower concentration ratio of these ions. Moreover, it has been reported that the pH change of the culture solution during the growing period of the mating type strain has some influence on an exhaustion of the nutrients⁵). Since it became clear that a specific ratio of ammonium to nitrate in the culture solution is necessary for the perithecial growth as shown in the previous experiment, the pH value of the culture filtrate was measured after 96, 144 and 216 hrs.

The pH value decreased to less than the initial one in 96 hrs. with an increase

Table 3. pH value at various culture durations on the media with the various amount of either ammonium or nitrate. Initial pH; 5.8-6.0.

CF	Conc. grade										
	Culture duration	1	2	3	4	5	6	7	8	9	10
+CF	96 hrs.	6.0	4.2	4.4	4.2	4.2	4.2	3.8	3.8	3.8	4.0
	144	4.9	4.7	4.9	4.9	4.6	4.5	4.7	4.7	4.7	4.5
	216	6.5	6.6	6.2	6.2	3.7	4.7	4.5	4.7	5.5	5.7
-CF	96	6.0	4.2	4.2	4.2	4.2	4.2	3.4	3.4	3.8	4.0
	144	4.7	4.5	4.5	4.3	4.3	4.3	4.5	4.5	4.3	4.3
	216	6.4	6.7	6.8	6.0	6.0	5.9	5.5	5.5	5.5	5.5
±CF	96	4.0	3.6	3.8	3.8	4.0	3.8	3.4	3.4	3.8	4.0
	144	5.1	4.7	4.7	4.7	4.5	4.5	4.7	4.7	4.7	4.7
	216	3.6	3.8	3.7	3.4	3.6	3.4	3.8	4.6	4.6	4.7

of the amount of ammonium in either the +CF or -CF, and then returned gradually to the initial value in 216 hrs.

Discussion

For understanding the significance of both ammonium and nitrate ions for the growth of the perithecia, the present work was designed chiefly to investigate two main points, (1) the pattern of utilization of both NH_4^+ and NO_3^- ions in the media by these strains, and (2) the effect of the culture filtrate obtained from the media containing these ions, on the growth of perithecia. As shown in Table 1-(I), the average diameter of the perithecium in the medium with 10^{-2} M. nitrate ion was larger than in that with 10^{-2} M. ammonium ion. Similarity between the two experimental lots was recognized below the probability 0.05. The ammonium ion as the sole nitrogen source was more effective for the perithecial growth in concentrations below 10^{-2} M. than in those above 10^{-2} M. (Table 2). On the other hand, the nitrate ion was effective evenly over these ranges of concentrations and the its efficacy was almost equivalent to that of ammonium ion at the middle concentration grade. Consequently it may be stated that the ammonium ion may be utilized relatively well for the perithecial growth in the lower concentration range; on the other hand, the nitrate ion may act effectively over a wide range of concentration, but with little effect upon the size of perithecia. The negative correlation coefficient of growth of the perithecium with the amount of ammonium ions was higher than that of nitrate ions. The growth rate of perithecia rose progressively with a decrease in the amount of ammonium ion to zero (Table 1). This fact suggested also that ammonium ions acted in an inhibitory way, while nitrate ions acted productively.

In order to clarify the mechanism of utilization of these ions for the growth of perithecia, the residual amount of these ions and the pH change of the culture media were measured. No correlation was recognized between the perithecial growth revealed at the concentration grades 0.2, 4.5 and 6 in +CF and at the concentration grades 7 and 10 in -CF, and the residual amounts of these ions at these concentration grades. The significance of the ammonium minimum medium for the formation of perithecia lay in the +CF and the -CF, as was shown in a previous paper³). The effect of the ammonium ion on the growth of perithecium seemed to lie in the CFs (Table 1.). The pH value 4.6~4.8 of the medium during the vegetative growth decreased in 96 hrs. and returned to the initial value after 144 hrs. followed by a

slight rise after 216 hrs. In study on the perithecial formation of *Sordaria fimicola*, Lilly and Barnett⁶⁾, have stated that perithecia were formed only after the pH value of the medium had risen above 6 in a medium containing 25g. glucose and casein hydrolysate (equivalent to 20 g. casein), but this pH alone was not sufficient to insure sexual reproduction. Esser⁷⁾ confirmed the increase of pH value of the medium at the time of the spore formation in *Podospira anserina*. Ryan *et al.*⁸⁾ stated that the decrease of the pH value during the growth of mycelia was due to the removal of NH_4^+ , while the pH value rose when only NO_3^- was used for growth. It is reasonable to assume that the decrease of pH value during the early growing stage observed in the author's experiment (Table 3) may depend upon the exhaustion of NH_4^+ ; on the other hand its increase in the later growing stage may be due to the utilization of NO_3^- . Therefore, it may be concluded that a small amount of NH_4^+ is used during the early stage of growth, while NO_3^- is used during the later growing stage for the production of some substance which stimulates the perithecial formation.

Summary

The present report deals with the effect of two nitrogen ions, ammonium and nitrate, on the perithecial growth in *Neurospora crassa*.

The growth of perithecia is activated in media with both ammonium tartrate (0.5×10^{-3} M. $\sim$ 0.5×10^{-2} M.) and potassium nitrate (10^{-2} M.) rather than in those with ammonium tartrate (0.5×10^{-2} M.) or potassium nitrate (10^{-3} M. $\sim$ 10^{-2} M.). It is also made evident that the perithecial enlargement should be related to some stimulative substance produced by either A(+) or a(-) strain in the media of the special ionic concentration. Ammonium ions may be utilized in the relatively lower concentration range ($< 10^{-2}$ M.) at the early growing stage, while nitrate ions may act effectively over a wide range of concentration at the later growing stage.

References

- 1) Hirsch, H. M., *Physiologia Plantarum* 7: 72 (1954).
- 2) Ito, T., *Bot. Mag. Tokyo* 69: 369 (1956).
- 3) —, *ibid.* 72: 852 (1959).
- 4) Westergaard, M., and Mitchell, H. K., *Amer. Jour. Bot.* 34: 573 (1947).
- 5) Hawker, E. L., *Camb. monographs in Exp. Biol.* 6 (1957).
- 6) Lilly, V. G., and Barnett, H. L., *Amer. Jour. Bot.* 34: 131 (1947).
- 7) Esser, K., *Compt. rend. Lab. Carlsberg, Ser. Physiol.*, 26: 7 (1960).
- 8) Ryan, F. G., Beadle, G. W., and Tatum, E. L. *Amer. Jour. Bot.* 30: 784 (1943).

摘 要

伊藤太郎：アカパンカビの子実体形成 IV.

培地中におけるアンモニウムイオンと硝酸イオンの被子器の直径に対する影響

本報は *Neurospora crassa* の被子器生長におよぼす二種の窒素源イオン、アンモニウムならびに硝酸イオンの影響に関する研究報告である。

被子器生長はアンモニウム最小培地（酒石酸アンモニウム 0.5×10^{-3} — 0.5×10^{-2} M. 硝酸加里 10^{-2} M.）で、とくに NH_4^+ と NO_3^- の濃度比が 10:10, 5:10, 3:10 および 0:10 の場合に良好である。この添加窒素源の特定量添加のさいにみられる生長作用は、 NH_4^+ と NO_3^- の特定濃度比を有する合成培地中で A(+) 系および a(-) 系が、それぞれその反対性型系、すなわち、a(-) 系および A(+) 系菌系に原被子器 protoperithecia 形成を誘起し、さらにその生長を助長する性ホルモン様物質を生成するためであることが明らかとなった。 NH_4^+ は比較的低濃度で成育初期に、 NO_3^- は比較的大い濃度範囲で成育後期に有効に利用されると思われる。（帯広畜産大学酪農学教室）