Ca2+イオンによるAphanomyces euteiches Drechslerの卵胞子形成の誘導

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Induction of Oospore Formation of *Aphanomyces euteiches* Drechsler by Calcium Ion

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Abstract

Abundant oospores of *Aphanomyces euteiches* Drechsler were formed in corn meal broth culture, however, none in nutrient broth culture. The extract of mycelial mat in corn meal broth culture induced mycelial mat in nutrient broth culture to form oospores. This indicated the possibility of an inducer in the extract to form oospores. This was further supported by the fact that inorganic fractions from the extract induced oospore formation. Among the inorganic compounds that are possibly included in inorganic fractions, CaO, $CaCO_3$ and $CaCl_2$ have induced oospore formation. From these results, it was thought that Ca^{2+} might play a role in inducing oospore formation. Ca^{2+} concentrations of corn meal broth and nutrient broth were 1.3×10^{-4} and 8.4×10^{-6} M, respectively. The eight inorganic compounds, containing Ca^{2+}, were able to induce oospore formation and of these, $CaCl_2\cdot2H_2O$ was the most effective at 10^{-4} M. Oospore formation did not occur in corn meal broth with a Ca^{2+} content lower than 7×10^{-5} M by the addition of chelating reagents. We conclude from these facts that Ca^{2+} is indispensable for the oospore formation in *A. euteiches*.

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Key words: *Aphanomyces euteiches*, Ca^{2+} ion, oospore formation.

INTRODUCTION

Oospores of the plant pathogenic oomycetous fungi are important as survival structures[11] and as organs of heredity. Oospores have been investigated from several view points including observation of oosporogenesis with light[2,3,5] and electron[2,3,6] microscopies, the effects of nutrients and ions[1,4,6,12,17], cyclic nucleotides[9] and sexual hormones[15] on oospore formation, as well as existence of mating types[7].

It is important to understand the mechanism of oospore formation in order to prevent diseases caused by oomycetous fungi.

Abundant oospores of *Aphanomyces euteiches* Drechsler are produced in the host, *Pisum sativum* L. and in corn meal broth. However, there is no report on suitable chemically defined medium for inducing oospore formation by this fungus. In preliminary studies, we failed to induce oospore formation with several chemically defined media generally used to stimulate oospore formation by oomycetous fungi.

In this paper, we report on the result of the identification of constituents needed for oospore formation by *A. euteiches*.

MATERIALS AND METHODS

Media. The following eight chemically defined media (without agar) were used: Leal's medium[10], Sakai's medium[16], GG medium[5], Czapek Dox medium, Haglund's medium[8], Papavizas and Davey's medium[14], modified Papavizas and Davey's medium[13] and Yang and Schoulties's medium[18].

As the natural media, corn meal broth (CMB) and nutrient broth (NB) were used. CMB was prepared from Difco corn meal agar (CMA). Agar was removed from CMA (17 g) by extractions performed three times with 300 ml of distilled water (DW). During the extraction, CMA was stirred in DW for 15 min and then centrifuged for 10 min at 3000 rpm. The supernatant from each extraction was collected and made up to 1000 ml with DW and then sterilized. NB was prepared with Difco nutrient broth.

Culturing. A 7-mm diam. CMA culture disc of the test fungal isolate was inoculated to 25 ml each of

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culture media in a petri dish (9-cm diam.). Before inoculation, the nutrient of inoculum disc was removed by repeated dipping of the disc in sterile distilled water (SDW). *A. euteiches* isolate, AE-F3 used throughout this experiment was incubated at 25°C for 6–7 days in the case of CMB and 4–5 days in the case of NB unless otherwise stated.

Addition of chelating reagents to CMB. Three chelating reagents, EDTA (ethylendiaminetetraacetic acid). GEDTA (glycoetherdiaminetetraacetic acid) and Quin 2 (8-amino-2-((2-amino-5-methylphenoxy)methyl)-6-methoxyquinoline-*N,N,N′,N′*-tetraacetic acid, tetrapotassium salt) were used. Each reagent was added to CMB to make concentrations from 5×10^{-5} to 10^{-3} M and then the media were sterilized. Final Ca^{2+} concentration in the medium was checked by a pH-Ion Meter (Denki Kagaku Keiki 10 L-30) before use.

Preparation of mycelial exudate and extract.

Exudate. A mycelial mat in CMB culture (MM-C) was washed three times with SDW and kept for 24 hr in 5 ml of SDW at 5°C to collect the exudate. The MM-C was then removed and the water containing exudate was filtered through a membrane filter (3 μm in pore size) and made up to 5 ml with SDW. This solution was designated as the original exudate solution.

Extract. A washed MM-C was homogenized for 5 min at 8000 rpm together with 5 ml SDW. Then the homogenate was centrifuged for 10 min at 3000 rpm. The resulting supernatant was made up to 5 ml with SDW. This solution was designated as the original extract solution.

The experiment was conducted aseptically.

Purification of the inducer of oospore formation from MM-C. Mycelial mats harvested from cultures in 1500 plates were throughly washed with DW and used for obtaining the inducer. The inducer was purified according to the procedure shown in Fig. 1.

Oospore formation test. After the inoculum disc was removed from mycelial mat in NB culture (MM-N), the mycelial mat was washed three times with 20 ml of SDW. About 6-cm diam. of thin fungal mat uniformly adhered to the bottom of petri dish was finally obtained. Five milliliters of the test solution was added to this mycelial mat. Formation of oospore was checked after 2 days of incubation at 25°C. The number of mature oospores from each mycelial mat in four microscopic fields (2.1 mm in diam. 50×) were counted. The test was conducted three times. In each test, oospore formation on MM-C which fluctuated from 250 to 280 per microscopic field, was considered as the control. The effect of the test sample was expressed as an equation : (mean no. of oospores per microscopic field in the test sample/mean no. of oospores per microscopic field in MM-C)× 100.

RESULTS

Selection of test media

Selection of suitable media for this experiment were performed using eight chemically defined media and two natural media. Growth and oospore formation of five isolates of *A. euteiches* (F 3, Ma-3, O-33, E-31, HA) were

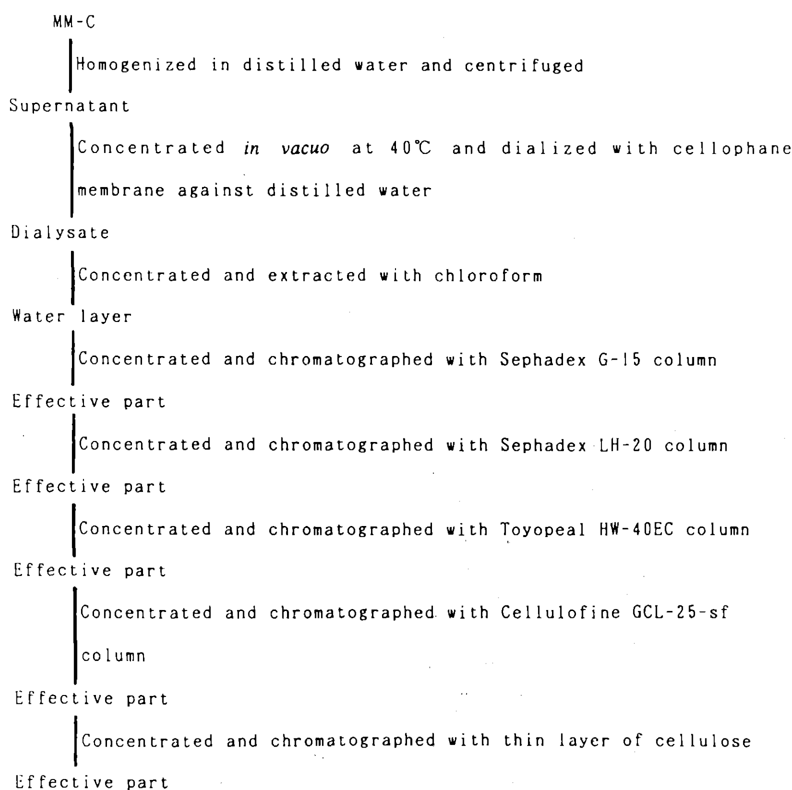

Fig. 1. Procedure to purify inducer of oospore formation from mycelial mat in corn meal broth culture (MM-C).

observed for 3 weeks after the inoculation.

Among the chemically defined media, Czapek Dox medium, Papavizas and Davey's medium, modified Papavizas and Davey's medium and Yang and Schoulties's medium supported poor growth forming irregular and thick colonies but the other four did not. In modified Papavizas and Davey's medium and Yang and Schoulties's medium, aborted oospores were rarely formed and in other two media, no oospore was formed at all.

CMB supported good growth and oospore formation. NB gave thin and uniform mycelial growth but no oospore formation.

No difference was observed among the five isolates on the growth rates and oospore formation ability.

CMB and NB were selected to evaluate the factors related to oospore formation. Especially the mycelial mat grown in NB was suitable for the direct microscopic observation.

Effects of exudate and extract from MM-C on oospore formation

In MM-C, 279 oospores per microscopic field were formed whereas in MM-N, no oospore was formed even after a long period of incubation.

It was considered that the fungus synthesized or absorbed the substance that induces oospore formation from CMB. To make sure of the presence of the inducer of oospore formation in MM-C, its exudate, extract or those diluted solutions were added to MM-N. The result is shown in Table 1.

Oospore formation was induced by the original exudate solution and it's 2 fold diluted solution, but not in the case of 5 and 10 fold diluted solutions. Oospore formation was induced strongly by the original extract solution, but only slightly by it's 2 and 5 fold diluted solutions. This result ascertained the presence of the inducer of oospore formation in MM-C.

Identification of inducer of oospore formation in MM-C

With the procedure used to purify the inducer (Fig. 1), five effective fractions were detected on the thin layer chromatography of cellulose. There was no sign of organic substance in any of these fractions by the analysis with H′-NMR (JEOL 400 MHz) spectra. Most parts of each fraction did not burn. These fractions were then analyzed with ESCA (Shimadzu ESCA-850). The detected elements in five fractions are C, O, Ca, Mg, Na, Cl, I and S. In these, C, O, Ca, Mg were detected in all fractions.

Inorganic compounds which are probable to be included in these fractions, are $NaHCO_3$, NaCl, Na_2CO_3, $MgCO_3$, MgO, CaO, $CaCl_2$ and $CaCO_3$.

Oospore formation was observed after adding 10^{-3} to 10^{-5} M solutions of these compounds and elements (S and I) to MM-N.

CaO, $CaCl_2$ and $CaCO_3$ induced oospore formation, but others did not. This result suggested that Ca^{2+} might be the inducer of oospore formation present in MM-C.

Certification of Ca^{2+} as an inducer of oospore formation

To prove if Ca^{2+} is the inducer of oospore formation, the following tests were performed:

1) Ca^{2+} concentrations in CMB and NB. Concentrations of Ca^{2+} in CMB and NB were 1.3×10^{-4} M and 8.4×10^{-6} M, respectively, determined with a pH-Ion Meter.

2) Effect of inorganic compounds containing Ca^{2+} on oospore formation. Several concentrations of solutions of eight inorganic compounds containing Ca^{2+} ($CaSO_4 \cdot 2H_2O$, CaO, $CaCO_3$, $CaMoO_4$, $CaCl_2 \cdot 2H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, $CaHPO_4 \cdot 2H_2O$ and $Ca(OH)_2$) were added separately to MM-N and the oospore formation was tested. The result is summarized in Table 2.

All eight compounds induced oospore formation. A solution of 10^{-4} M was the most effective for all the compounds except CaO, in which 10^{-5} M was the most effective. With $CaSO_4 \cdot 2H_2O$, there was little difference in the effectiveness between 10^{-4} M and 10^{-5} M solutions. $CaCl_2 \cdot 2H_2O$ was the most effective followed by

Table 1. Induction of oospore formation with exudate and extract of mycelial mats in corn meal broth culture (MM-C) at different dilutions[a)]

Sample	Oospore formation (%)[b)]			
	1	1/2	1/5	1/10
Exudate	14.22	4.53	0	0
Extract	24.49	0.46	0.15	0

a) Exudate or extract derived from a MM-C was made up to 5 ml with sterile distilled water (SDW). This original solution designated as 1 in concentration was diluted at 1/2, 1/5 or 1/10 with SDW. Five milliliters of each solution was added to mycelial mat in nutrient broth culture.

b) Expressed as: (a number of oospores per microscopic field in the test sample/a number of oospores per microscopic field in MM-C) ×100.

Table 2. Effect of Ca^{2+} containing inorganic compounds on oospore formation

Compound	Oospore formation (%)[a)] at different concentrations (M)				
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
$CaSO_4 \cdot 2H_2O$	—[b)]	—	10.95	7.01	0
CaO	—	—	0.17	13.36	6.70
$CaCO_3$	—	—	14.97	7.34	9.17
$CaCl_2 \cdot 2H_2O$	3.10	43.18	50.11	4.42	0
$Ca(NO_3)_2 \cdot 4H_2O$	0.06	4.30	18.12	2.01	0
$CaHPO_4 \cdot 2H_2O$	—	—	6.36	0.23	3.21
$CaMoO_4$	—	—	8.66	3.50	0.06
$Ca(OH)_2$	—	—	7.11	3.56	0.40

a) Expressed as: (a number of oospores per microscopic field in the test sample/a number of oospores per microscopic field in the mycelial mat cultured in corn meal broth) ×100.

b) Not tested because of incomplete of dissolution of the compound.

Table 3. Oospore formation in mycelial mat in corn meal broth culture containing various chelating reagents

Concentration of chelating reagent (M)	EDTA		GEDTA		Quin 2	
	Oospore (%)[a)]	Concentration of Ca^{2+} (M)[b)]	Oospore (%)	Concentration of Ca^{2+} (M)	Oospore (%)	Concentration of Ca^{2+} (M)
10^{-3}	0	2.7×10^{-6}	0	6.3×10^{-5}	0	1.2×10^{-7}
5.0×10^{-4}	0	8.2×10^{-6}	63.1	6.8×10^{-6}	0	4.7×10^{-7}
2.5×10^{-4}	0	3.2×10^{-5}	97.5	9.7×10^{-5}	0	3.4×10^{-5}
10^{-4}	81.0	1.0×10^{-4}	97.4	1.1×10^{-4}	76.4	7.6×10^{-5}
5.0×10^{-5}	84.2	1.3×10^{-4}	80.8	1.3×10^{-4}	81.8	1.0×10^{-4}

a) Expressed as: (a number of oospores per microscopic field in the test sample/a number of oospores per microscopic field in mycelial mat cultured in corn meal broth) $\times100$.
b) Concentrations of Ca^{2+} in CMB supplemented chelating reagents were measured by pH-Ion Meter before inoculation.

$Ca(NO_3)_2\cdot4H_2O$. $Ca(OH)_2$ was the least effective.

3) Addition of chelating reagents to CMB. To reduce the availability of Ca^{2+} in CMB, three chelating reagents, EDTA, GEDTA and Quin 2 were added separately to CMB and oospore formation was tested. The result is shown in Table 3.

In media containing more than 2.5×10^{-4} M EDTA or Quin 2 growth was inhibited completely and oospore formation was slightly inhibited at concentrations below 10^{-4} M.

In the medium containing 10^{-3} M GEDTA, mycelial growth also was inhibited completely. Oospores were well formed at the concentrations from 5×10^{-5} to 2.5×10^{-4} M, but inhibited in some degree at 5×10^{-4} M GEDTA.

Inoculum that failed to grow in the media containing chelating reagents, grew and formed oospores when reinoculated to CMB.

These results indicate that more than 7×10^{-5} M of Ca^{2+} is required for oospore formation.

4) Oospore formation in NB added Ca^{2+}. Ca^{2+} was added to NB as $CaCl_2\cdot2H_2O$ to make the Ca^{2+} concentrations from 10^{-5} to 10^{-2} M. No oospore was formed at all concentrations. Some inhibitory factors for oospore formation other than Ca^{2+} deficiency may be involved.

DISCUSSION

In this study, we proved the requirement of Ca^{2+} for oospore formation by *A. euteiches*. *Pythium* species[17] and *Saprolegnia diclina* Humphrey[12] are known to require Ca^{2+} for oogonium formation. Requirement of Ca^{2+} for the oospore maturation was suggested in *Phytophthora cactorum* Schroeter[4] and *S. diclina*[6].

There are few reports describing the detailed role of Ca^{2+} in the process of sexual organ formation of oomycetous fungi. Fletcher[6] observed the high rate of abortion of the sexual organs of *S. diclina* in Ca^{2+} deficient condition. In our experiment, Ca^{2+} seemed to regulate the oospore number but the proportion of aborted oospores was quite low regardless of the Ca^{2+} concentration. To understand the effects of Ca^{2+} on oospore formation as a whole, the observations like those performed with *S. diclina*[6], the Ca^{2+} distribution in mycelium, the biochemical effects of Ca^{2+} and combinations of these aspects are required.

In *A. euteiches*, oospore formation was induced most effectively at 10^{-4} M of Ca^{2+}. In *Pythium debaryanum* Hesse[17], the optimum level of Ca^{2+} for oospore formation was found to be 10^{-2} M. The maturation of oospores in *P. graminicola*[17] and *P. cactorum*[4] was promoted effectively at 3.4×10^{-4} and 10^{-3} M of Ca^{2+}, respectively. These differences in effective Ca^{2+} concentration for sexual organ formation, probably, are derived from many factors, such as constituents of medium and growth stage of fungus when Ca^{2+} is provided.

We tried to ascertain the effectiveness of Ca^{2+} on oospore formation with several methods such as concentration of Ca^{2+} in CMB compared to NB, inhibition of oospore formation in CMB with chelating reagents and the induction of oospore formation with inorganic compounds containing Ca^{2+}. The results of all these methods led us to conclude that Ca^{2+} might be the inducer of oospore formation. However, when Ca^{2+} was added to NB, *A. euteiches* failed to form oospores. As NB contains abundant proteins and amino acids, it is possible that these substances made complexes with Ca^{2+} rendering it unavailable for use by the fungus.

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和 文 摘 要

横沢菱三・国永史朗・佐久嶋明世・関崎春雄：Ca^{2+}イオンによる *Aphanomyces euteiches* Drechsler の卵胞子形成の誘導

Aphanomyces euteiches Drechsler は corn meal broth (CMB) で卵胞子を旺盛に形成するが，nutrient broth (NB) では全く形成しない。CMB 培養菌体抽出物を NB 培養菌体に与えると卵胞子形成が誘導されることを利用し，CMB 培養菌体中の卵胞子形成誘導物質を調べた。分離と分析の結果，CMB 培養菌体中の無機成分が卵胞子形成を誘導した。この無機成分中に含まれる元素を分析し，それらの元素の組合せから生じる無機化合物の卵胞子形成の誘導を調べると，$CaCO_3$, $CaCl_2$ および CaO のみにその効果が見られた。このことから Ca^{2+} が卵胞子形成を誘導すると考えられた。CMB と NB の Ca^{2+} 濃度は，それぞれ 1.3×10^{-4} M, 8.4×10^{-6} M であった。Ca^{2+} を含む 8 種類の無機化合物を種々の濃度で NB 培養菌体に与えると，いずれの化合物も卵胞子形成を誘導した。この中で $CaCl_2$ が最も作用が強く，10^{-4} M が卵胞子形成の誘導に最適な濃度であった。キレート剤の種々の量を CMB に加えて，有効な Ca^{2+} を減じると，Ca^{2+} の濃度が 7×10^{-5} M より低いと卵胞子の形成は認められなかった。これらのことから，*A. euteiches* の卵胞子形成には Ca^{2+} が不可欠であることが示された。