Evaluation by specific inhibitors of metabolic dependence of asexual differentiation processes in *Phytophthora capsici*

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Summary : Metabolic dependence of differentiation processes during asexual life cycle of *Phytophthora capsici* was evaluated by the use of specific inhibitors whose modes of the action have been known. Actinomycin D, an RNA synthesis inhibitor, interfered with vegetative growth, and formation and germination of zoosporangia of the fungus, but had negligible adverse effects on swimming of zoospores and germination of encysted zoospores. Cycloheximide, a protein synthesis inhibitor, inhibited germination of encysted zoospores besides the processes inhibited by actinomycin D. Oligomycin, an oxidative phosphorylation inhibitor, inhibited all the differentiation processes. Antimycin A, an electron transport inhibitor, however, failed in inhibiting vegetative growth and germination of encysted zoospores. This was probably due to impermeability of the inhibitor through the plasma membrane and qualitative difference in the membrane at those antimycin A-insensitive phases of the fungus was suggested. The difference in the plasma membrane was also indicated by the sensitivity to nystatin, a membrane sterol inhibitor : nystatin did not inhibit vegetative growth and germination of encysted zoospores, but did more and less other processes. Vinblastin, a microtubule inhibitor, inhibited strongly formation and germination of zoosporangia, and swimming of zoospores, and slightly vegetative growth. None of the differentiation processes evaluated was found to be very sensitive to cytochalasin B, a microfilament inhibitor. From these results, possible requirements of the specific metabolisms or functions for the individual differentiation process was postulated. Furthermore, no requirement of RNA and protein synthesis for swimming of zoospores, as indicated by insensitivity to the inhibitors, was confirmed by tracer experiments.

Introduction

A genus of plant pathogenic fungi, *Phytophthora*, undergoes various morphogenic processes in its sexual and asexual life cycle[10] and appears to provide a superior organism for studying the differentiation mechanism involved in eukaryotic cells. We are beginning to study some biochemical parameters that would control asexual differentiation processes of this fungus.

Researches up to now on this aspect in *Phytophthora* have been mostly directed toward the effects of circumstantial factors such as moisture, oxygen, light, and temperature[10], and only little toward the biochemical mechanisms though the requirement of sterols for zoosporangial formation and its indirect germination has been extensively studied[3]. Another

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recent biochemical finding on the differentiation mechanisms of this fungus is one by Yoshikawa and Masago[8] that initiation of zoosporangial formation in *P. capsici* is under the control of catabolite repression which can be reversed by cyclic AMP.

The present report aims to show, as assessed by the use of specific metabolic inhibitors, that different morphological processes involved in asexual life cycle of *P. capsici* require to differential degrees the *de novo* RNA and protein syntheses, microtubule and microfilament functions, and sterols in membrane, besides continuous active respiration throughout the cycle. The results on RNA and protein synthesis requirements were further confirmed by direct measurements of the synthetic activity which was evaluated by incorporation of radioactive amino acid and nucleoside.

Materials and methods

Phytophthora capsici Leonian was maintained as stock cultures on V-8 juice agar (10 % Campbell Soup Corp. V-8 juice, 0.1 % $CaCO_3$, 1 % agar) and used throughout the experiments.

Inhibitors used were as follows: actinomycin D (Schwarz/Mann), an RNA synthesis inhibitor; cycloheximide (P-L Biochem.), a protein synthesis inhibitor; antimycin A (P-L Biochem.), an electron transport inhibitor; oligomycin (Sigma), an oxidative phosphorylation inhibitor; nystatin (serva), a membrane sterol inhibitor; cytochalasin B (Aldrich), a microfilament inhibitor; vinblastin (Sigma), a microtubule inhibitor[4]. Inhibitors were dissolved or finely suspended in 80 % ethanol as concentrated stocks and added to the assay medium at appropriate concentrations. Final concentrations of ethanol in the assay medium were 0.5 % or less that were non-toxic to all fungal differentiation processes examined.

The effect of the inhibitors on mycelial growth was evaluated in small glass cells on 0.2 ml V-8 juice agar containing the inhibitors at appropriate concentrations. Other differentiation processes except zoosporangial formation were evaluated as above but in water. Zoosporangia were synchronously produced according to the method developed by Yoshikawa *et al*[9] in 5 ml of 20 mM phosphate buffer containing the inhibitors. Zoospores were produced by incubating at 20°C in water the zoosporangia obtained by the method of Miyata *et al*[7] and encysted zoospores by shaking vigorously zoospore suspension for 30 sec. Inhibitory rates of mycelial growth were measured from radial growth, and those of other differentiation processes were evaluated under microscope after fixing samples by adding one tenth volumes of formal calcium solution (10 % formalin, 2 % calcium acetate).

Incorporations of radioactive precursors into RNA and protein were conducted as follows. Ten ml spore suspension in water was incubated at 20°C with 1 μCi of ^{14}C-lysine (Amersham, 348 mCi/mmol, uniformly labeled) for protein synthesis measurement or with 1 μCi of ^{14}C-uridine (Amersham, 524 mCi/mmol, uniformly labeled) for RNA synthesis measurement. After appropriate periods of incubation, 2 ml of samples were withdrawn and poured into 2 ml of 10 % ice-cold trichloroacetic acid (TCA), and left for 30 min at 0°C. The TCA insoluble materials were collected on glass filters (GF/A) and referred to as protein or RNA fraction. Radioactivity was measured by a liquid scintillation spectrometer (Packard Tri-Carb) after drying the filters and placing the filters into vials containing toluene

scintillation fluid (5 g PPO, 0.2 g POPOP per liter of toluene).

Protein content was determined by the method of Lowry *et al*[6].

Results

The asexual life cycle of *P. capsici* is illustrated in Fig. 1. Mycelial growth was sensitively inhibited by cycloheximide and oligomycin, and partially by actinomycin D and vinblastin (Table 1). Thus, the vegetative growth of this fungus was assumed to require active respiration, protein and RNA syntheses, and function of microtubule. Failure of antimycin A in interfering with the vegetative growth presumably arises from impermeability of the inhibitor across the plasma membrane since it is unlikely that electron transport system is not operating in this growth phase of the fungus. It follows that the plasma membrane of the vegetative phase may somehow differ from that of the reproductive phase of the fungus, and the difference appears to be correlated with the sensitivity with nystatin. This is in support for the proposal that membranes of vegetative phase of this fungus do not contain

Table 1. Effects of various specific inhibitors on different asexual differentiation processes of *Phytophthora capsici*

Inhibitor (cellular function interfered)	Concn. (ug/ml)	Per cent of inhibition				
		Asexual differentiation process[a]				
		A	B	C	D	E
Actinomycin D	1	0[b]	6.7[c]	7.4[d]	17.1[e]	2.8[f]
(RNA synthesis)	5	19.3	0.3	19.8	12.3	4.7
	10	38.5	48.9	30.2	15.4	4.0
Cycloheximide	0.5	96.2	89.3	36.9	19.3	82.2
(protein synthesis)	1	100.0	96.5	49.9	15.5	91.0
	5	100.0	100.0	78.6	20.8	99.6
Antimycin A	0.05	3.9	18.8	21.4	17.7	4.0
(electron transport)	0.2	0	22.4	55.9	57.1	0
	1	3.9	44.6	69.4	64.8	10.4
Oligomycin	1	59.9	71.5	57.1	79.0	6.9
(oxidative phosphorylation)	5	100.0	96.0	65.0	85.7	34.1
	10	100.0	98.1	65.6	88.6	89.8
Nystatin	5	0	47.8	16.1	13.6	19.0
(membrane, sterol)	20	0	73.6	21.7	10.6	16.3
	50	0	68.7	36.2	26.4	11.2
Vinblastin	10	15.6	14.3	19.1	22.5	15.6
(microtubule)	50	26.4	69.8	40.8	31.4	15.7
	100	30.3	98.2	74.5	92.5	13.4
Cytochalasin B	10	3.9	37.1	11.5	14.6	4.6
(microfilament)	50	3.9	14.6	17.6	16.3	10.4
	100	0	13.1	16.2	23.2	17.1

a A-E: refer to Fig. 1.
b The values are inhibition rates in radial growth of mycelia (24 hr incubation at 28°C).
c The values are inhibition rates in the number of zoosporangia formed (24 hr incubation at 28°C).
d The values are inhibition rates in per cent germination of zoosporangia (1 hr incubation at 20°C).
e The values are rates of per cent bursting of zoospores (1 hr incubation at 20°C).
f The values are inhibition rates in per cent germination of encysted zoospores (3 hr incubation at 28°C).

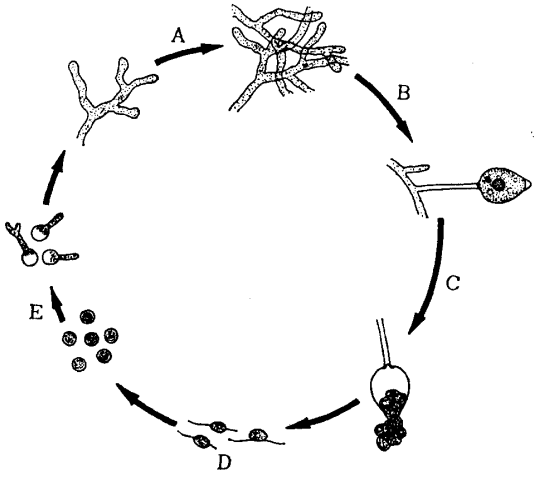

Fig. 1. Asexual life cycle in *Phytophthora capsici*.
A-E: differentiation processes
A: mycelial growth (vegetative growth)
B: zoosporangial formation
C: germination of zoosporangia
D: swimming of zoospores
E: germination of encysted zoospores

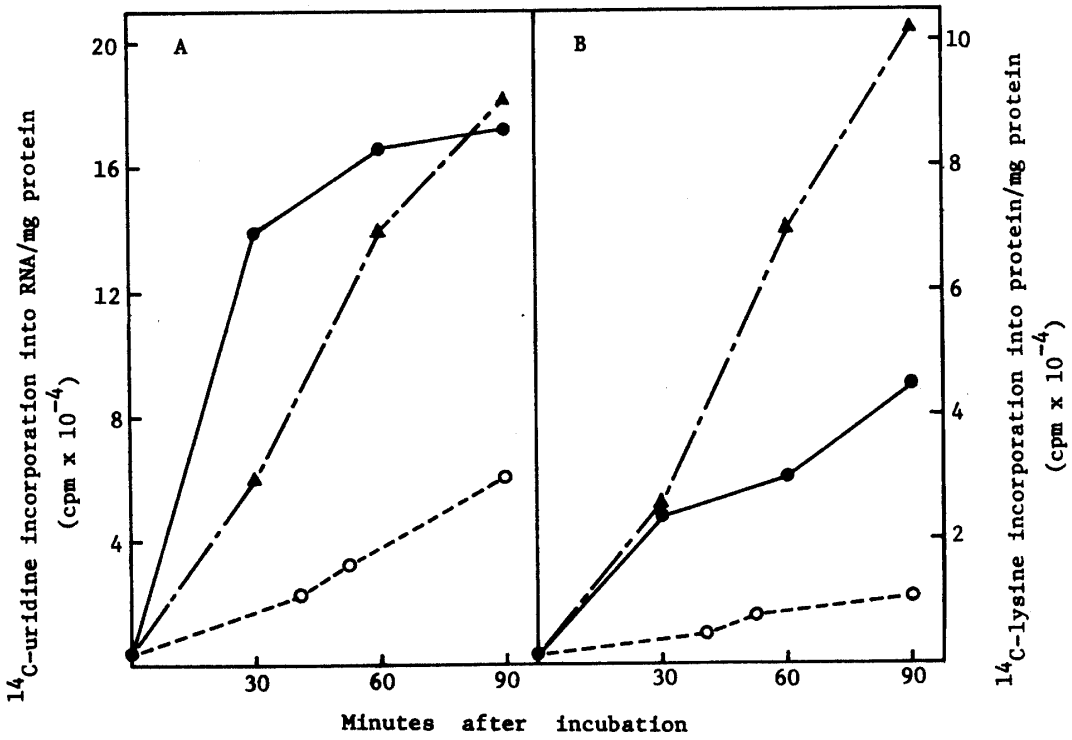

Fig. 2. RNA (left, A) and protein (right, B) synthetic activity as assessed by ^{14}C-precursor incorporations during germination of zoosporangia (●——●) and encysted zoospores (▲—·—▲), and swimming of zoospores (○·····○) in *Phytophthora capsici*.

sterols but do those of reproductive phases[3].

The process of zoosporangial formation appeared, from the sensitivity to the inhibitors, to require RNA and protein syntheses, and sterol and microtubule functions besides respiration. Requirements of RNA and protein syntheses in this process are consistent with observations on *Achlya*[2], but not on *Allomyces*[1]. The similar trends in the metabolic requirements were revealed in the process of zoosporangial germination. Swimming of zoospores were found to be sensitve only to respiratory and microtubule inhibitors, and partially to membrane sterol inhibitor, but not affected at all by inhibitors of RNA and protein syntheses. This stage of the fungus were, thus, not apparently associated with active RNA and protein syntheses.

This was confirmed by direct assessment of levels of RNA and protein syntheses (Fig. 2), in which only low levels of ^{14}C-uridine and lysine incorporations were revealed at the swimming stage when compared with those at the germination stages of zoosporangia and encysted zoospores. Dependence of the swimming on microtubule is well predicted since zoospores of this fungus possess typical microtubule-containing bi-flagella.

Active protein synthesis, but not RNA synthesis seemed to be required for germination of encysted zoospores. The results are in agreement with the germination of encysted zoospores of Blastochladiella[5] where the author found that RNA synthesis did not initiate in advance of the germination and the RNA synthesis *per se* was not required for the germination. Antimycin A again failed in inhibiting this process, and concomitantly nystatin insensitivity was observed.

Discussion

There have been several reports that assessed the dependence of differentiation processes on RNA and protein syntheses in fungi[1,2,5], but no such attempts have been made in the genus *Phytophthora*. Furthermore, no researches have appeared in evaluating microtubule and microfilament functions in the differentiation processes in fungi. The use of specific inhibitors for evaluating such metabolic dependence has been employed by many workers and proved to be an useful tool. Cares have to be, however, taken to assess the results: ineffectiveness of certain inhibitors would not necessarily implys that the metabolism referred to is not operating because the inhibitors may not permeate into the cells, as was the case of antimycin A in this study.

The present investigations revealed that metabolic dependence during the differentiation of *P. capsici* differs from one process to the other. RNA synthesis, as assessed by actinomycin D sensitivity, was presumed to be required for vegetative growth, and formation and germination of zoosporangia, but not for swimming of zoospores and germination of encysted zoospores. Protein synthesis would be required throughout all the asexual differentiation processes except for swimming of zoospores. All the processes, as can be predicted, required active respiration. Presence and function of sterol was also suggested, from nystatin sensitivity, during reproductive phases of the fungus such as zoosporangium and zoospore phases. This is of interest when referring to the sterol requirement for zoosporangial formation[3]. It was also suggested that some processes such as formation and germination of zoosporangia, and swimming of zoospores were largely dependent upon microtubule function. We believe that these results have given some preliminary insight for the differentiation mechanisms and the pre-requisite metabolism for the individual differentiation process on which further detailed researches should be focussed by employing more direct assay methods.

Literature cited

1) Burke, D. H., T. W. Seale, & B. J. McCarthy (1972): J. Bactriol. 110: 1065-1072.

2) Griffin, D. H. & C. Breuker (1969): J. Bacteriol 98: 689-696.

3) Hendrix, J. W. (1970): Ann Rev. Phytopathol. 8: 111-130.

4) Hepler, P. K. & B. A. palevitz (1974): Ann. Rev. Plant Physiol. 25: 309-362.

5) Lovett, J. S. (1968): J. Bacteriol. 96: 962-969.
6) Lowry, O. H., N. J. Rosebrough, A. L. Farr, & R. J. Randall (1957): J. Biol. Chem. 193: 265-275.
7) Miyata, Y., K. Katsura, & T. Murokawa (1970): Sci. Rept. Kyoto Pref. Univ. Agr. 22: 27-30.
8) Yoshikawa, M. & H. Masago: Submitted to Can. J. Bot.
9) Yoshikawa, M., A. Nishida, & H. Masago: Submitted to Can. J. Bot.
10) Zentmyer, G. A. & D. E. Erwin (1970): Phytopathology 60: 1120-1127.

摘 要 作用機作がすでに知られている各種特異的阻害剤を用いて，*Phytophthora capsici* の無性的生活環における種々の分化の過程の代謝依存性を推察した。RNA 合成阻害剤であるアクチノマイシン D は菌糸伸長，遊走子のう形成，ならびにその発芽を抑制したが，遊走子の遊泳や被のう胞子の発芽にはほとんど影響しなかった。蛋白合成阻害剤であるシクロヘキシミドはアクチノマイシン D で阻害を受けた過程と，さらに被のう胞子の発芽をも強く阻害した。酸化的リン酸化の阻害剤であるオリゴマイシンはすべての分化過程を阻害したが，同じ呼吸阻害剤であるアンチマイシン A（電子伝達系阻害剤）は菌糸伸長ならびに被のう胞子の発芽を阻害することができなかった。このことは，それらの過程で電子伝達系が作動していないと考えるよりは，アンチマイシンがそれらの過程において菌の細胞内にとり込まれにくいと考えるほうが妥当である．それと同時に，それらの過程においては物質の透過性をつかさどる菌の原形質膜に何らかの差異があることをも示唆する。このことは菌糸伸長ならびに被のう胞子の発芽過程がナイスタチン（細胞膜のステロール類と結合することにより膜機能を阻害する）に不感受性であることからも示唆される。microtubule 阻害剤であるビンブラスチンは遊走子のう形成ならびにその発芽を強く阻害し，また菌糸伸長をも若干阻害した。また microfilament 阻害剤であるサイトカラシン B は分化のどの過程に対しても顕著な阻害を示さなかった。これらの結果より，それぞれの分化の過程における特定の代謝の依存性が推察された。また，アクチノマイシン D とシクロヘキシミドに対する不感受性によって示された遊走子の遊泳期における RNA ならびに蛋白合成の不必要は ^{14}C のアミノ酸またヌクレオシドの取り込み実験からも支持された。