I. ULTRASTRUCTURE OF PERONOSPORA TRIFOLIORUM MYCELIUM IN ALFALFA LEAVES
II. STORAGE OF PERONOSPORA TRIFOLIORUM SPORANGIA

by 2214

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B. S., Kansas State College of Pittsburg, 1970

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

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I. ULTRASTRUCTURE OF PERONOSPORA TRIFOLIORUM MYCELIUM IN ALFALFA LEAVES.

The development and application of electron microscopy techniques has permitted a basic understanding of the ultrastructural organization of the interface between host and pathogen. Electron microscopy of obligate parasites has primarily dealt with haustoria and the sheaths surrounding them (1,3,4,13,18). The ultrastructural organization of the interface between the host and the completely intracellular obligate parasite, Plasmodiophora brassicae, is also known (19,20).

To our knowledge, the ultrastructure of an intercellular obligate plant parasite that does not produce haustoria has never been reported. Peronospora trifoliorum d By. offers this opportunity. Fraymouth (8) found a few haustoria of P. trifoliorum in small disintegrating areas of infected alfalfa (Medicago sativa L.) leaves collected in May and June and found none in infected leaves collected in August. During preliminary investigations with a light microscope we found no haustoria in infected alfalfa leaves.

This paper deals with the ultrastructure of P. trifoliorum, the interface between it and alfalfa leaf cells, and changes that occur in those host cells.

MATERIALS AND METHODS

Potted alfalfa plants with 5 days regrowth were placed at an 8-hr photoperiod with 500 ft-c of fluorescent and incandescent lighting at 18 C for 5 days. They were sprayed until run-off with a sporangial suspension (10^5sporangia/ml) (15), placed in the dark at 100% relative humidity for 16 hr, and then returned to the above conditions for 11-16 days.

Plugs were cut from chlorotic areas of infected leaves and from corresponding areas of control plants with a punch made from an 18 gage hypodermic needle. The material was immediately fixed in either 4% unbuffered $KMnO_4$ for 30 min at 0 C, or in 1% glutaraldehyde in 0.05 M S-collidine buffer pH 7.4 for 2 hr and the latter post-fixed for 1 hr in 1% osmium in 0.05 M S-collidine buffer pH 7.4 at 0 C. An ethyl alcohol dehydration series was used. The tissue was stained 30 min in saturated uranyl acetate in the 70% alcohol step. The material was embedded in Arildite Epon embedding medium (10), sectioned with glass knives on a Reichert ultra-microtome, mounted on 300 mesh copper grids, and examined with an RCA EMU-4 electron microscope.

RESULTS AND DISCUSSION

Peronospora trifoliorum sporangia germinate by germ tube which forms an appressorium and penetrates directly through the leaf surface within 12 hr after inoculation (15). The vegetative hyphae grow throughout the leaf conforming to intercellular spaces.

Fungal fine structure.--The fine structure of the vegetative hyphae of P. trifoliorum is similar to that reported for other Oomycetes (1,5,13,16). Numerous mitochondria were evenly distributed throughout the cytoplasm (Fig. 1-B; Fig. 2-B). Dictyosomes were also abundant (Fig. 1-B; Fig. 2-B). Endoplasmic reticulum was present in the form of paired parallel membrances (Fig. 2-B). Lomosomes, described by Moore and McAlear (11), were not found.

An extensive cytoplasmic membrane complex that consisted of rolls of interconnected membranes lying side by side was found (Fig. 1-B; Fig. 2-A). The complex width was from 0.3-0.5μ and was at least 5μ long. Similar cytoplasmic membrane complexes have been reported in species of Streptomyces (9), Actinomyces (12), Sporobolomyces (14), Penicillium (21), and Armillaria (2). A similar structure in Aphanomyces was referred to as a Golgi apparatus (16). Similar structures in other organisms are believed to be variations of the endoplasmic reticulum (14), involved in cross-wall formation (9,21), or involved in nuclear division (21).

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Fig. 1. A-D) Intercellular mycelium of Peronospora trifoliorum in alfalfa leaves. Host nucleus(N); chloroplast(C); host mitochondrion(M); host endoplasmic reticulum(ER); host plasma membrane(P); lipid bodies(L); host cell wall(W); fungal cell wall(w); fungal wall projection(wp); fungal plasma membrane(p); fungal mitochondrion(m); fungal dictyosome(d); membrane complex(c); fungal nucleus(n). A) Glutaraldehyde-osmium fixation (X11,000). B) Glutaraldehyde-osmium fixation (X15,000). C) Potassium permanganate fixation (X7,000). D) Potassium permanganate fixation (X57,000).

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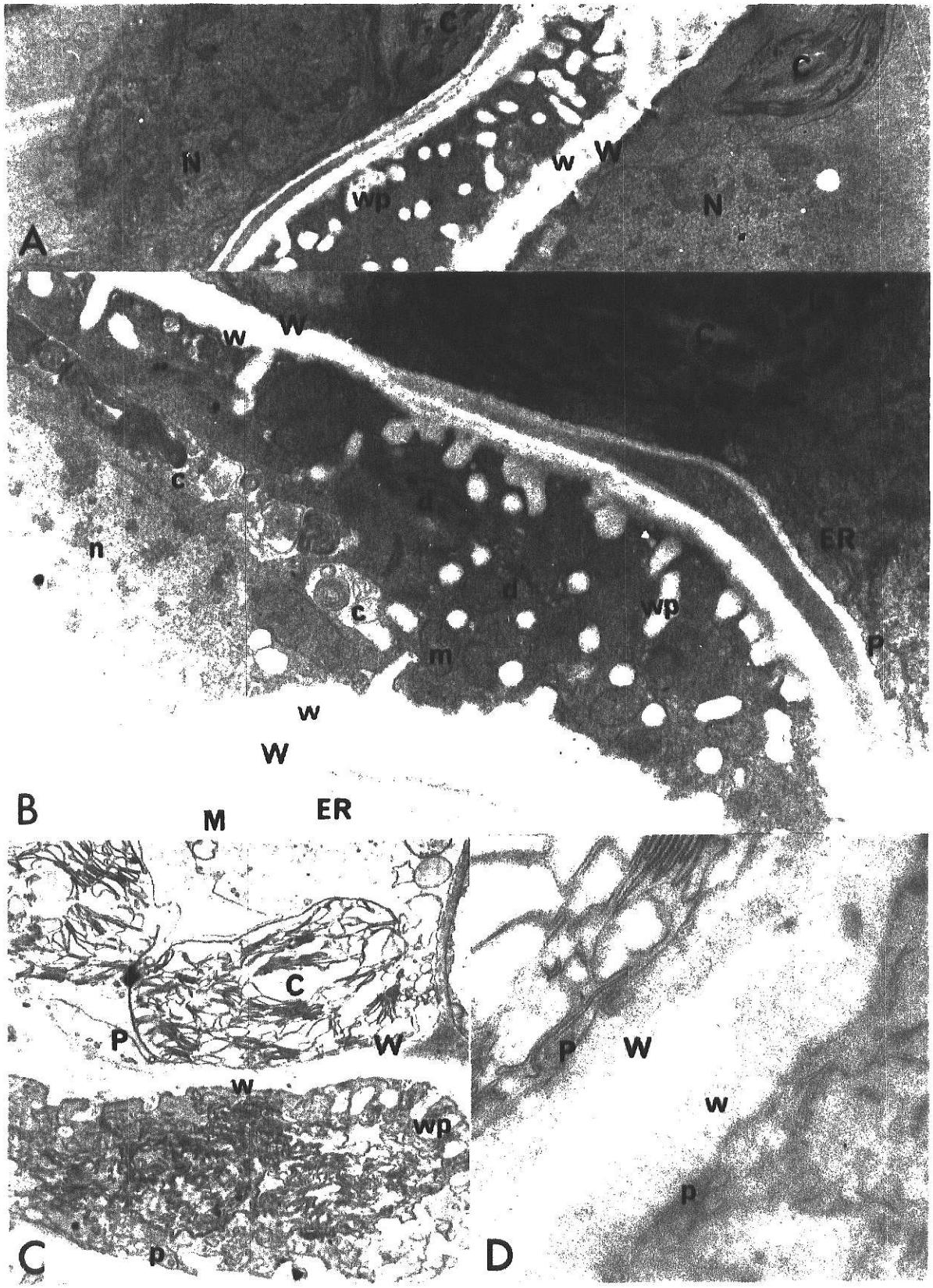
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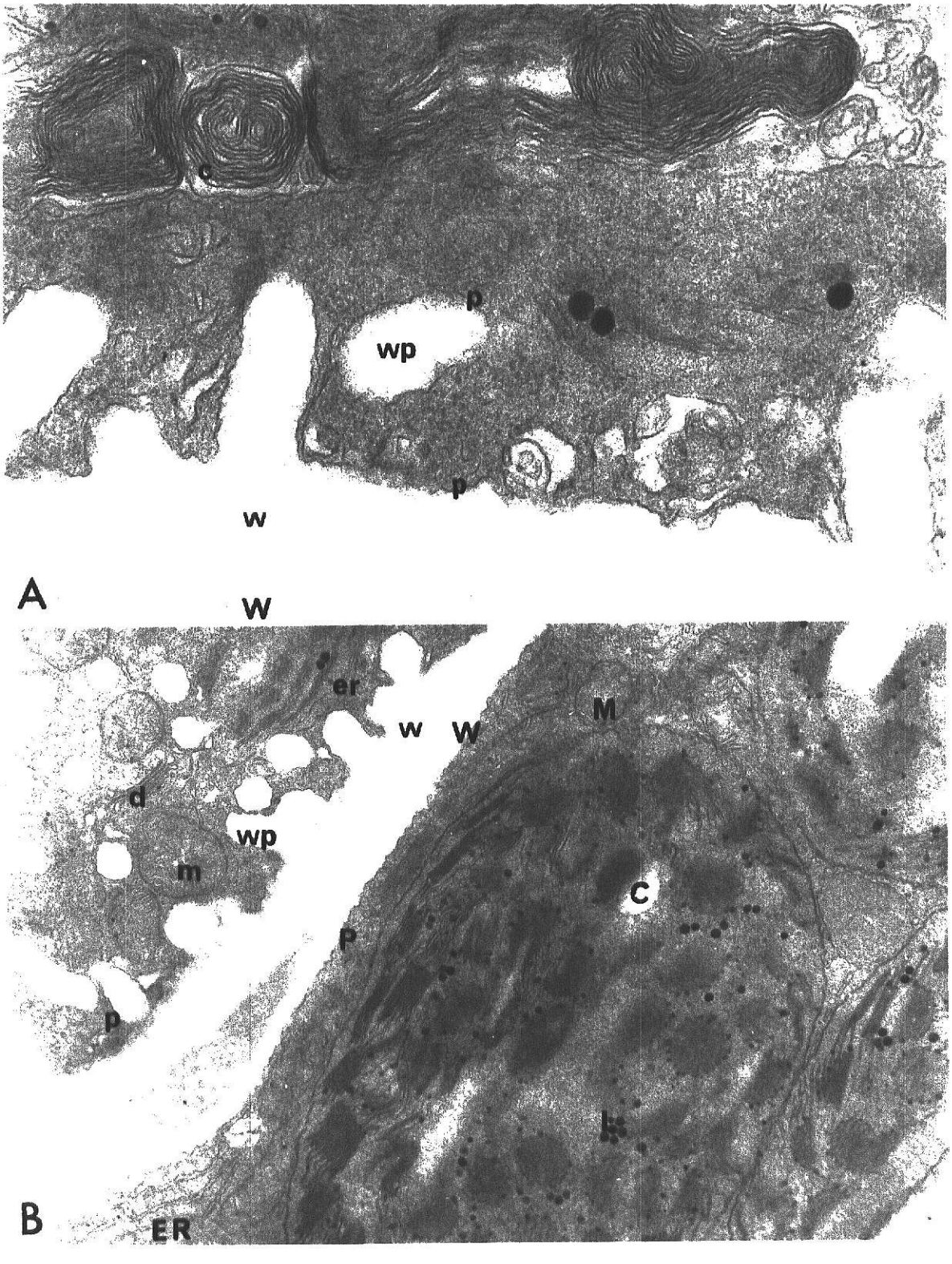
Fig. 2. A,B) Intercellualr mycelium of Peronospora trifoliorum in alfalfa leaves; glutaraldehyde-osmium fixation. Chloroplast(C); lipid bodies(L); host endoplasmic reticulum(ER); host mitochondrion(M); host plasma membrane(P); host cell wall(W); fungal cell wall(w); fungal wall projection(wp); fungal plasma membrane(p); membrane complex(c); fungal mitochondrion(m); fungal endoplasmic reticulum(er); fungal dictyosome(d). A) (X68,000). B) (X28,000).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The fungal wall is a single electron transluscent layer with finger-like projections as deep as 1μ into the fungal cytoplasm. The wall projections are surrounded by the fungal plasma membrane (Fig. 1-A-C; Fig. 2-A,B), and thus cause invaginations of the membrane. Fungal wall projections in haustoria-producing obligate parasites have not been reported. Perhaps the increased surface area of the plasma membrane created by these invaginations has made haustoria unnecessary for P. trifoliorum.

Effects of infection on host cytoplasm.--Increases in the number of mitochondria, granularity of the cytoplasm, the amount of endoplasmic reticulum, and the presence of lipid bodies in the chloroplasts were noted in host cells adjacent to intercellular mycelium (Fig. 1-B; Fig. 2-B). The chloroplasts and nuclei were often found near an area of contact between the host and parasite walls (Fig. 1-A,B,C; Fig. 2-B). Such responses are common in host cells containing haustoria of obligate parasites (1,7,17,18). Therefore these reactions are not merely responses to the presence of haustoria or their sheaths.

Host-parasite interface.--The interface between P. trifoliorum mycelium and the host is the area of physical contact between the fungus and host cell walls (Fig. 1-A-D; Fig. 2-A,B). No encapsulation or sheath was seen in this area, but the host nucleus and chloroplasts were often closely associated with the interface (Fig. 1-A,B,C; Fig. 2-B). These organelles have been associated with the interface

of other obligate parasites (17,18). The nucleus of P. trifoliorum was never found in close association with the interface.

The area of actual contact between the host and fungal wall is the true interface of this obligate parasite and its host. All physiological activities necessary for obligate parasitism are occurring without the production of haustoria. Autoradiography of ^{14}C transfer from parasite to host showed that labeled molecules do not accumulate in the haustorium sheath of Puccinia graminis (6), but in areas of the host where actual mechanical contact was being made with the intercellular hyphae. That evidence along with the information reported here indicates that haustoria of some of the obligate parasites may not be as physiologically active, compared to other fungal structures, as presently believed.

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II. STORAGE OF PERONOSPORA TRIFOLIORUM SPORANGIA

Downy mildew of alfalfa (Medicago sativa L.), caused by the obligate parasite Peronospora trifoliorum d By., is a sporadic disease in much of the temperate areas of the U.S. Thus the disease and the pathogen have been difficult to study. Rockett (7) developed a method to maintain P. trifoliorum on alfalfa seedlings in the laboratory. Large numbers of sporangia are produced but the sporangia are short-lived so weekly plantings, inoculations, and the continuous use of growth chambers are necessary merely to maintain cultures. A means of storing sporangia would reduce those requirements as well as reduce the chances of contamination and possible genetic changes.

Melhus and Patel reported that P. trifoliorum sporangia, frozen 7 and 10 days in water germinated 10 and 0% respectively (4). Sporangia of P. effusa frozen in water at -10 F lost viability in 1 month but when stored 6 months on diseased spinach leaves at -10 F in closed jars 3% germinated (6). Bromfield and Schmitt (1) developed a procedure for storing sporangia of P. tabacina in liquid nitrogen (LN) for prolonged periods. There are advantages to deep-freeze and LN storage and this paper deals with both storage methods for P. trifoliorum sporangia.

MATERIALS AND METHODS

Sporangia of P. trifoliorum were produced on alfalfa seedlings as described by Rockett (7). For deep-freeze storage studies, two-week-old mildewed 'Buffalo' seedlings were harvested and sealed in 10-ml screw-top tubes and stored at -22 C in a freezer.

For LN studies, the diseased seedlings were placed in distilled water in a closed jar and shaken to dislodge the sporangia. The suspension was poured through a tea strainer to remove host material and the sporangia were concentrated by slow speed centrifugation. Sporangia were resuspended to 5×10^5 sporangia/ml in either distilled water or 15% dimethyl sulfoxide (DMSO). From there 3/4-ml portions were placed in 1-ml NS 33 Snapule ampules (Wheaton glass Co. Millvile, New Jersey) and sealed with a cross-fire oxygen-gas torch. After storage and thawing, the sporangial suspensions were poured onto 2% water agar plates, incubated 16 hr at 18 C in the dark and the per cent germination determined. In preliminary work we noted that sporangial germination was reduced about 50% if the 15% DMSO was not removed after thawing, so in all work reported here the sporangia were removed from the DMSO by centrifugation and washed twice in distilled water immediately after thawing.

Data reported are the means of three experiments of three replications each.

RESULTS AND DISCUSSION

Deep-freeze storage.--Tubes of frozen seedlings were removed from the freezer periodically, water was added and they were shaken to dislodge the sporangia from their sporangiophores. The average germination after 6, 10, 14, and 24 weeks of storage was 31, 22, 17, and 11%, respectively. Rate of thawing or shocking the sporangia in a 40-C water bath during or immediately after thawing did not affect their germination. There was no evidence that storage reduced the virulence of viable sporangia. Therefore, the deep-freeze offers a very simple and reliable means of preserving P. trifoliorum sporangia for several months.

LN storage.--The advantages, and often the necessity, of a protective freezing medium for LN storage of fungal spores and a slow rate of prestorage freezing of about 1 C/min down to at least -22 C are well documented (1,2,5). However, not all fungi require these precautions. For example, Puccinia graminis urediospores are preserved in LN without a protective medium or prestorage freezing (3).

In preliminary trials, P. trifoliorum sporangia survived LN storage by the method outlined for P. tabacina (1). However, we thought it worthwhile to investigate some alternative treatments to hopefully improve survival and, or, simplify the procedures for storing P. trifoliorum sporangia in LN.

To determine the effects of various freezing and thawing methods and freezing media on sporangial viability, ampules of sporangia in water or 15% DMSO were: (I) stored at -4 C; (II) stored at -22 C; (III) stored in LN; (IV) kept 1 hr at -4 C and stored at -22 C; (V) kept 1 hr at -4 C and stored in LN; (VI) kept 1 hr at -4 C, transferred to -22 C for 1 hr and stored in LN; or (VII) kept 1 hr at -22 C and stored in LN. The ampules of sporangia were stored 2 days and thawed in air at room temp or agitated in a 40-C water bath for 30 sec. After thawing, sporangia were incubated on 2% water agar 16 hr at 18 C in the dark and the per cent germination was determined.

Sporangia survived LN storage only in DMSO and only when frozen at -22 C before LN storage and thawed in a 40-C water bath afterwards (Table 1). It was apparent that a prestorage slow freeze in a protective medium was required. The criticalness of the prestorage freezing rate was apparent as the sporangia held 1 hr at -22 C before LN storage germinated 32%, whereas those given the same treatment except preceded by 1 hr at -4 C did not germinate. However, sporangia held 2 days at -4 C germinated 53%. In subsequent experiments, germination of sporangia stored at -4 C in 15% DMSO dropped from 55% to 2% in one month and none germinated after 2 months.

To further study the critical prestorage freezing period, ampules of sporangia were kept at -8, -12, -15, or -22 C for 1 hr, immediately transferred to LN for 1 day, and thawed in a 40-C water bath for 30 sec. Germination was 0, 5, 8, and 57% respectively. Sporangia germinated 55% prior to prestorage treatment.

Table 1. Effects of various freezing and thawing methods, and freezing media (15% dimethyl sulfoxide(DMSO) or water) on germination of Peronospora trifoliorum sporangia.

	Freezing process(C)[a]	Storage temp(C)	Freezing medium	% germination[b] Thawed in air(25 C)	% germination[b] Thawed in 40-C water bath
I.		-4	DMSO	54	53
			H_2O	46	49
II.		-22	DMSO	0	0
			H_2O	0	0
III.		-196	DMSO	0	0
			H_2O	0	0
IV.	-4	-22	DMSO	2	12
			H_2O	0	0
V.	-4	-196	DMSO	0	0
			H_2O	0	0
VI.	-4, -22	-196	DMSO	0	0
			H_2O	0	0
VII.	-22	-196	DMSO	0	32
			H_2O	0	0

[a] Sporangia were held for 1 hr at these temp., starting from left to right, before storage.

[b] Sporangia germinated 44% before freezing.

Because -22 C was markedly superior to the other freezing temp tried we next determined the optimum time required for prestorage freezing at -22 C. Ampules of sporangia after varying periods of time at -22 C were stored 1 day in LN and the germination determined. The prestorage period in hr at -22 C and the resulting germination were: 1/4.0%; 1/2,0%; 1,42%; 2,45%; 4,40%; 8,20%; 16,17%; and 24,0%. From 1 to 4 hr at -22 C was the optimum prestorage treatment.

To determine the effects of variation in the thawing procedures, ampules of sporangia from LN were thawed: (I) in a 40-C water bath for 30 sec; (II) in air at room temp; (III) at room temp and placed in a 40-C water bath for 30 sec; and (IV) at room temp, DMSO was removed, sporangia were washed and resuspended in water, and placed in the 40-C water bath for 30 sec. Average germination on 2% water agar was 24, 0, 8, and 10%,respectively. Apparently low temp induced a dormancy in some P. trifoliorum sporangia as none thawed at room temp germinated but if they were then placed in the 40-C water bath, 10% germinated.

In long term experiments, ampules of sporangia frozen 1 hr at -22 C and stored in LN were removed periodically, thawed in a 40-C water bath, and the germination and virulence were determined. Sporangia after 1 year germinated 19% and were virulent.

To determine if sporangia could be stored in LN without a freezing medium as reported for Puccinia graminis (3), ampules of mildewed seedlings were flame-sealed and placed in LN for 1 day and thawed in a 40-C water bath. None germinated.

None of the prestorage or thawing treatments we used surpassed those outlined by Bromfield and Schmitt for storing P. tabacina sporangia (1).

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

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MASTER OF SCIENCE

Department of Plant Pathology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

ABSTRACT

Peronospora trifoliorum, an intercellular obligate parasite, did not produce haustoria in *Medicago sativa* leaves. Mycelial ultrastructure was similar to that of other Oomycetes, except *P. trifoliorum* possessed a membrane complex and inward projections of the hyphal wall into the fungal cytoplasm. The increase in surface area of the plasma membrane created by the invaginations could be an adaptation that has enabled *P. trifoliorum* to survive without haustoria.

A sheath or an encapsulation was not found between the intercellular hyphae and the host cell wall. However, host cell responses to *P. trifoliorum* mycelium were similar to those reported for host cells containing haustoria of other obligate parasites. Therefore, such responses are not necessarily a reaction to only the haustorium or sheath, but also to the intercellular hyphae. Perhaps the haustoria of many obligate parasites are not as physiologically active, compared to other fungal structures, as presently believed.

Several methods of preserving *P. trifoliorum* sporangia at low temp were investigated. Storage was most successful at -22 C or in liquid nitrogen. Sporangia stored at -22 C on alfalfa seedlings for 6, 10, 14 and 24 weeks germinated 31, 22, 17 and 11%, respectively. Optimum survival in liquid nitrogen occurred when the sporangia were suspended in 15% dimethyl sulfoxide, frozen for 1 to 4 hr at -22 C, stored in liquid nitrogen, and thawed in a 40-C water bath. Sporangia stored 1 year under these conditions germinated 19% and were virulent.