Formation and Germination of Chlamydospores of *Phytophthora parasitica* Under Various Oxygen and Carbon Dioxide Tensions

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ABSTRACT

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Mycelial mats of *Phytophthora parasitica* from tomato were incubated in continuous-flow atmospheres containing various concentrations of O_2, CO_2, and N_2, and the numbers of sporangia and chlamydospores produced after 2 wk were determined. Total sporulation (sporangia plus chlamydospores) was maximal at O_2 concentrations from 21 to 1% and at all CO_2 concentrations from 0.03 or less to 6.5%. Low CO_2 combined with O_2 concentrations decreasing from 21 to 2.5% favored production of abundant sporangia with few chlamydospores (sporangium to chlamydospore ratio about 85:15). At 1.25% O_2, sporangia and chlamydospores were produced in about equal numbers, but at 0.3% O_2, formation of both spore types was reduced to nearly nil. Increased CO_2 from 1 to 6.5% inhibited formation of sporangia and stimulated chlamydospore formation (sporangium to chlamydospore ratio 5:95). Above 6.5% CO_2, production of chlamydospores decreased, and at 21% CO_2, it was reduced by 30%. At 2–2.5% CO_2, chlamydospore formation was inhibited below 1% O_2. Chlamydospores incubated in atmospheres of various concentrations of O_2 and CO_2 on cornmeal agar (CMA) germinated predominantly by means of mycelium-producing germ tubes. In soil extract (SE) or on SE agar, chlamydospores germinated predominantly by means of sporangia. This type of germination and sporangium production from mycelium was maximum in atmospheres containing 0.03% CO_2 and 2.5–21% O_2. Below 2.5% O_2, chlamydospore germination in SE (sporangium production) was reduced, and at 0.2% O_2, it was nearly nil. Germination on CMA (mycelium-producing germ tubes) was maximal from 0.5 to 21% O_2. At 0.2% O_2, germination still was reduced about 50%. In constant 9–10% O_2 and CO_2 increasing above 0.03%, chlamydospore germination, both on CMA and SE, was maximal at 0.03% CO_2 and decreased with each increment in CO_2 concentration to nearly nil at 28% CO_2. At 0.03% CO_2, 90% of chlamydospores germinated by means of sporangium-producing hyphae, whereas at 2–10% CO_2, 60–100% of the germinating chlamydospores formed mycelium instead of sporangia.

The chlamydospore is a primary unit of survival for many soilborne *Phytophthora* spp. and with heterothallic species may be more important than the oospore (16). *P. parasitica*, the main cause of a severe root and crown rot disease of tomato in California, is heterothallic; its populations in tomato field soils appear to be composed of a single mating type (5). Thus for this species, the chlamydospore may be the only long-term survival structure in soil. Therefore, factors affecting its formation, survival, and germination are of paramount importance in epidemiology of the disease.

Tsao (14,15) reported that the types of asexual spores (sporangia or chlamydospores) produced by *P. parasitica* are determined to a great extent by environmental factors. Under conditions of high relative humidity, adequate aeration, and relatively high temperatures (25 C), abundant sporangia are formed within a few days. Poor aeration and low temperatures (15–18 C) prevent formation of sporangia and favor development of chlamydospores (14,15). From these observations, Tsao (15) developed a method (submersion of washed mycelial mats in a 50-mm-deep layer of water) for mass production of chlamydospores in sporangium-free liquid cultures of *P. parasitica*.

Several investigators (1,2,9,11,12) have reported the influence of O_2 and CO_2 on growth, sporangium formation, and other stages of the life cycle of *P. parasitica* and other *Phytophthora* spp. Formation and germination of chlamydospores, however, were not examined in these studies. In our studies on the water relations of *P. parasitica* (6), mycelial mats or colonized tomato tissues produced neither sporangia nor chlamydospores when buried in saturated soil, indicating that O_2 was a limiting factor for both types of asexual reproduction.

On rich agar or broth media, chlamydospores usually germinate by single or multiple germ tubes that grow and branch to form mycelium, whereas in natural soils and soil extracts, chlamydospores typically germinate by producing sporangia (14). The latter, and perhaps more important, type of chlamydospore germination also was inhibited in saturated soil (6), further indicating the importance of aeration for germination of *P. parasitica* chlamydospores in soil.

These observations (6) and the reports by others (9,11,12,14,15) led us to investigate in more detail the influence of various O_2 and CO_2 tensions on formation and germination of *P. parasitica* chlamydospores.

MATERIALS AND METHODS

Preparation of gas mixtures. Mixtures

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of gases were prepared by metering together appropriate amounts of compressed air, compressed CO_2, and liquid N_2, as described elsewhere (7). The actual concentrations of O_2, CO_2, and N_2 (percentage of gas volume) were determined by gas chromatography (7). Mycelial mats or chlamydospores of *P. parasitica* were exposed to the different gas mixtures in 4-L cylindrical glass chambers fitted with metal covers with inlet and outlet openings. Gas mixtures were humidified and introduced into the sample chambers at flow rates of about 5 L/hr. The incubation chambers were maintained in the dark in a controlled-temperature room at 18 C for chlamydospore formation or 22 C for chlamydospore germination.

Formation of sporangia and chlamydospores. The tomato isolate DM30-2 of *P. parasitica* (5,6) was used in all experiments. To produce mycelial mats, 1-mm-diameter inoculum plugs taken from the edge of a 5-day-old colony growing on cornmeal agar (CMA) were placed in 60-ml glass prescription bottles containing 5 ml of V-8 juice broth (V8JB) (10,15). After incubation at 24 C in the dark for 25 hr, the bottles were shaken vigorously by hand and incubated horizontally as stationary cultures at 24 C for 6 days (15). Mycelial mats were harvested aseptically, washed three times for 10 min in sterile distilled water (SDW) or in sterile buffer solution, and placed in 60-mm-diameter petri plates containing 5 ml of SDW or buffer. Four buffer systems, all pH 6.5, were tested: 0.05 M K_2HPO_4:KH_2PO_4 (phosphate), 0.01 M phosphate, 0.01 M Sorensen's citrate, and 0.01 M 2-(*N*-morpholino)-ethanesulfonic acid (MES) (3). The 0.05 M phosphate and 0.01 M citrate buffers inhibited formation of both sporangia and chlamydospores, thus their use was discontinued.

Plates containing washed mycelial mats were incubated in gas mixtures that included seven concentrations of O_2, from 0.3 to 21% (normal air), and eight concentrations of CO_2, ranging from 0.03% (normal atmospheric level) to 21% in various combinations (Fig. 1A–C). The numbers of spores (sporangia and/or chlamydospores) produced were determined after 1 and 2 wk of incubation as described elsewhere (5,6). At each reading, three replicate plates were examined; results are expressed as average number of spores per mycelial mat.

Germination of chlamydospores. Chlamydospore suspensions in SDW containing about 5×10^4 viable spores per milliliter were prepared as described by Tsao (14,15) and Mircetich et al (10). The viability of chlamydospores was determined by staining with rose bengal (10). Portions (0.2 ml) were transferred to 60-mm-diameter petri plates containing CMA medium buffered at pH 6.5 with 0.05 M phosphate buffer. (In tests conducted in normal air, this buffer did not affect the germination of chlamydospores.) Plates were incubated uncovered in atmospheres of the desired composition; after 12, 18, and 24 hr, chlamydospore germination was assessed by microscopic examination of 100 spores on each of three replicate plates. A spore was considered germinated if a microscopically visible germ tube had developed (5).

Chlamydospore germination also was studied by incubation in 10% soil extract (SE), where they usually germinated by producing short germ tubes with apical sporangia (10,14,15). The SE, prepared as described elsewhere (5), was used either unsterilized or autoclaved. The pH of unsterilized SE was 7.1, but after sterilization, it had increased to 7.3. Part of the sterilized SE was adjusted to pH 6.5 with 0.05 M phosphate buffer. Chlamydospores (2.5×10^4 spores per milliliter) were suspended in the respective SE preparation, and 0.2-ml portions were pipetted into 0.5-ml microbeakers in petri plates containing a double layer of moist filter paper and incubated in atmospheres of the desired composition. Chlamydospore germination via sporangia or germ tubes was assessed after 12, 18, and 24 hr (5).

In some experiments, the influence of O_2 on the germination of chlamydospores was examined using the same autoclaved SE described before but it was solidified by adding agar at 15 g/L (SE agar). Germination on this substrate was compared with CMA and liquid SE. All three media were buffered with 0.05 M phosphate buffer, pH 6.5.

RESULTS

Formation of sporangia and chlamydospores. The numbers of sporangia determined after 1 wk of incubation in the different atmospheres were about equal to the respective numbers of sporangia determined after 2 wk. Most chlamydospores, however, were formed during the second week of incubation, so for both spore types, only results obtained after 2 wk of incubation are presented. Sporulation in SDW was generally better than in either 0.01 M phosphate or 0.01 M MES buffers, but the relative effects of different O_2 and CO_2 concentrations were similar with all three media. Thus the numbers of spores determined after 2 wk in the three substrates were pooled; their averages are shown in Figure 1A–C.

In atmospheres of constant CO_2 concentration at or below the normal atmospheric level of 0.03%, all O_2 concentrations from 21 to 2.5% favored formation of sporangia rather than chlamydospores (sporangium to chlamydospore ratio 85:15) (Fig. 1A). Decrease of O_2 concentration below 2.5% decreased sporangium formation and increased chlamydospore formation. Thus at 1.25% O_2, the sporangium to chlamydospore ratio was about 50:50 and the total number of spores produced was equal to production at higher O_2 levels (Fig. 1A). At 0.7% O_2, chlamydospore numbers remained high but very few sporangia were produced; the total number of spores was reduced to about 50% of those at higher O_2 levels. At 0.3% O_2, formation of both spore types was completely inhibited (Fig. 1A).

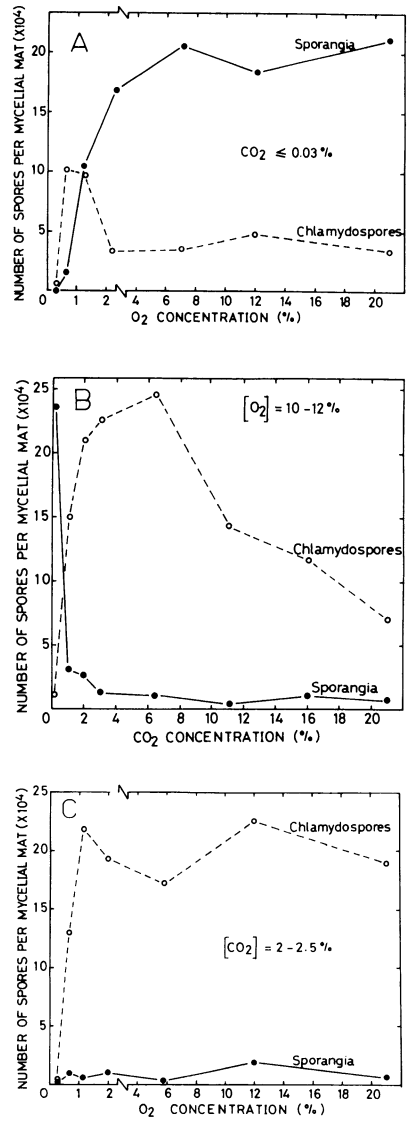

Fig. 1. Effects of various concentrations of O_2 and CO_2 on sporangium and chlamydospore formation by *Phytophthora parasitica*. Washed mycelial mats were incubated in petri plates containing a shallow layer of sterile distilled water or one of two buffer solutions at pH 6.5, and the number of spores produced was determined after 15 days of incubation at 18 C in the dark. Figures shown are averages obtained in the three incubation media, five replicates each. **(A)** CO_2 was held near constant at or below 0.03% and O_2 concentration decreased from 21 to 0.03%. **(B)** O_2 concentration was held nearly constant at 10–12% and CO_2 concentration increased from 0.03 to 21%. **(C)** CO_2 concentration was held nearly constant at 2–2.5% and O_2 concentration decreased from 21 to 0.5%.

The influence of CO_2 was examined in atmospheres containing constant 10–12% O_2 concentration and various concentrations of CO_2. In atmospheres containing 0.03% CO_2, the fungus produced abundant sporangia but very few chlamydospores (Fig. 1B). Small increases in CO_2 concentration resulted in a reduction in sporangium formation and an increase in chlamydospore formation. Total sporulation (sporangia plus chlamydospores) was not affected by CO_2 levels up to 6.4%, but the sporangium to chlamydospore ratio was reduced from 95:5 at 0.03% CO_2 to about 5:95 at 6.4% CO_2 (Fig. 1B). Sporangium formation was reduced to insignificant levels at all CO_2 concentrations higher than 1%. Chlamydospore formation was maximal at CO_2 concentrations between 2 and 6.4%. At CO_2 levels higher than 6.4%, chlamydospore formation decreased gradually but considerable numbers (about 30% of the maximal) were still produced even at 21% CO_2 (Fig. 1B).

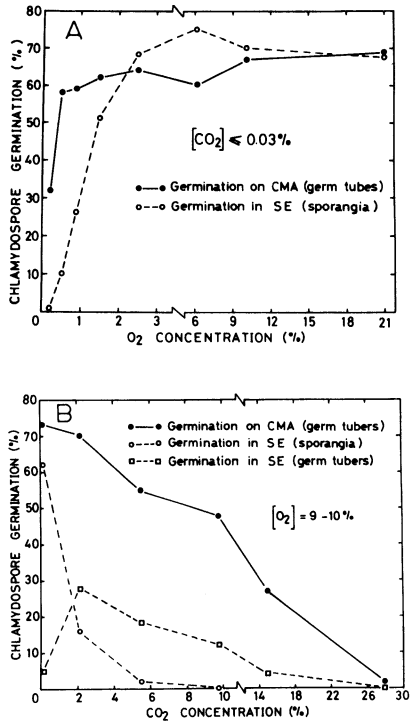

Fig. 2. Germination of chlamydospores of *Phytophthora parasitica* in atmospheres of various concentrations of O_2 and CO_2. Chlamydospores were incubated on cornmeal agar (CMA) or in sterilized soil extract (SE) (both media buffered at pH 6.5 with 0.05 M phosphate buffer) at 20–22 C in the dark. Germination on CMA and in SE was assessed after 12 and 18–24 hr, respectively. **(A)** CO_2 concentration was held nearly constant at or below 0.03% and O_2 concentration decreased from 21 to 0.2%. **(B)** O_2 concentration was held nearly constant at 9–10% and CO_2 concentration increased from 0.3 to 28%.

The O_2 requirement of *P. parasitica* for chlamydospore formation was determined by exposing mycelial mats to various concentrations of O_2 and 2–2.5% CO_2. At this level of CO_2, formation of sporangia was inhibited regardless of the level of O_2 (Fig. 1C). Under these conditions, all O_2 concentrations between 21 and 1% were optimal for formation of chlamydospores. At less than 1% O_2, production of chlamydospores was sharply reduced and was nil at 0.25% O_2 (Fig. 1C).

The initial pH of 6.5 varied less than 0.3 unit after 2 wk of exposure to different gas mixtures. In unbuffered cultures (initial pH about 7), however, final pH varied from 7.5 in atmospheres of low CO_2 to 5.8 in atmospheres of high CO_2. Final pH measurements were made immediately after removing cultures from the incubation chambers. This was particularly important for cultures that had been exposed to high CO_2 concentrations. In such cultures, any delay in pH measurement, especially if combined with agitation, resulted in considerable increase in pH values, probably because of CO_2 escape to the atmosphere. In several cases, pH increased by up to one unit when samples were agitated for 0.5 min before pH measurement. Different concentrations of O_2 did not have any appreciable effect on the final pH.

Germination of chlamydospores. At various concentrations of O_2 and a near constant CO_2 concentration at or below the normal atmospheric level (0.03%), chlamydospores incubated on CMA germinated almost exclusively by means of mycelium-producing germ tubes. The percentage of this type of germination was most readily assessed after 12 hr of incubation (Fig. 2A). In the same atmospheres, chlamydospores incubated in all three SE preparations germinated predominantly via production of sporangia. This type of germination required 18–24 hr of incubation for completion. Results obtained with autoclaved SE buffered at pH 6.5 are shown in Figure 2A. Similar results were obtained with the other two SE preparations.

Germination on CMA (germ tube production) was maximal at all O_2 concentrations from 21 to 0.5%. At 0.2% O_2 (O_2 content of the nitrogen source used in this experiment), germination was reduced about 50% (Fig. 2A). In SE, chlamydospore germination (sporangium production) was more sensitive to reduced O_2 concentrations than on CMA. Thus maximal germination occurred at O_2 concentrations between 21 and 2.5%; O_2 concentrations lower than 2.5% caused a decrease in germination, and at 0.2% O_2, practically no chlamydospores germinated.

To determine whether the observed difference in O_2 requirements for chlamydospore germination on CMA and in SE (Fig. 2A) was due to the different rates of O_2 diffusion in liquid (SE) vs. solid (CMA) substrates, an experiment was conducted using both liquid and solidified (by addition of agar) SE substrates. The type and percentage of germination on these two substrates, as well as on CMA, were determined after 24 hr of incubation in normal air (21% O_2) and in a nitrogen atmosphere containing 0.15% O_2 (Table 1). In normal air, chlamydospore germination was about 70% with all three substrates. As expected, germination on CMA was predominantly by means of mycelium-producing germ tubes, whereas in both liquid and solid SE substrates, most chlamydospores germinated via production of sporangia (Table 1). In 0.15% O_2, chlamydospores failed completely to germinate when incubated either in liquid or on solid SE substrates, but 26% of those incubated on CMA germinated by means of mycelium-producing germ tubes (Table 1). Thus the two types of chlamydospore germination appear to have different O_2 requirements (Fig. 1A, Table 1).

In atmospheres containing a near constant 9–10% O_2 concentration and various levels of CO_2, >99% of chlamydospore germination on CMA was by means of mycelium-producing germ tubes (Fig. 2B). The percentage of germination was maximal at 0.03% CO_2 and decreased gradually in a near linear pattern with increasing CO_2 concentrations. Germination was reduced by 50% at about 12% CO_2 and was nearly nil at 28% CO_2 (Fig. 2B). Germination in SE was also maximal at 0.03% CO_2, where most germinating chlamydospores (92.5%) produced sporangia. At 2% CO_2, however, the percentage of chlamydospore germination was reduced significantly and more than 60% of the germinating chlamydospores formed mycelium-producing germ tubes rather than sporangia (Fig. 2B). Sporangium production from germinating chlamydospores was reduced to insignificant levels

Table 1. Type and percentage of germination of *Phytophthora parasitica* chlamydospores on three substrates after 24 hr of incubation in normal air and in a nitrogen atmosphere containing 0.16% O_2

	Type and percentage of germination in					
	Normal air (21% O_2)			Nitrogen (0.16% O_2)		
Substrate[a]	Germ tube	Sporangia	Total	Germ tube	Sporangia	Total
Soil extract (liquid)	5	65	70	0	0	0
Soil extract agar	1	71	72	0	0	0
Cornmeal agar	68	5	73	26	0	26

[a] All substrates were buffered with 0.05 M phosphate buffer, pH 6.5.

at 5.5% CO_2. Germination by germ tubes also was reduced by CO_2 concentrations higher than 2%, but complete inhibition of this type of germination was observed only at 28% CO_2 (Fig. 2B).

The pH values of buffered cultures varied less than 0.1 pH unit during incubation in the different atmospheres. In unbuffered SE (initial pH 7.1–7.3), however, final pH varied from 6.3 in atmospheres of high CO_2 concentrations to 7.5 in atmospheres of 0.03% CO_2.

DISCUSSION

Results from our tests on the effects of O_2 and CO_2 on sporangium formation from mycelium of *P. parasitica* (Fig. 1A–C) agree closely with results reported by Mitchell and Zentmyer (12). In addition, we found that sporangium formation from germinating chlamydospores (Fig. 2A, B) is as sensitive to decreased O_2 and increased CO_2 concentrations as sporangium formation from mycelium (Fig. 1A–C). The similarity in aeration requirements of mycelia and chlamydospores is consistent with their similar water requirements for sporulation in soil and their inability to produce sporangia in saturated soil (6).

In the laboratory, vegetative mycelium of *P. parasitica* is known to produce abundant chlamydospores, instead of sporangia, when incubated under conditions of reduced aeration (14,15). However, neither sporangia nor chlamydospores were produced from mycelium or colonized tomato tissues buried in unsterilized saturated soil, indicating that aeration under saturated soil conditions was inadequate for both types of asexual sporulation (6). Indeed, in our study, O_2 was essential for formation of both spore types, although production of sporangia was slightly more sensitive to decreased O_2 levels than was production of chlamydospores (Fig. 1A,C). Thus there was a very narrow range of decreasing O_2 concentrations between 2.5 and 1% that were partially inhibitory to sporangium but still favorable for chlamydospore formation. These O_2 levels, combined with a near normal atmospheric level of CO_2, induced production of mixtures of sporangia and chlamydospores (Fig. 1A).

The two types of asexual reproduction differed greatly in their tolerance to increasing CO_2 concentrations. At CO_2 levels higher than the normal atmospheric concentration, especially in the range from 1 to 6.5%, formation of sporangia was nearly nil and the mycelium was converted into abundant chlamydospores (Fig. 1B). Thus mass production of chlamydospores in sporangium-free liquid cultures of *P. parasitica* under conditions of reduced aeration, described by Tsao (15), must be attributable more to increased CO_2 than to decreased O_2 levels. Although gas composition in such cultures was not determined, pH measurements at the end of the incubation period provided indirect evidence of CO_2 accumulation. The pH of the liquid culture medium was between 7.2 and 7.4 if determined immediately after culture bottles were opened but increased by up to 1 pH unit if determined after aeration (by agitation) for 0.5 min, apparently because dissolved CO_2 had escaped (15).

The mode of chlamydospore germination (via germ tube or sporangium production) is known to be influenced by nutrient availability (10,14). In our study, chlamydospores plated on CMA germinated predominantly by means of mycelium-producing germ tubes, whereas in SE, the predominant type of germination was by means of sporangium production. Germination in SE (sporangium production) was more sensitive to decreasing O_2 and increasing CO_2 concentrations than was germination on CMA (by mycelium-producing germ tubes) (Fig. 1A,B). The higher O_2 requirement for germination in SE was not due to reduced O_2 diffusion in the liquid medium, because similar results were obtained when chlamydospores were incubated on a solid SE agar substrate (Table 1). Increasing CO_2 concentrations reduced the total percentage of chlamydospore germination in SE and induced most of the germinating chlamydospores to form mycelium-producing germ tubes instead of sporangia (Fig. 2B). Different concentrations of O_2 and CO_2 also have been reported to affect the type of sporangium germination (direct vs. indirect) in other *Phytophthora* spp. (16). Similar morphogenic effects of O_2 and CO_2 have been noted with other fungi (4,13).

Concentrations of O_2 and CO_2 that partially inhibited chlamydospore germination on CMA did not appreciably affect further germ tube elongation, branching, and mycelial growth from germinated chlamydospores. Similarly, Klotz et al (9) reported that growth of zoospore germ tubes was not affected at 0.1% O_2 and that some mycelial growth of *P. parasitica* occurred even at 0.04% O_2. Mitchell and Zentmyer (11) also found that mycelial growth of *P. parasitica* was not severely affected at 1% O_2 and that it tolerated very high concentrations of CO_2. Similar results have been reported for *P. parasitica* var. *nicotianae* (2).

Results from this study are consistent with reported results on the behavior of *P. parasitica* in vitro (15) and in soil (6,14) and may be useful in understanding the epidemiology of diseases caused by this pathogen. Using these findings in disease control appears difficult, however, because levels of O_2 and CO_2 that favor formation of both sporangia and chlamydospores as well as germination of chlamydospores are commonly encountered in agricultural field soils (8).

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