An Improved Technique for Inoculating Plant Surfaces with Fungal Zoospores

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ABSTRACT

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Infection of bean leaves by zoospores of *Synchytrium macrosporum* was enhanced by Gelgard, a highly cross-linked hydrophilic polyacrylamide gel mixed with the inoculum. Infection loci were more uniformly distributed and occurred at a higher frequency on the leaf surface when Gelgard was added. The gel restricted zoospore motion, shortened their swimming time, and held the inoculum in place on the plant surfaces. Gelgard or other similar materials that maintain moisture near infection sites and prevent zoospores from being washed off the plant surface should prove useful for enhancing infection by zoosporic fungi.

Additional key words: chytrids, gall formation.

Gelatin, agar, and a variety of surfactants have been added to aqueous inoculum suspensions in order to study or enhance the infection process by various plant pathogens (1,3). These materials have been especially beneficial in studying infections arising from germination of nonmotile, fungal spores. Various inoculation techniques have also been used with motile fungal spores. These include atomization, flooding, immersion, and applying discrete drops of inoculum on plant parts (3,6–8,12,13).

Although zoospores of many fungi infect young plants through their roots (10,11,13), zoospores of *Synchytrium macrosporum* Karling infect only tissues containing chlorophyll, where they induce small self-limiting galls on the plant surface (J. S. Karling, Purdue Univ., *personal communication*). Thus, while trichomes of tomato, root hairs of cabbage seedlings, and the chlorophyll-less regions in leaves of *Coleus* are not infected by *S. macrosporum* zoospores, the green leaves, stems, or petioles of these plants are susceptible (C. M. Montecillo and C. E. Bracker, *unpublished*). However, inoculation of these tissues with an aqueous suspension of zoospores is difficult because their surfaces tend to be hydrophobic; inoculum may be easily washed from the host surface or unevenly distributed, and zoospores easily become desiccated prior to penetration.

This study reports the increased effectiveness and efficiency of fungal zoospore inoculum when it is mixed with a polyacrylamide gel and experimentally used to infect leaf surfaces.

MATERIALS AND METHODS

Host plants. Five bean (*Phaseolus vulgaris* L., 'Pinto') seeds were planted in 15-cm-diameter clay pots and grown in the greenhouse on an open bench. When seedlings were in the primary-leaf stage, those in each pot were thinned to one plant. The first trifoliolate leaf of each seedling was inoculated 12–14 days after planting when plants were in the second trifoliolate-leaf stage. Three to seven trifoliolate leaves were used for each treatment, and the experiment was repeated four times. In separate experiments, young unopened leaves and fully opened leaves were inoculated and compared.

Inoculation. Treatments included distilled water and 0.15% (w/v) aqueous Gelgard separately applied to leaf surfaces. (Gelgard is a fully polymerized, highly cross-linked polyacylamide gel marketed also as "Norbak" by Dow Chemical, Midland, MI 48640); aqueous *S. macrosporum* zoospore suspension; and *S. macrosporum* zoospores in an aqueous suspension of 0.15% (w/v) Gelgard. The 0.15% Gelgard concentration was selected because its thickened fluid consistency was adequate to retain inoculum on the leaf surface.

Zoospores were obtained from bean plants infected with *S. macrosporum*. Dried leaves were soaked in water for 5–7 days, and then prosori were teased from the host tissue, gently rinsed with distilled water, transferred to a petri plate lined with moistened filter paper, and incubated in the laboratory for 2–3 wk or until the prosori germinated and the mature sori appeared bright orange (6).

Sori were transferred individually with a fine needle into drops of distilled water on a glass slide, covered with a glass coverslip, and gently broken by pressing lightly with a pencil eraser to release the zoosporangia. After 1 to 2 hr, swimming zoospores could be seen with a light microscope. The zoospores were collected in 10-ml beakers 2.0–2.5 hr after zoosporogenesis was induced and most of the zoosporangia had released their zoospores. Two 10-ml aqueous zoospore suspensions were prepared. The first was used to inoculate bean leaves directly, whereas 15 mg of Gelgard was mixed with the second zoospore suspension before inoculation. The density of the zoospore inoculum was determined with a standard hemacytometer count and adjusted to 1.5×10^5 zoospores per milliliter. The aqueous zoospore inoculum was pipetted onto the entire upper surface of the trifoliolate leaves. The zoospore and Gelgard mixture was applied on the leaves with a wide-mouth glass pipette prepared by filing off the tip of a Pasteur pipette near its widest part. The inoculum was pipetted onto the leaf surface and then thinly smeared over the entire leaf surface. Young trifoliolate leaflets inoculated with the gel mixture tended to droop from the weight and required support to prevent breakage.

All the treated plants were covered with polyethylene bags for 48 hr to provide high humidity and incubated in the greenhouse (23–30 C) or in the laboratory (23 C under fluorescent lamps) for 48 hr before the polyethylene bags were removed. The plants were watered and examined daily for symptoms of disease development. Infected leaves were removed from plants as they senesced or withered, and then pressed and dried. The galls on each leaflet were then counted.

The behavior and shape of zoospores in vitro with and without Gelgard treatment was observed by Nomarski interference-contrast optics on a Zeiss WL microscope.

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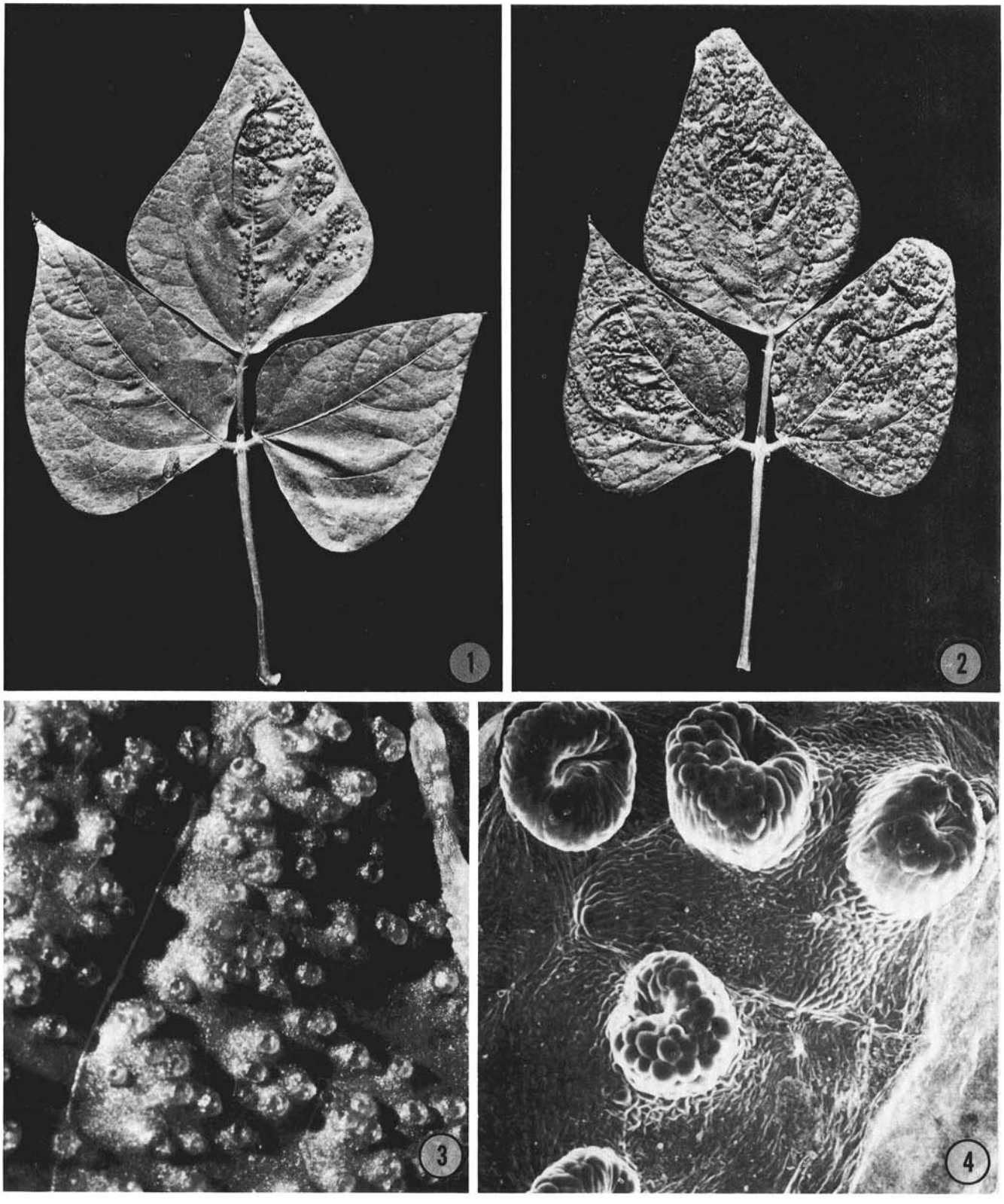
RESULTS

Light microscopy. The zoospores of *S. macrosporum* normally swim for 1–2 hr in water after their release from the zoosporangium. Within 2–3 min after Gelgard was added, the zoospores began to swim more slowly and for shorter distances. Within 5 min, the zoospores had stopped swimming, withdrawn their flagellum and were spheroidal. Five to 10 min later, they had assumed an irregular shape. Undifferentiated zoosporangia continued to differentiate into zoospores in the Gelgard suspension. These zoospores were also sluggish, soon stopped moving, withdrew their flagellum, and changed from spheroidal to



Figs. 1–4. Galling of bean leaves after inoculation with zoospores of *Synchytrium macrosporum* in inoculum suspensions **1,** lacking polyacylamide gel (note localization and nonuniformity of infection distribution) (×0.8) and **2,** with polyacrylamide gel in the inoculum suspension (note the increased numbers and more uniform distribution of the infections) (×0.7). Close-up views of galls (which consist of hyperplastic and hypertrophied host cells) as seen under **3,** reflected light microscopy (×8) and **4,** scanning electron microscopy (×45).

an irregular shape.

Infection of leaves. Infection by zoospores of *S. macrosporum* resulted in tiny protrusions barely visible to the naked eye on the leaf surfaces 5–7 days after inoculation. These small cellular protrusions developed into 0.2–0.5 mm diameter galls with various shapes and complexities 3–4 wk after inoculation (Figs. 1 and 2). The galls appeared rosettelike with a central depression (Figs. 3 and 4). A resting-spore of the fungus developed in the infected host cell near the center of each gall during disease development. The aqueous zoospore inoculum without Gelgard usually resulted in unevenly distributed infections. These infections usually aggregated along the veins or clustered at the tip of a leaflet (Fig. 1). The individual galls in clusters were generally much smaller than those that developed separated from each other. Sometimes two to seven galls coalesced, in which case the resulting resting spores were usually small.

The addition of Gelgard to the inoculum increased the average number of galls per leaflet (Table 1, Figs. 1 and 2) and resulted in a more uniform distribution of infections on the inoculated leaf surface. The center leaflet of the trifoliolate leaf developed significantly more galls than either of the side leaflets (whether or not Gelgard was used with the inoculum). When only the center leaflets were compared, the difference in infection frequency between inoculum with and without Gelgard was even more pronounced than indicated in Table 1. The range in the number of galls among all the leaflets was much narrower, and the number of galls per unit area of leaf surface was significantly greater when Gelgard was added to the inoculum than when it was omitted (Table 1). Although the surface area of leaflets inoculated with zoospores alone was significantly reduced compared with controls, leaf surface area was not significantly correlated with the severity of infection (number of galls per leaflet) ($r = 0.44$) in the ranges evaluated.

The number of galls produced was also influenced by leaf age, leaf topography, and inoculum concentration. Young unexpanded leaves inoculated with zoospores in water developed more galls than did leaves inoculated after expansion. However, galls were more numerous on opened leaves than on unopened leaves when Gelgard was added to aqueous zoospore inoculum. High concentrations of galls occurred at localized areas of the opened leaflets (around ridges and depressions) inoculated with zoospores without Gelgard. At zoospore concentrations of 10^3, 10^4, 10^5, 10^6 zoospores per milliliter with Gelgard, we obtained the largest number of galls with the highest inoculum concentration.

DISCUSSION

S. macrosporum, a chytridiomycetous fungus, is an obligate plant parasite that infects only the chlorophyllous parts of its host. Zoospores of *S. macrosporum* mixed with a polyacrylamide gel and inoculated on opened leaves infected the host more effectively and efficiently than when applied in aqueous suspension. The increased infection rate on unopened leaves inoculated with aqueous suspensions of zoospores is probably due in part to the retention of moisture on the convoluted juvenile leaves and is consistent with a previous report by Karling (6). By providing a moist environment around the zoospores on opened leaves and preventing them from running off the leaf surface during inoculation, Gelgard would have a similar effect. The increasing number of galls with increasing zoospore concentration, and the high frequency and uniform distribution of galls on the inoculated opened leaves indicate that Gelgard had no adverse effect on infection. In addition to maintaining a continuous moisture film around the zoospores, Gelgard limited motility of the zoospores on the host surface, probably because of its higher viscosity. Zoospsores usually swim around before they settle down and penetrate a host surface (2,5,10,13), but when Gelgard was added to swimming zoospores of *S. macrosporum* they stopped swimming, withdrew their flagella, and changed their shape. We are uncertain of any other physical or physiological effects that Gelgard may exert on the zoospores. Peripheral experiments in which we inoculated opened leaves of bean plants with zoospores of *S. macrosporum* mixed with gelatin in concentrations of 0.15% (w/v) and 0.5% (w/v) indicated possible physiological effects of Gelgard, since the number of infections obtained with the gelatin was similar to that obtained with the aqueous zoospore inoculum alone. This is in contrast to the increased Septoria leaf blotch of barley (4) obtained when 0.5% (w/v) gelatin was used to hold spores on leaf surfaces.

Although infection by zoospores of *Synchytrium* can be established with simple aqueous suspensions of zoospores as inoculum without adding Gelgard (6,7, and *unpublished*), infections were sporadic and limited. For instance, in this study, infections were more numerous near the leaf veins and margins where large droplets of the inoculum had accumulated. Leaf veins provided a ridging effect that permitted the inoculum to accumulate. Multiple and aggregated infections also occurred at the tips and marginal areas of leaves where the inoculum had collected after flowing from the leaf surface. The increased incidence of galls with greater zoospore density was consistent with the correlation of infection level with inoculum concentration of other pathogens (9,12).

Since Gelgard is hygroscopic, the dry powder should be stored in moistureproof impermeable containers to prevent sorption of water and contaminants that may be detrimental to biological systems. Adsorbed chemical contaminants may reduce or halt infections when mixed with zoospore suspensions. However, in contrast, this may also provide a vehicle for testing the effectiveness of various fungicides or other pesticide formulations.

In summary, the successful infections and enhanced infection rates obtained by adding Gelgard to zoospore inoculum offer several important advantages. Moreover, it is possible to extend limited amounts of inoculum to infect larger plant areas when Gelgard is added. The uniform distribution and retention of inoculum over the inoculated surface greatly facilitates studying the infection process and pathogenesis. This material and other inert substances with similar properties that maintain moisture near infection sites and prevent inoculum from being washed off plant surfaces should prove useful for enhancing infection by zoosporic fungi.

TABLE 1. The frequency of galls caused by *Synchytrium macrosporum* on bean leaves inoculated by different treatments[x]

Inoculation treatment	Galls/cm^2 (mean)	Galls/leaflet (mean)	Galls/leaflet (range)
Water alone	0 a[y]	0 a	0
Gelgard suspension alone[z]	0 a	0 a	0
Zoospores in water	10 b	184 b	1–930
Zoospores in Gelgard[z]	38 c	701 c	137–1,710

[x]Data are from two separate experiments with 30 leaflets for each treatment.
[y]Means not followed by the same letter are significantly different, $P = 0.01$.
[z]Polyacrylamide gel/water, 0.15% (w/v).

LITERATURE CITED

1. Andersen, A. L., and Henry, B. W. 1946. The use of wetting and adhesive agents to increase the effectiveness of conidial suspensions for plant inoculations. Phytopathology 36:1056-1057.
2. Bimpong, C. E., and Clerk, G. C. 1970. Motility and chemotaxis in zoospores of *Phytophthora palmivora* (Butl.) Butl. Ann. Bot. (Lond.) 34:617-624.
3. Eide, C. J., and Lauer, F. I. 1967. Testing potatoes for field resistance to late blight. Am. Potato J. 44:9-16.
4. Green, G. J., and Dickson, J. G. 1957. Pathological histology and varietal reactions in Septoria leaf blotch of barley. Phytopathology 54:1152-1153.
5. Karling, J. S. 1964. *Synchytrium.* Academic Press, New York.
6. Karling, J. S. 1974. Further induced infectivity by *Synchytrium macrosporum.* Bull. Torrey Bot. Club 101:311-316.
7. Lange, L. 1978. *Synchytrium endobioticum* and Potato Virus X. Phytopathol. Z. 92:132-142.

8. Lapwood, D. H., and McKee, R. K. 1966. Dose-response relationships for infection of potato leaves by zoospores of *Phytophthora infestans*. Trans. Br. Mycol. Soc. 49:679-686.
9. Lippincott, J. A., and Lippincott, B. B. 1978. Tumor initiation complementation on bean leaves by a mixture of tumorigenic and nontumorigenic *Agrobacterium rhizogenes*. Phytopathology 68:365-370.
10. MacFarlane, I. 1958. A solution-culture technique for obtaining root-hair, or primary, infection by *Plasmodiophora brassicae*. J. Gen. Microbiol. 18:720-732.
11. MacFarlane, I. 1970. *Lagena radicicola* and *Rhizophydium graminis*, two common and neglected fungi. Trans. Br. Mycol. Soc. 55:113-116.
12. Mitchell, D. J., Kannwischer, M. E., and Moore, E. S. 1978. Relationship of numbers of zoospores of *Phytophthora cryptogea* to infection and mortality of watercress. Phytopathology 68:1446-1448.
13. Williams, P. H., Aist, S. J., and Aist, J. R. 1971. Response of cabbage root hairs to infection by *Plasmodiophora brassicae*. Can. J. Bot. 49:41-47.