A Microinjection Technique for Conidia of *Erysiphe graminis* f. sp. *hordei*

Hideyoshi Toyoda, Yoshinori Matsuda, Ryuzo Shoji, and Seiji Ouchi

Laboratory of Plant Pathology, Faculty of Agriculture, Kinki University, Kowakae 3-4-1, Higashi-Osaka 577, Japan.
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ABSTRACT

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A microinjection technique was adapted to conidia of *Erysiphe graminis* f. sp. *hordei*. By using glass pipettes (outer tip diameter of 0.1 μm), silicon oil was effectively injected into conidia on an agar layer or on barley coleoptile inner epidermis under a constant injection pressure. Successful injection was easily confirmed by light microscopy because injected silicon oil formed a light-scattering, spherical droplet within conidia. When silicon oil was injected for 3 sec, droplets were 1 μm in diameter and did not affect germination or formation of appressorium or haustorium of the conidia. Moreover, the injected conidia produced elongated secondary hyphae after haustorium formation. Injected conidia developed these infection structures at rates comparable to those of noninjected conidia. Under the same injection conditions, fluorescein-isothiocyanate-conjugated albumin was successfully introduced into the cytoplasm of conidia. These results suggest that microinjection is a potentially useful technique for investigating plant host/fungal parasite interactions.

Microinjection techniques have been developed mainly for mammalian (4–6) or higher plant (7,8,10) cell systems as a means of introducing foreign genetic materials directly into their cytoplasm or nuclei. In fungal systems, however, few papers have dealt with the application of this technique except for work with hyphal cells of *Neurospora crassa* for artificial transfer of protoplasm between genetically marked strains (11–13). The establishment of a microinjection technique for fungal pathogens of plants will thus be useful for analyzing genetically specified molecular interactions between host cell and parasite. In the present study, we adapted a microinjection technique to conidia of the powdery mildew fungus of barley and determined whether injection per se affects fungal development on the inner epidermis of barley coleoptiles on which fungal structures can be observed without any staining (1–3).

MATERIALS AND METHODS

Plant and fungus. Primary leaves of 10-day-old barley seedlings (*Hordeum vulgare* L. 'Kobinkatagi', susceptible to race I) were inoculated with *Erysiphe graminis* DC f. sp. *hordei* Em. Marchal, race I, and incubated at 20 C under continuous illumination of 6,000–8,000 lux. Conidia were harvested 6 days after inoculation and used for microinjection. The inner epidermis of barley coleoptiles was separated from the outer epidermis by a razor blade (9) and used for experiments.

Microinjection of conidia. Injection slides were devised as shown in Figure 1; 2% agar was uniformly spread (20 × 20 × 0.2 mm) on a glass slide and framed with three layers of filter paper. Conidia were dusted directly onto the 2% agar layer or onto a barley coleoptile laid on the agar layer. The filter paper frame was supplied with distilled water to prevent the agar layer or coleoptile from drying. Conidia were microinjected by using an Olympus Injectoscope IMT-YF II, a phase-contrast and inverted microscope designed for injection (10,14). Glass pipettes (outer tip diameter of 0.1 μm) for piercing the conidia were made by controlling an electric current and voltage of a needle drawing apparatus (Olympus IMP). The outer diameter of the glass pipette was determined by enlarging the pipette tip portion with a television monitor linked with the Injectoscope (max ×4,250).

The injection material was either silicon oil (SO) or a solution of fluorescein isothiocyanate (FITC)-conjugated albumin (Sigma Chemical Co., St. Louis, MO) at 5 mg/ml. The injection material was pushed out of the pipette by applying pressure to SO in a tube linked with the pipette (Fig. 1). The pressure for injection was generated by a motor-driven syringe injector before injection and controlled at 2.5 kg/cm^2 by a pressure gauge. Constant pressure was maintained between injections. For injection, the sterilized pipette was focused at the cell surface, moved downward about 2 μm, and kept there for 1–30 sec within the cytoplasm of conidia. About 150 conidia were injected in each experiment. After injection, conidia on the agar layer or barley coleoptiles were incubated at 20 C in a moist chamber until observation. The viability of injected conidia was determined by observing the formation of infection structures (germ tubes, appressoria, haustoria, and elongating secondary hyphae) by these conidia.

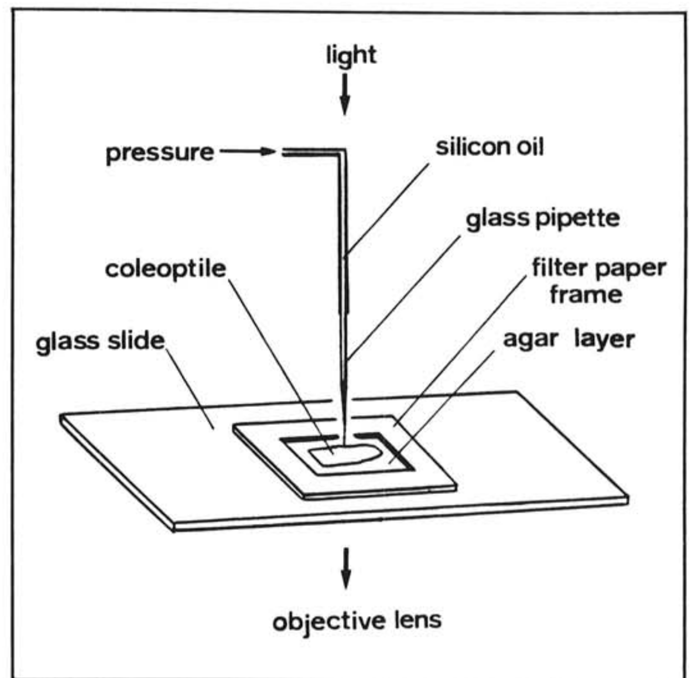

Fig. 1. Injection slide used for microinjection of conidia of *Erysiphe graminis* f. sp. *hordei*, race I, on 2% agar layer or barley coleoptile tissue laid on agar.

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RESULTS AND DISCUSSION

Injection of SO into conidia on an agar layer. Because injected SO forms a light-scattering, spherical droplet within the cytoplasm (10), microinjection of SO is useful for monitoring introduction of foreign materials into cells. In the present study, SO was successfully injected into conidia on an agar layer. Under a constant injection pressure, the volume of SO droplets increased as the injection time increased (Fig. 2A–C). Because quantification is essential for the application of this method to biological systems (6,14), the volumes of injected SO were calculated by measuring the diameters of the droplets, assuming that SO droplets injected into the fungal cells were true spheres. The results are shown in Figure 3. This calibration curve enabled us to determine the exact volumes of injected material and to estimate the duration of injection required for introducing a specified volume of material under a constant injection pressure.

Viability of injected conidia. The relationship between the volume of injected SO and the viability of SO-injected conidia is shown in Figure 4. When droplets of SO 10 μm in diameter were injected into conidia, more than 50% of the conidia burst soon after injection. Many conidia burst between 3 and 6 hr after injection when SO droplets 5 μm in diameter were introduced. Droplets 1 μm in diameter were formed (5.25×10^{-10} μl) when SO was injected into conidia for 3 sec under the present experimental conditions. The rates of bursting were lower for the conidia in which the smaller amounts of SO were introduced (Fig. 4A). Figure 4B shows the germination rates of the conidia injected with various volumes of SO. The highest rates of germination were observed in conidia injected with the smallest amounts of SO (1-μm droplets), the rates being comparable to those of noninjected conidia. Moreover, these conidia produced elongated germ tubes within 6 hr after injection (Fig. 2D–F). Germination rates decreased as the volumes of injected SO increased.

Development of SO-injected conidia on barley coleoptiles. Infection structures developed from conidia injected with SO (injection time of 3 sec) on barley coleoptiles (Fig. 5). The SO-injected conidia germinated and developed appressoria (Fig. 5A) within 10 hr, haustoria (Fig. 5B) at about 18 hr, and elongating secondary hyphae (Fig. 5C) within 96 hr after injection. The time courses of appressorium and haustorium formation of injected conidia were similar to those of noninjected conidia, although the

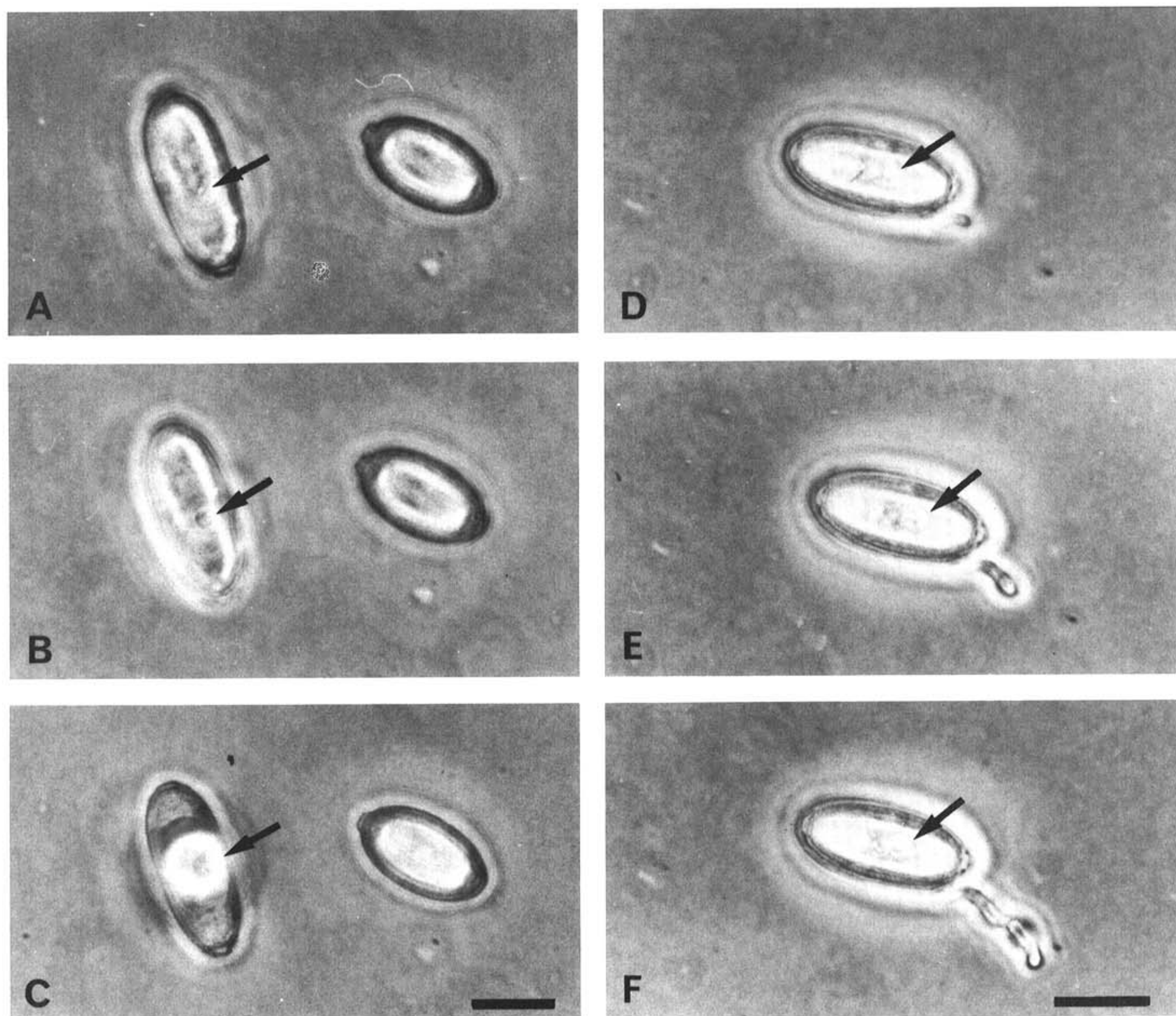

Fig. 2. Injection of silicon oil (arrows) into conidia of *Erysiphe graminis* f. sp. *hordei* on agar layer. **A–C,** Increase with time in diameter of silicon oil droplet injected into conidium under constant pressure (2.5 kg/cm^2) at 2, 7, and 30 sec after injection, respectively. **D–F,** Elongation of germ tube from conidium having 1-μm silicon oil droplet photographed at 1, 3, and 6 hr after injection, respectively. Bar = 10 μm.

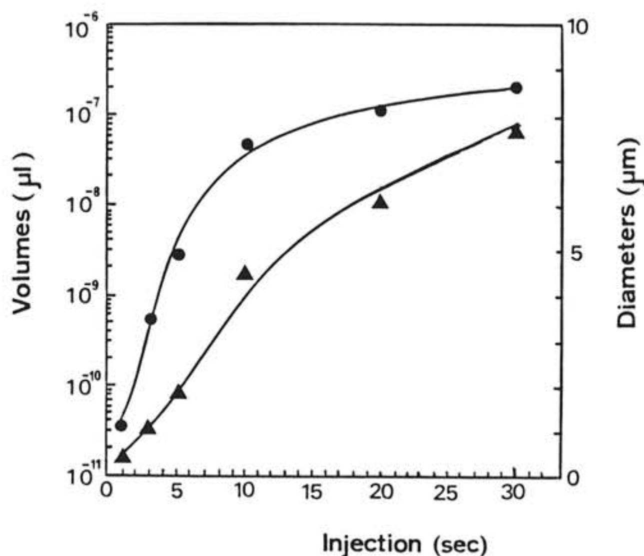

Fig. 3. Increase of volume of silicon oil in conidia of *Erysiphe graminis* f. sp. *hordei* relative to injection time. Volumes (circles) were calculated by measuring diameters (triangles) of spherical silicon oil droplets formed within conidia.

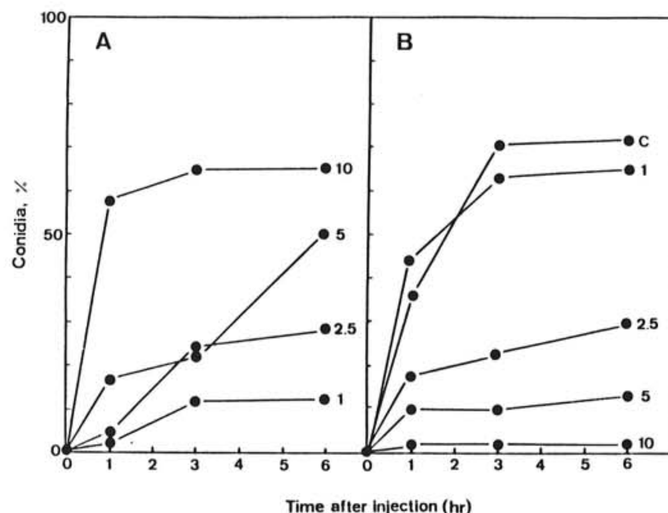

Fig. 4. Bursting rates **(A)** and germination rates **(B)** of conidia of *Erysiphe graminis* f. sp. *hordei* injected with various volumes of silicon oil. Numbers in figures represent diameters of silicon oil droplets; C represents noninjected controls. Experiments were carried out on 2% agar layers.

rates were a little lower in injected conidia than in noninjected ones (Fig. 6). Moreover, there were no statistically significant differences in the rates of haustoria forming elongating secondary hyphae (injected, 24.2% ± 7.29; noninjected, 29.33% ± 8.08) and the length of elongating secondary hyphae (injected, 104.5 μm ± 7.36; noninjected, 102.2 μm ± 25.64) between the injected and the noninjected conidia.

Injection of FITC-albumin. Under the same injection conditions, conidia were injected with FITC-albumin and observed with an Olympus fluorescence microscope (B excitation, B absorption, and O-515 barrier filters). Figure 7 shows successful introduction of FITC-albumin into a conidium on barley coleoptile tissue. Autofluorescence was not observed either in noninjected conidia or in 50 conidia injected with nonconjugated albumin (5 mg/ml).

The present method enabled us consistently to inject 100–150 conidia an hour with more than 80% of the SO-injected conidia developing normal infection structures. These rates are high enough for measurement of infection efficiencies. Thus, this system for microinjection can be used to effectively introduce foreign

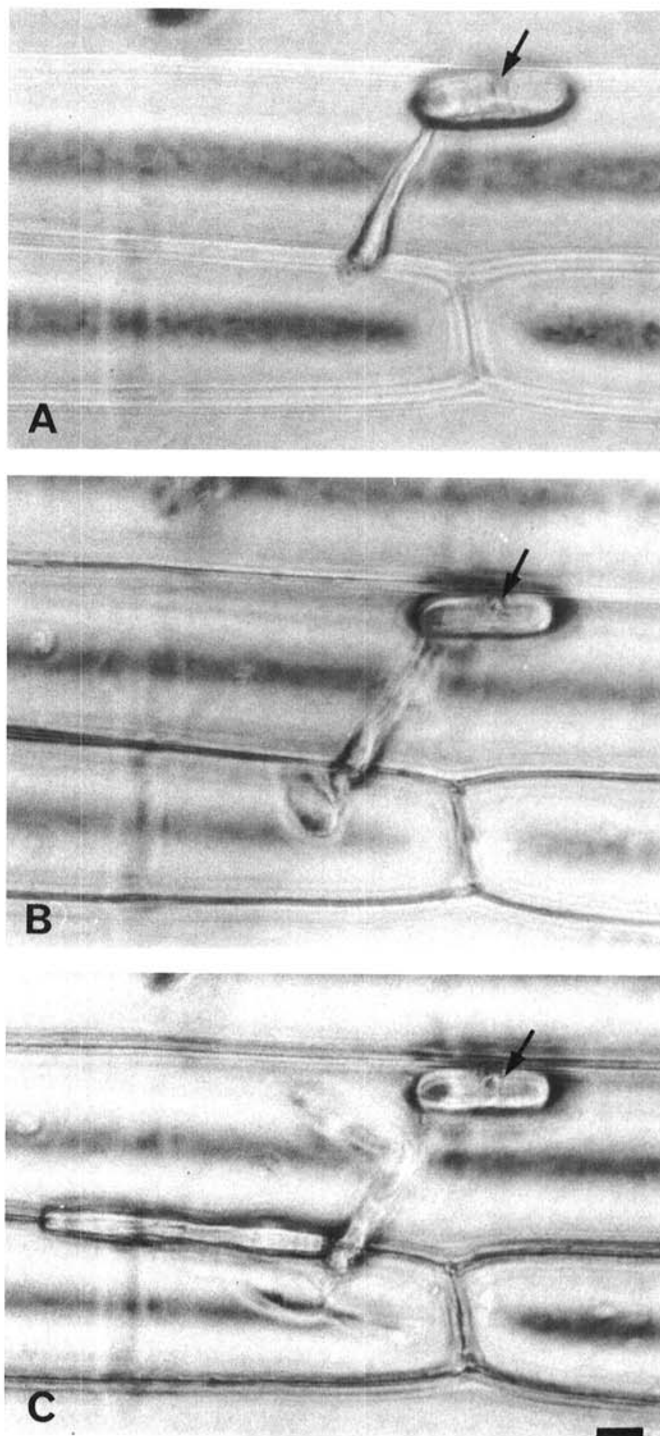

Fig. 5. Development of infection structures by conidium of *Erysiphe graminis* f. sp. *hordei* on barley coleoptile tissue injected with silicon oil (arrows). Injected conidium developed appressorium **(A)**, haustorium **(B)**, and elongating secondary hyphae **(C)**. Photographs were taken at 10, 18, and 96 hr after injection. Bar = 10 μm.

materials into powdery mildew conidia without detrimental effects on subsequent fungal development. The method can be used to introduce function-defined chemicals, such as fungicides or antibiotics, into conidia. Such applications will allow workers to determine the involvement of the corresponding metabolites in fungal development at the molecular level without affecting metabolism of host plant cells on which the fungus lives.

Another possible application is transfer of genetic materials from the pathogenic fungus (*E. g.* f. sp. *hordei*) to other Erysiphaceae that are nonpathogenic on barley coleoptiles. With

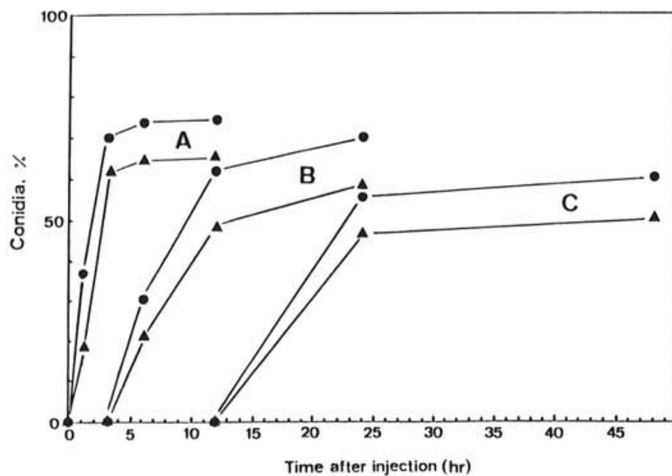

Fig. 6. Time course of infection structure formation by conidia of *Erysiphe graminis* f. sp. *hordei* on barley coleoptiles injected (triangles) and not injected (circles) with silicon oil (1 μm diameter). **A** represents rate of germination, **B** of appressorium formation, and **C** of haustorium formation. Injected and noninjected conidia (each 150 conidia) were observed at the various stages after injection.

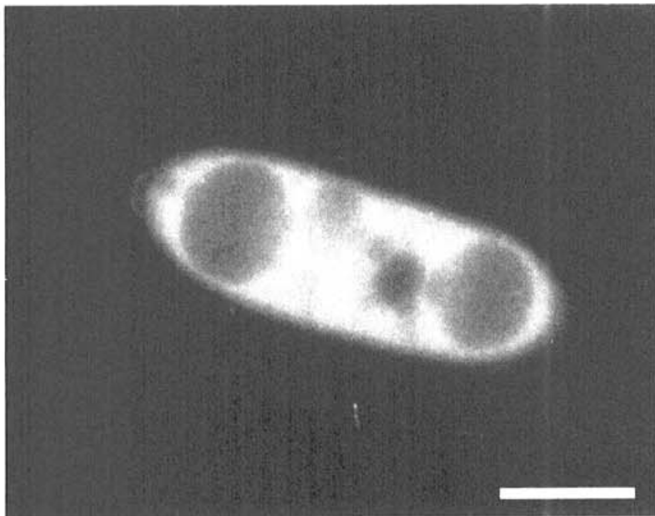

Fig. 7. Fluorescence micrograph of conidium on barley coleoptile injected with fluorescein-isothiocyanate-conjugated albumin (5 mg/ml) for 3 sec. Bar = 10 μm.

our microinjection system, it is very easy to suck cellular contents from one conidia and subsequently to inject them into another conidia on coleoptile tissue by electrically reciprocating the injector syringe. If genetic factors mediating fungal pathogenesis are successfully introduced and transformed, it would be easy to detect nonpathogenic fungi that have established the infection on nonhost barley coleoptiles. Thus, the microinjection technique is a feasible method for analyzing plant host/fungal parasite interactions and for manipulating genes in fungal pathogens.

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