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Temperature and Structural Effects on Transfer of Double-Stranded RNA among Isolates of the Chestnut Blight Fungus (*Cryphonectria parasitica*)

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***Cryphonectria parasitica* is a unique fungus which can serve as a model for understanding transfer of genes between eukaryotic microorganisms. We studied transfer of double-stranded RNA (dsRNA) between compatible and incompatible strains of *C. parasitica* to determine whether hyphal types or temperature could restrict that exchange. Hyphal connections between incompatible strains occurred at about 30% of the frequency of connections between compatible strains and differed morphologically. Gel electrophoresis and in situ hybridization confirmed that dsRNA was transferred through substrate hyphae but not through aerial hyphae. Freezing temperatures resulted in the loss of dsRNA from the new mycelium of the donor colony and stimulated the production of virulent pycnidiospores. These temperature and structural restrictions may help to explain the lack of spread of the dsRNA despite its presence in the field.**

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Cryphonectria (= *Endothia*) *parasitica* (Murr.) Barr is the causal agent of chestnut blight, the disease responsible for transformation of the American chestnut (Castanea dentata (Marsh.) Borkh.) from a dominant overstory tree to an understory, immature shrub (1, 8, 11, 12, 14). The fungus attacks the tree, girdling it within 5 to 20 years and killing the above-ground portion of the tree. The tree then resprouts, and the process is repeated (1).

In the 1960s, European pathologists noted that some of the trees recovered and discovered that the fungus in these trees was hypovirulent, i.e., capable of surviving but incapable of killing the host. The hypovirulence was associated with a cytoplasmic genetic element, a double-stranded RNA (dsRNA) virus (4). Despite the rapid spread of the fungus, the dsRNA appeared to spread slowly or not at all. The mechanisms for this low rate are not clear despite the presence of dsRNA in *C. parasitica* in many forests (1). The dsRNA is localized in cytoplasmic membrane-bound vesicles (7) and can be transferred between individuals (1). Therefore, we hypothesized that this cytoplasmic element could serve as a model for the transfer and escape of engineered genes in a eukaryotic microorganism. Specifically, we hypothesized that the transfer of dsRNA between both compatible and incompatible isolates of *C. parasitica* can be influenced by structural characteristics or temperature.

MATERIALS AND METHODS

The isolates of *C. parasitica* used were chosen because their genetic and physiological characteristics have been studied extensively (6). These isolates included both virulent (EP155 and EP2001) (without dsRNA) and hypovirulent (EP113) (with dsRNA) strains. An additional hypovirulent isolate (EP2001F) was derived by transferring the dsRNA from EP113 into EP2001 (6). Isolates EP113, EP2001, and EP2001F are compatible, whereas EP155 is incompatible. Isolates were grown on PDAmys medium (described by Hansen et al. [7]) at 25°C in the light unless otherwise indicated. The effects of temperature on dsRNA transfer were examined at 25 and 30°C. Other isolates were frozen at −10°C for 24 h and then returned to 25°C. Five replicates for each study were conducted.

To microscopically examine the interface between two isolates, the colonies were grown side by side. Segments of the contact zone between the two colonies were cut out with a sterile scalpel and were trimmed into small blocks of agar with both substrate and aerial hyphae. The blocks of agar and fungal hyphae were fixed, dehydrated, and embedded in epoxy resin by a standard protocol for plant tissue (2). The plastic blocks were mounted, and 4- to 6-μm sections were cut across the interface with a microtome. The sections were examined microscopically for the presence, structure, frequency, and locations of connecting hyphae. The frequency of hyphal connections was estimated by a line intercept method in which the number of clear hyphal fusions was calculated for a given area for each compatibility group. In addition, the interface zone of actively growing colonies was observed directly for antagonistic interactions as indicated by autofluorescence. Autofluorescence was observed with a Leitz Laborlux II microscope with a violet-blue excitation filter and a long-pass suppression filter (filter cube no. H3).

Gene transfer between strains was detected by three methods. First, the recipient hyphae were examined for changes in morphology that occurred after the colonies came into contact. In these isolates, color can be correlated with the presence of the dsRNA (7), so a change in hyphal color from yellow prior to contact to white after contact with the donor fungus suggested the presence of the dsRNA. Second, the transfer of the dsRNA was examined by colony blot hybridization as described by Martin and Van Alfen (10). This procedure uses a biotin-labeled probe that attaches to the dsRNA in the fungi growing on GeneScreen Plus (New England Nuclear, Boston, Mass.). The GeneScreen Plus was

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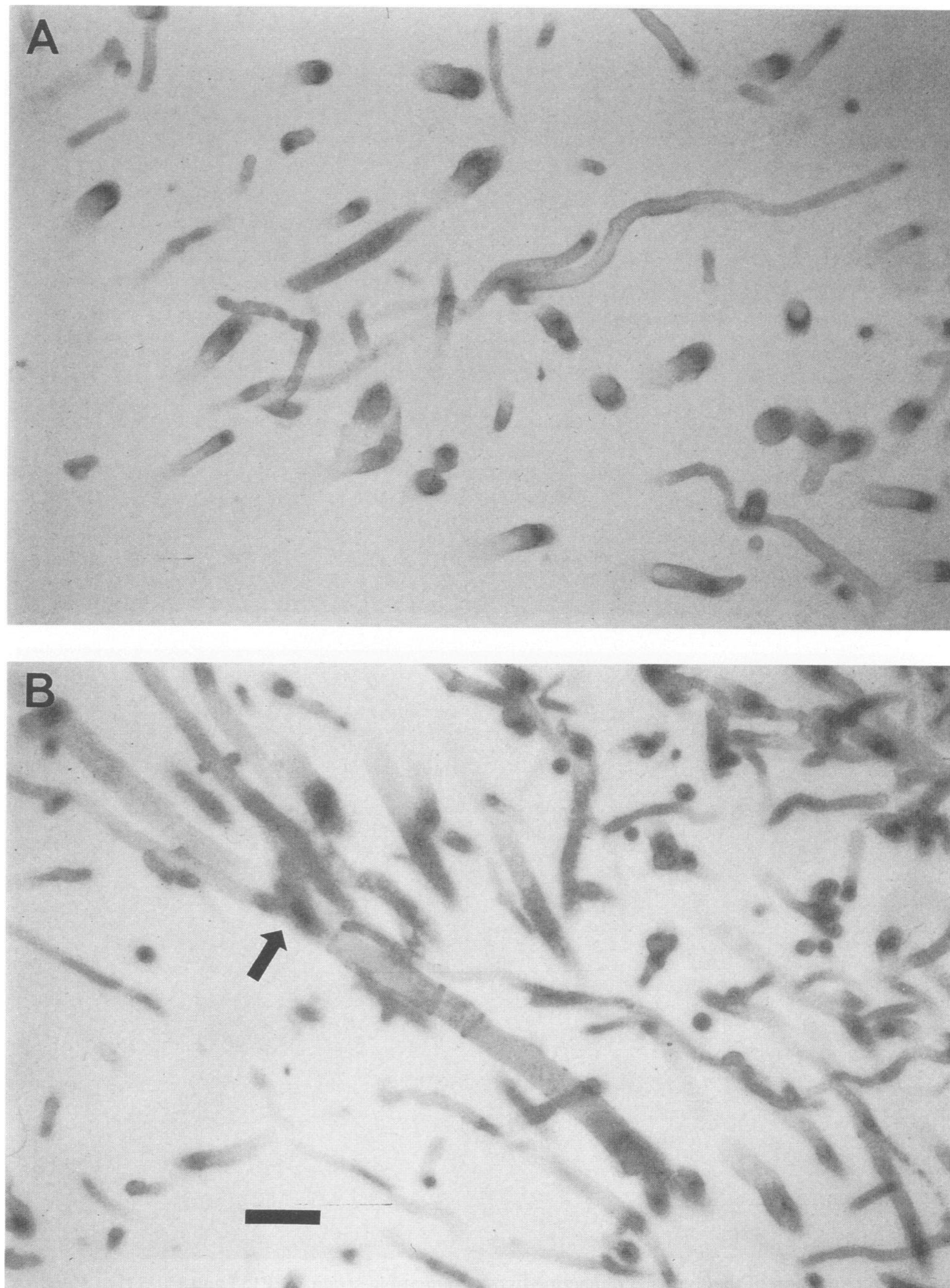

FIG. 1. Hyphal anastomosis in the substrate hyphae of *C. parasitica*. Magnification, ×600; bar = 10 μm. Shown are the initial connections among compatible isolates (EP113 and EP2001) (A) and hyphal intertwining (arrow) between mature incompatible isolates (EP155 and EP113) (B).

placed directly over the inoculated plate, and the fungus was allowed to grow through the screen at 25°C. The screen was isolated after 5 days when the fungal colonies had grown over the GeneScreen Plus. The membrane then was peeled away from the agar and processed through a series of standard washes. The nucleic acid bound on the membranes then was denatured, incubated in prehybridization solution, and probed with a biotin-labeled cDNA clone (10). Detection and visualization reagents were applied as described in the BluGENE nonradioactive nucleic acid detection kit (catalog no. 8279SA; Bethesda Research Laboratories). The presence of the dsRNA also was verified by reisolating the hyphae of interest and growing it in pure liquid culture. After several days of growth, the dsRNA was purified, and its presence was detected by polyacrylamide gel electrophoresis as described elsewhere (7).

The ability of the dsRNA to migrate between aerial hyphae and between surface or subsurface hyphae was tested by using split plates. To test for aerial hyphal transport, colonies were placed on both sides of a barrier. The

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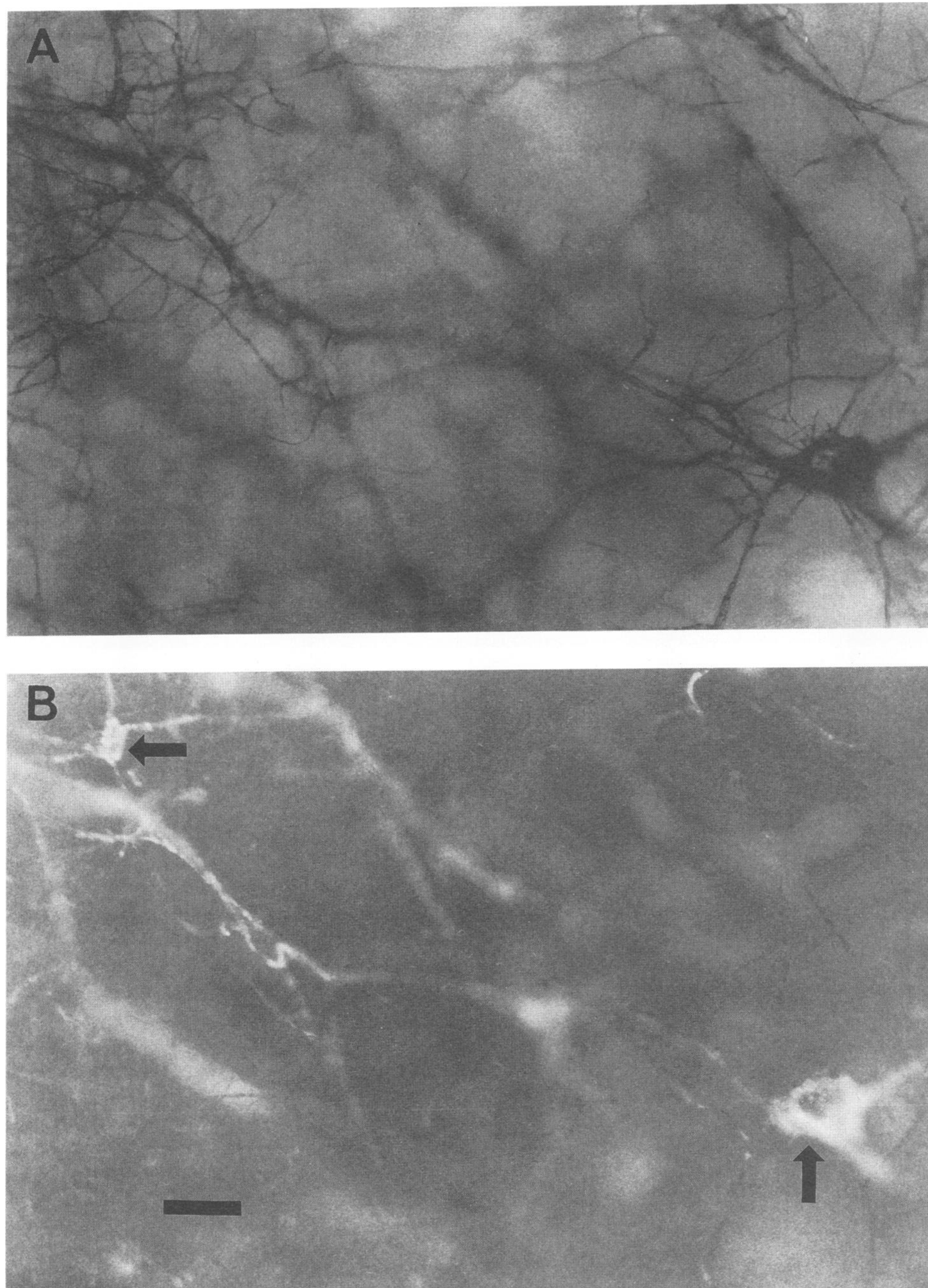

FIG. 2. Autofluorescence reaction of the hyphae connecting two incompatible strains (EP155 and EP113) of *C. parasitica*. Magnification, ×160; bar = 100 μm. Shown are the hyphae without autofluorescence under white light (A) and the autofluorescing hyphae (arrows) where intertwining networks were present (B). The autofluorescence was observed with a Leitz microscope as described in the text.

hyphae were allowed to connect only over the barrier. To test for surface transfer, another set of isolates was grown on barrier plates as described above, except that the barrier had a notch cut in it such that the surface and subsurface hyphae could connect only through that point. The movement of dsRNA was monitored by colony blot hybridization as described above. To examine the aerial hyphae directly for the presence of dsRNA, these hyphae were isolated and grown on PDAmys medium. These colonies were then assessed for the presence of dsRNA by electrophoresis (7).

RESULTS

Hyphae of both compatible and incompatible isolates were observed to fuse. When hyphae of compatible fungi intersected, single hyphae were observed to fuse (Fig. 1). The mean number of hyphal connections (± standard error) per 100 μm^2 between incompatible fungi (4.17 ± 1.06) occurred at about 30% of the estimated frequency of the connections between compatible fungi (12.09 ± 1.45). These pairings were quite different morphologically (Fig. 1). With incom-

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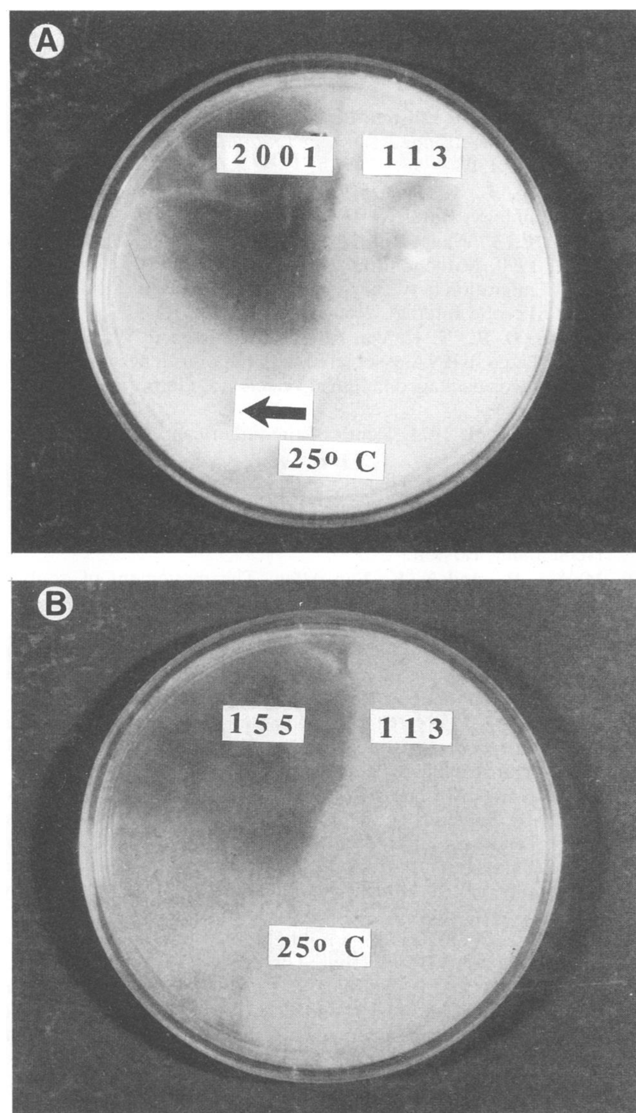

FIG. 3. Pigment transformations of the recipient colony after transfer of dsRNA in a compatible interaction (arrow; EP113 and EP2001) (A) and an incompatible interaction (EP113 and EP155) (B) between fungal isolates grown at room temperature.

patible isolates, groups of four to six individual hyphae from each isolate merged to form an intertwining network (Fig. 1). These networks of the incompatible fungi autofluoresced (Fig. 2). No autofluorescence between compatible isolates was observed. All connecting hyphae were found only on the surface or in the agar medium. No interconnections occurred in the aerial hyphae of compatible or incompatible interacting fungi.

All methods to test for transfer of the dsRNA between isolates provided the same results. The dsRNA was transferred between both compatible and incompatible isolates. The color of the recipient colony after contact and transfer of the dsRNA resembled that of a hypovirulent strain in each case (Fig. 3). The colony blot hybridization and electrophoresis confirmed that the dsRNA was transferred to the receiver fungus following hyphal connections between all isolate pairings. The patterns were identical for compatible and incompatible fungi.

The fate and transfer of the dsRNA between compatible and incompatible fungal isolates, as well as the growth rate and asexual reproduction of the organism, were affected by temperature. After the isolates were frozen, the dsRNA disappeared from the donor colony; this disappearance was indicated by a return of a yellow pigmentation to the actively growing outer region of the affected colonies. Some isolates failed to survive the freezing temperatures. The environmental stress of freezing also appeared to stimulate abundant asexual sporulation, but the colonies arising from spores did not contain dsRNA. At room temperature, transfer of dsRNA occurred between strains as described above. At 30°C, dsRNA was transferred between the compatible fungi but not between incompatible strains.

The dsRNA was not detected in aerial hyphae after direct isolation of those hyphae, nor was the dsRNA transferred when only the aerial hyphae grew over the barrier and into the recipient mycelium. However, the dsRNA was transferred when the barrier was breached, allowing contact of surface and subsurface hyphae.

DISCUSSION

The data presented here confirm the observation that dsRNA can be transferred among compatible and incompatible isolates of *C. parasitica* (6). However, pairings of incompatible isolates exhibit an antagonistic autofluorescence response coupled with complex hyphal interactions and fewer hyphal connections than occur among compatible isolates. The antagonistic interactions between incompatible isolates support the hypothesis that these fungi act as individuals, thus restricting their interactions. This may help explain the lack of transfer of nuclear or mitochondrial DNA between incompatible isolates previously observed (6). Nevertheless, the dsRNA passed that barrier and rapidly spread to the incompatible colony.

Despite the fact that dsRNA migrated between all isolates tested, there were structural and temperature constraints. Firstly, not all hyphae transfer dsRNA. Since the aerial hyphae apparently did not contain dsRNA, they could not transfer the dsRNA in either compatible or incompatible pairings. The features differentiating the aerial and submerged hyphae and causing this response are unknown and should be the basis for further study. In addition, incompatible hyphal connections, which were found at a reduced frequency, are antagonistic and probably limit the movement of dsRNA in nature. Finally, environmental factors such as temperature appear to affect the transfer and fitness of the dsRNA. Freezing temperatures apparently cause a loss of dsRNA. Also related to freezing is the stimulated production of asexual pycnidiospores that contained no detectable dsRNA.

These data suggest potentially significant temperature and morphological constraints on the spread of the hypovirulence-inducing dsRNA. The American chestnut was once a major forest tree in the eastern United States (14) and could reemerge if the hypovirulence-inducing dsRNA could move freely in nature among virulent forms of the fungi. Potential vectors of dispersal of the chestnut blight fungus include insects and birds (1), and the fungal propagules that these organisms transmit are most likely to be the asexual spores and the aerial hyphae growing out of the bark surface. However, these propagules may not contain dsRNA. Furthermore, freezing temperatures such as those found in

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temperate climates of northeastern deciduous forests could adversely affect the dsRNA in the fungi on trees. While these results provide insight into structural and temperature constraints on hypovirulence, further work on other environmental factors regulating movement of extranuclear elements such as dsRNA is needed.

The major problems with the movement of extranuclear elements and the introductions of engineered microorganisms reside in our inability to predict their responses in the environment, even in a general sense (5). Indeed, most recent discussions deal with the potential for application of these organisms but avoid discussion of how they will be controlled in the environment. It is clear that the transfer of cytoplasmic genes in fungi can be very complicated. In a narrow view, incompatible isolates of a fungus species are individuals incapable of exchanging materials and genes to a large degree (13). According to this view, there should be little concern for the horizontal transfer and subsequent escape of an introduced microorganism. One could simply monitor the original microbe and assume that the engineered gene is encased only in that organism. At the other extreme, the view that microbial genes are readily exchanged between organisms has been often expressed (3, 9, 15). Simple assessments, such as the view that genes will always be transferred to other organisms or that they will never be transferred, are inadequate. A much better understanding of the basic population ecology of fungi is needed before predictions of the potential transfer and escape of engineered genes can be made.

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