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Set-up of a biocontrol experiment with genetically characterized hypovirus-infected *Cryphonectria parasitica* strains

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Abstract

For a better understanding of its dissemination, the *Cryphonectria* hypovirus has been artificially introduced into two different *C. parasitica* populations. Chestnut blight cankers were treated with a hypovirus-infected strain. In one population (Choëx, Valais) hypovirulence was absent before the treatment, whereas in the other (Claro, Ticino) it was already naturally present. The aim of the experiment was to track the introduced virus as well as its fungal carrier and to survey the dynamic of the disease in these stands. In Choëx, where no other viruses were present, the virus is easily detected by verifying the virus-associated culture morphology of the isolated strains. In Claro, RFLPs of two fragments amplified from the reverse transcribed viral genome (RT-PCR) serve as markers to distinguish the introduced virus from the resident hypoviruses. Similarly, a DNA fingerprinting method is used to identify the introduced fungal strain.

Introduction

*Cryphonectria (*Syn. *Endothia) parasitica* (Murill) Barr., an ascomycete, is the causal agent of chestnut blight. After the introduction into the eastern USA at the beginning of this century it virtually eliminated the American chestnut trees (*Castanea dentata*) in their natural range (Anagnostakis 1987). A similar result was anticipated when chestnut blight was found in Europe near Genova (Italy) in 1938. However, the unexpected appearance of transmissible hypovirulence prevented the European chestnut (*C. sativa*) from succumbing to the blight (reviewed by Heiniger and Rigling 1994).

Hypovirulence is caused by an unencapsidated double-stranded (ds) RNA virus, which is transferred between fungal strains via hyphal anastomosis (Choi and Nuss 1992, Day et al. 1977, Nuss 1992). Infection by the hypovirus causes a reduction of fungal virulence (hypovirulence) ranging from avirulence to almost normal virulence (Bazzigher et al. 1981, Rigling et al. 1989). Furthermore, infected strains show reduced sporulation and pigmentation, resulting in the typical white cultural appearance.

The hypovirus is of great interest for biological control. However, the exact mechanisms of its dissemination are still unclear. Several factors restrict the dissemination of the hypovirus in fungal populations: i) vertical transmission occurs only into asexually produced conidia, but not into sexually produced ascospores, ii) the rate of transmission into conidia varies considerably, iii) conidia production is reduced in hypovirulent strains and iv) a system of vegetative incompatibility limits horizontal transmission of the virus among fungal strains (Anagnostakis 1988, Liu and Milgroom 1996). Nevertheless, the hypovirus is disseminated quite efficiently in many European *C. parasitica* populations.

For a better understanding of the dissemination of the hypovirus, an experiment in two different plots was started. Biocontrol treatments with selected hypovirus-infected strains were performed. Subsequently, the temporal and spatial movement of the introduced hypovirus and its fungal carrier will be followed by using genetic markers.

Materials and Methods

The two experimental plots in Choëx and Claro are *C. sativa* coppice stands. Choëx is located at the edge of the occurence of chestnut blight in Switzerland. The disease was first recorded in this region in 1986 (Heiniger and Stadler 1990). Claro is situated in southern Switzerland, where chestnut blight has been present since at least 1948 (Gäumann 1951).

Isolations of *C. parasitica* were performed as described in Bissegger et al. (1997), with the modification that tannic acid malt extract agar (Rigling 1995) was used as isolation medium. The isolates were assigned to vegetative compatibility groups (VCGs) according to the merging/barrage response (Anagnostakis 1988). Culture morphology and dsRNA extractions were used to identify hypovirus-infected isolates. The inoculum source had to be prepared after selecting a hypovirus (the same for both plots) and fungal strains (one for each plot) adapted to the resident populations. The selected fungal strains (carriers) were infected with the hypovirus by pairing them in vitro with a strain infected with the selected hypovirus. The hypovirulent inoculum for the biocontrol treatment was grown in liquid culture with EP complete medium (Day et al. 1977) at 25 °C for 5 days. Cankers were treated by making holes at the margin of the infection using a cork borer (5 mm in diameter). The holes were filled with the inoculum and covered with tape.

RFLPs of two fragments amplified from the reverse transcribed viral genome were used to identify the introduced hypovirus. dsRNA was isolated by cellulose chromatography (Morris and Dodds 1979). First strand cDNA was synthesized from the purified dsRNA using random primers. The cDNA was used as template to amplify two fragments (one from ORF A and one from ORF B) of the viral genome. These fragments were digested separately with different four-cutter restriction enzymes. RFLPs were visualized by running the digests on agarose gels. Similarly, a DNA fingerprinting method developed by Milgroom et al. (1992) was used to identify the introduced fungal strain. This method consists of probing Southern blots of *Pst*I-digested total DNA with a multilocus probe (pMS5.1). Instead of radioactivity, DIG-labelling and detection by chemiluminescence was used.

Results and Discussion

The fungal population in Choëx was very clonal. Only one vegetative compatibility group (VCG) was found among 140 isolates tested. The same VCG was found among 23 isolates from the surrounding area. Almost all DNA fingerprints were identical. Only 3 out of 56 isolates showed a slightly different pattern. This can be explained by the recent introduction of the disease in this region (Heiniger and Stadler 1990). Most probably, only a very few genotypes were responsible for the infection in Choëx. Furthermore, no hypovirus-infected strains were isolated, i.e. no hypoviruses were present. The presence of only one VCG in this plot greatly favors the application of biocontrol treatments. One single hypovirus-infected strain has the potential to convert all other strains in the plot, thus rising the prospects of a good establishment of hypovirulence. The clonal fungal population without any hypovirus infection also facilitates the tracking of both, the hypovirus and its fungal carrier. The

introduced hypovirus can easily be detected by verifying the virus-associated culture morphology of the isolated strains. A fungal strain was selected from the culture collection (at the Swiss Federal Institute for Forest, Snow and Landscape Research, CH-8903 Birmensdorf) that fulfilled the criteria shown in Table 1.

Table 1. Criteria for the selection of the hypovirus and its fungal carrier for the biocontrol experiment

Hypovirus		
	Virulence	The hypovirus should not deblilitate its fungal host too much. The hypovirus-infected strain must still be able to form cankers and to produce asexual spores.
	Identity	The hypovirus must be genetically distinguishable from the resident viruses (RT-PCR/RFLP).
Fungal strain		
	VCG	The fungal strain must be in the dominating VCG of the plot. This is crucial for the horizontal transmission of the hypovirus and prevents emergence of new VCGs by outcrossing.
	Identity	The fungal strain must be genetically distinguishable from the resident strains (DNA fingerprinting).

The fungal population in Claro shows a completely different picture compared to Choëx. Twelve different VCGs were detected among 170 isolates tested. More than 90% of the isolates could be assigned to only four major VCGs. Similarly, a high diversity in DNA fingerprints was detected. Fourty different patterns were observed among 58 isolates. Almost 50% of the isolates were hypovirus-infected. Comparable diversity was found in other populations in southern Switzerland (Bissegger et al. 1997). Therefore, for subsequent tracking in the field, the hypovirus and the fungal strain had to be selected carefully according the criteria shown in Table 1.

Biocontrol treatments were performed in order to introduce the hypovirus-infected strains in the plots in fall 1996. In Choëx about 30% and in Claro about 20% of the cankers were treated. In spring 1997, a subset of the treated cankers was sampled to determine if the virus was still present after the winter period. Strains exhibiting the white culture morphology could be isolated from all of the sampled cankers, indicating a good establishment of the introduced hypovirus. The temporal and spatial movement of the hypovirus and its fungal carrier will now be followed by examining the population periodically. The results of this study will lead to a better understanding of the dissemination of the *Cryphonectria* hypovirus and thus be helpful to current attempts for a sustainable biological control of chestnut blight.

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