

Characterization of South African *Cryphonectria cubensis* Isolates Infected with a *C. parasitica* Hypovirus

S. W. van Heerden, L. M. Geletka, O. Preisig, D. L. Nuss, B. D. Wingfield, and M. J. Wingfield

First, third, fifth, and sixth authors: Tree Pathology Cooperative Programme, Forestry and Agricultural Biotechnology Institute, Department of Genetics, University of Pretoria, Pretoria, 0002, South Africa; and second and fourth authors: Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park 20742.

Accepted for publication 5 April 2001.

ABSTRACT

van Heerden, S. W., Geletka, L. M., Preisig, O., Nuss, D. L., Wingfield, B. D., and Wingfield, M. J. 2001. Characterization of South African *Cryphonectria cubensis* isolates infected with a *C. parasitica* hypovirus. *Phytopathology* 91:628-632.

Cryphonectria cubensis is the causal agent of a serious canker disease of *Eucalyptus* spp. in tropical and subtropical parts of the world. In this study, a South African *C. cubensis* isolate was transfected by electroporation with a synthetic RNA transcript corresponding to the full-length coding strand of the *C. parasitica* hypovirus (CHV1-EP713). Hypovirus infection resulted in pronounced morphological changes that included a striking increase in bright yellow-orange pigment production, a reduction

in mycelial growth rate, and reduced sporulation. Greenhouse studies revealed that the virus-containing strain was significantly less virulent than the original virulent *C. cubensis* isolate. Although the hypovirus was not transmitted through conidia produced by infected *C. cubensis*, the virus was readily transmitted via hyphal anastomosis to *C. cubensis* isolates representing a broad range of vegetative compatibility groups. These results suggest that vegetative incompatibility may not pose a strong barrier against virus transmission in South African isolates of *C. cubensis* and that hypovirus-mediated biological control could provide opportunities to reduce the impact of *Cryphonectria* canker in South Africa.

Additional keywords: *Eucalyptus* diseases, hypovirulence.

Cryphonectria cubensis (Bruner) Hodges, causes a serious stem canker disease on *Eucalyptus* spp. that reduces forest plantation yield. This disease is favored by high rainfall and temperatures above 23°C and has thus reduced the establishment of *Eucalyptus* spp. in the tropics and subtropics (1,15,19,30), including the KwaZulu-Natal area of South Africa. Various strategies have been implemented to reduce the impact of *Cryphonectria* canker. The most effective strategies are breeding and selection of resistant planting stock. Another promising prospect to reduce the impact of *Cryphonectria* canker is through biological control, involving hypovirulence mediated by double-stranded (ds) RNA mycoviruses.

dsRNA viruses in the family *Hypoviridae* can attenuate virulence (hypovirulence) of the chestnut blight fungus *C. parasitica* (Murr.) Barr, thereby contributing to reduced disease severity and biological control (3,6,12,16,17). Symptoms associated with hypovirus infection can include altered colony morphology (3,14), suppressed conidiation (13), suppressed pigmentation (3), and a reduction in oxalate accumulation (18,22).

For hypoviruses to serve optimally as biocontrol agents they have to disseminate efficiently through the fungal population. Hypoviruses can be transmitted in two ways, either vertically through asexual spores (conidia) or horizontally via hyphal anastomosis (27). However, the ability of *C. parasitica* strains to anastomose depends upon a vegetative incompatibility (*vic*) system (2) governed by five to seven genetic loci (4). Liu and Milgroom (24) have reported a negative correlation between the frequency of hypovirus transmission and the number of *vic* genes that differ between two fungal isolates. However, the correlation

is not absolute, i.e., hypovirus transmission can occur between fungal strains that are unable to form heterokaryons due to different alleles at a single *vic* locus (20,21).

The potential utility of dsRNA viruses for biological control has been enhanced by the construction of a cloned cDNA copy of the viral RNA genome. A full-length cDNA clone was constructed from the large dsRNA of the hypovirus CHV1-EP713 (9,11). Choi and Nuss (11) reported that hypovirus infection occurred when a virus-free *C. parasitica* strain was transformed with a plasmid, pXH9, containing this hypovirus cDNA clone. Chromosomal integration of a functional hypovirus cDNA copy introduced new modes for virus transmission. Essentially, all asexual spores generated from such transgenic fungal strains carry the integrated virus cDNA and cytoplasmically replicating viral RNA derived from the chromosomally integrated viral cDNA copy (8). Unlike the cytoplasmic hypovirus RNA, the integrated viral cDNA is transmitted to ascospore progeny via nuclear inheritance (8).

Chen et al. (9) were able to establish virus infection by electroporation of in vitro-produced RNA transcripts representing the coding strand of hypovirus CHV1-EP713 into fungal spheroplasts. The hypovirus transfection system was successfully employed to establish virus infection in three other species of *Cryphonectria* and one *Endothia* sp. These included the *Eucalyptus* spp. pathogens *C. havanensis* (Bruner) Barr and *C. cubensis*, a nonpathogenic fungus *C. radicalis* (Schw. ex Fries) Barr and the oak pathogen *Endothia gyrosa* (Schw. ex Fries) Fries. Although hypovirulence was reported only for transfected *Endothia gyrosa*, these results raised the possibility that hypoviruses could be used for the biological control of other pathogenic fungi such as *C. cubensis*.

The aim of this study was to transfect a well-characterized, virulent, South African *C. cubensis* isolate with the full-length coding strand transcript of *C. parasitica* hypovirus CHV1-EP713. The transfected isolate was characterized with respect to sporulation and ability to transmit viral RNA through conidia. A set of isolates representing the known range of vegetative compatibility

Corresponding author: S. W. van Heerden
E-mail address: schalk.vanheerden@fabi.up.ac.za

Publication no. P-2001-0507-02R
© 2001 The American Phytopathological Society

(VC) groups from the South African *C. cubensis* population were used to determine whether the dsRNA could be transferred to virus-free strains by hyphal anastomosis. Greenhouse inoculations were conducted in an initial effort to evaluate the effect of hypoviruses on virulence and thus their potential usefulness as bio-control agents for *Cryphonectria* canker in South Africa.

MATERIALS AND METHODS

Transfection. Virulent South African *C. cubensis* isolate (CMW 2113) was selected to be transfected with *C. parasitica* hypovirus CHV1-EP713 RNA (32). Full-length positive strand CHV-EP713 RNA was produced in vitro from *SpeI*-linearized plasmid pLDST using T7 RNA polymerase (9). The product RNA transcript was mixed with fungal spheroplasts and electroporated according to the protocol of Chen et al. (7).

Pathogenicity tests. *Eucalyptus grandis* clone TAG5, known to be moderately tolerant to *C. cubensis* infection (S. W. van Heerden, unpublished data), was used for the inoculation studies. Trees were grown in 1-liter planting bags and maintained in a glasshouse at temperatures ranging from 23 to 25°C until they were approximately 1 m high and 1 year old. Two isolates were used in the inoculations: transfected isolate CMW 2113-T and wild-type virus-free isolate CMW 2113.

Trees were wounded by removing a cambial disk with a 5-mm-diameter cork borer. A similar sized disk from an actively growing culture on 2% malt extract agar (MEA) was placed in each wound and wrapped with Parafilm to reduce desiccation. Fifteen plants were inoculated with each of the two isolates. Ten plants were inoculated in a similar manner with a sterile MEA disk as a control. After 4 weeks, the Parafilm was removed from the inoculated

stems and the cambial lesion lengths were measured. The entire trial was repeated once. Data were statistically analyzed using a one-way analysis of variance with SYSTAT (version 7.0.1, SPSS Inc., Chicago).

Conversion of virus-free virulent *C. cubensis* isolates. A previous study showed that the South African *C. cubensis* population is represented by 23 VC groups (32). Twenty-nine isolates representing all 23 characterized VC groups were selected for this experiment (Table 1). Prior to inoculation, 2% MEA agar surfaces were overlaid with sterile cellophane disks in 9-cm-diameter petri dishes to reduce the nutrient supply and, therefore, the growth of the fungus. The transfected isolate was paired against all 29 isolates according to the technique described by Anagnostakis and Day (5). One cube of agar (4 mm³) was transferred from the edge of a young, actively growing culture of the healthy isolate and placed 2 mm from a cube of agar representing the transfected isolate (CMW 2113-T) in the middle of the petri dish. Cultures were incubated in the dark at 25°C for 5 to 7 days. Determination of the conversion to the hypovirulent phenotype was made 7 days after incubation and was based on morphology and the presence of dsRNA in the test isolates subcultured from the middle of the fungal colony. It was further determined whether the isolates that were converted to the transfected phenotype could act as donors to other non-dsRNA containing isolates representing different VC groups.

Detection of dsRNA. The transfected isolate (CMW 2113-T) is bright yellow-orange, whereas the usual color of this fungus is a

TABLE 1. *Cryphonectria cubensis* isolates tested for conversion to the hypovirulent phenotype

VC group ^a	Isolate number	No. of isolates	Success of conversion	Converted by
SA1	CMW 6087	...	+	CMW 2113-T
	CMW 6088	2	-	...
SA2	CMW 6089	1	-	...
SA3	CMW 6090	1	-	...
SA4	CMW 6091	1	-	...
SA5	CMW 6092	1	+	CMW 6115-T
SA6	CMW 6093	1	-	...
SA7	CMW 6094	...	-	...
	CMW 6095	...	-	...
	CMW 6096	3	-	...
SA8	CMW 6097	1	-	...
SA9	CMW 6098	...	+	CMW 2113-T
	CMW 6099	2	+	CMW 6109-T
SA10	CMW 6100	1	-	...
SA11	CMW 6101	1	+	CMW 6115-T
SA12	CMW 6102	1	-	...
SA13	CMW 6103	...	+	CMW 2113-T
	CMW 6104	2	+	CMW 2113-T
SA14	CMW 6105	...	+	CMW 2113-T
	CMW 6106	2	-	...
SA15	CMW 6107	1	-	...
SA16	CMW 6108	1	+	CMW 6109-T
SA17	CMW 6109	1	+	CMW 2113-T
SA18	CMW 6110	1	-	...
SA19	CMW 6111	1	+	CMW 2113-T
SA20	CMW 6112	1	+	CMW 2113-T
SA21	CMW 6113	1	-	...
SA22	CMW 6114	1	+	CMW 2113-T
SA23	CMW 6115	1	+	CMW 2113-T

^a Vegetative compatibility (VC) groups identified by van Heerden and Wingfield (32). Isolates converted to the hypovirulent phenotype were recognized by a change to the yellow phenotype and were further used for verification of conversion using the reverse transcription polymerase chain reaction (RT-PCR) technique. The nonconverted isolates were used as controls for the RT-PCR amplification.

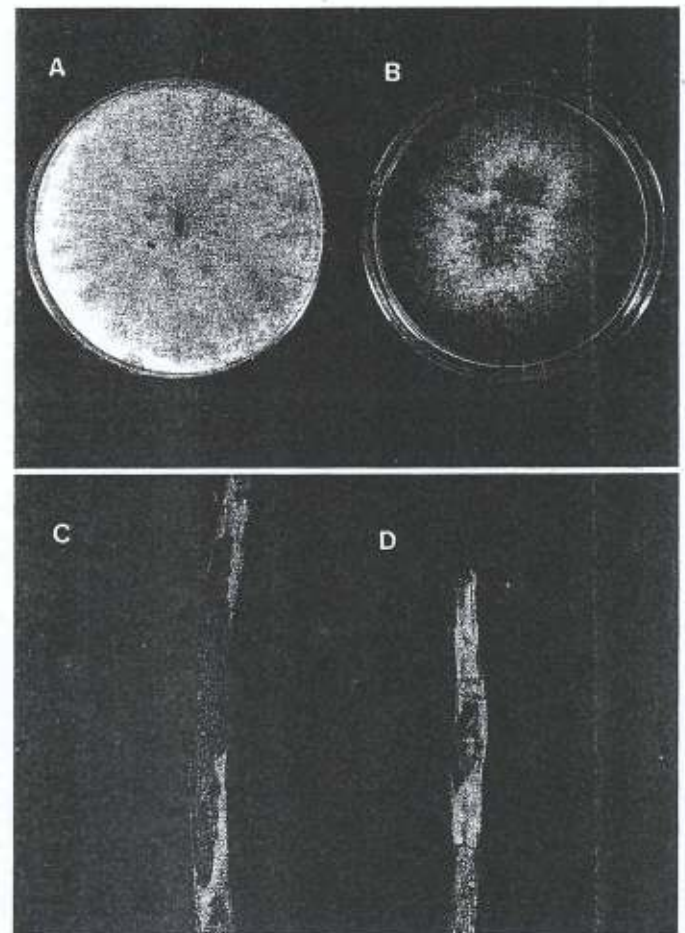


Fig. 1. Phenotypic characteristics associated with healthy virus-free isolate and transfected *Cryphonectria cubensis* isolate (CMW 2113-T). A, Wild-type virus-free isolate characterized by white mycelia. B, Transfected isolate characterized by bright yellow-orange hyphae penetrating deeply into the agar with an uneven colony margin. C, Lesions on *Eucalyptus grandis* clone (TAG5) associated with the wild-type isolate of *C. cubensis* and D, the transfected isolate.

much lighter orange. Transfer of dsRNA was therefore observed visually by the change in culture color. Two representative sectors from each converted isolate were subcultured to petri dishes containing 2% MEA. These two sectors were taken from the colony margin of the transfected isolate and the isolate against which it was paired. In addition, one isolate (CMW 6094) that did not show any morphological signs of virus transfer was tested for the presence of dsRNA and, thus, served as a control. The transfer of the hypovirus was verified by a reverse transcription polymerase chain reaction (RT-PCR). In this way, it was possible to correlate visual signs of conversion of the virulent to the hypovirulent phenotype with the presence of dsRNA.

Total nucleic acid extraction was performed on mycelium scraped from colonies of the selected isolates (Table 1). Nucleic acids were extracted according to the protocol described by Preisig et al. (29). A one tube RT-PCR system (Titan; Roche Molecular Biochemicals, Mannheim, Germany) was used for dsRNA detection with primer pair RSDS10 and BR43. Primer RSDS10 (5'-GCCTATGGGTGGTCTACATAGG-3') corresponds to the 5'-terminal sequence of CHV1-EP713 coding strand, and primer BR43 (5'-GGATCCACTGTAGTAGGATCAA-3') is complementary to nucleotide positions 566-545 of the CHV1-EP713 coding strand. RT was performed for 30 min at 50°C. Amplification conditions for PCR were one cycle at 94°C for 2 min, 10 cycles at 94°C for 30 s, 65°C for 30 s, 68°C for 1 min followed by 25 cycles at 94°C for 30 s, 65°C for 30 s, and 68°C for 1 min with a 5 s time increase per cycle. A prolonged final elongation time at 68°C for 10 min was used. The products obtained were separated with a 1.5% agarose gel.

Virus transfer to conidia. To determine whether the virus was present in the conidia of the transfected *C. cubensis* isolate, freshly cut stem sections (1 cm in diameter and 15 cm long) from the *Eucalyptus grandis* clone (TAG5) were used to culture the fungus. This was done instead of culturing the fungus on synthetic medium where the sporulation rate is usually low. Prior to inoculation, the stem pieces were surface disinfected by wiping the surfaces with 70% ethanol. The ends were coated with melted paraffin wax to reduce desiccation. The bark was removed with a 5-mm-diameter cork borer, and stem sections were inoculated under laboratory conditions with disks of agar taken from the actively growing margin of a 7-day-old fungal culture (CMW 2113-T). Fifteen stem sections were inoculated in this manner. The wounds were sealed with Parafilm to prevent drying and contamination. The stem pieces were placed in a plastic container lined with moist filter paper, incubated at 25 to 28°C, and regularly inspected for the presence of fruiting structures. After matured pycnidia formed, 84 single conidial cultures were made. The presence of the virus in these isolates was tested by RT-PCR as described previously.

RESULTS

Transfection and pathogenicity tests. Transfection of the South African *C. cubensis* isolate (CMW 2113) with the full-length coding strand of *C. parasitica* hypovirus CHV1-EP713 was successful. Hypovirus transfection resulted in distinct morphologi-

TABLE 2. Mean lesion length on *Eucalyptus grandis* clone (TAG5) after artificial inoculation with transfected isolate (CMW 2113-T) and wild-type isolate (CMW 2113) of *Cryphonectria cubensis* and control^a

Isolates	Mean lesion length (mm)	
	Replicate 1	Replicate 2
CMW 2113-T	9.0 ± 0.5	8.4 ± 0.5
CMW 2113	57.3 ± 4.9	62.9 ± 4.1
Control	6.1 ± 0.03	6.0 ± 0.001

^a Mean lesion width (millimeter). Values are reported as mean ± standard error.

cal changes that included the production of a bright yellow-orange pigment when grown on MEA (Fig. 1A). In addition, the hyphae penetrated deeply into the agar, and no fruiting structures were produced. This is in contrast with the wild-type virus-free isolates, which are much lighter, produce mostly fluffy aerial mycelium, and conidiate at moderate levels on synthetic medium (Fig. 1B).

Cambial lesions developed on all plants inoculated with both the transfected (CMW 2113-T) and the virus-free isolate (CMW 2113) (Fig. 1C and D). No lesions were associated with the control inoculations. Significant differences in the lesion length were observed for the transfected isolate compared with the nontransfected isolates in both replicates of the experiment ($F = 103.4$; $df = 2$; $P < 0.001$) ($F = 188.9$; $df = 1$; $P < 0.001$), with the transfected isolate causing lesions that were 84% (replicate 1) or 87% (replicate 2) smaller than lesions caused by the virus-free isolate (Table 2).

Conversion of virus-free, virulent *C. cubensis* isolates. Visual inspection of the 29 *C. cubensis* isolates initially paired against the transfected isolate (CMW 2113-T) showed that 10 recipient isolates acquired the yellow-orange phenotype typical of the transfected isolate. When these converted isolates were used as donors to convert other non-dsRNA-containing isolates representing different VC groups, another four isolates were converted to the transfected phenotype (Table 1). By this process, it was possible to spread the hypovirus to a large number of isolates residing in different VC groups. From these converted isolates, total nucleic acid was isolated and tested for the presence of hypovirus RNA by RT-PCR amplification of the 5' terminal of the hypovirus genome.

The RT-PCR resulted in a 600-bp product from all nucleic acid preparations of the converted isolates as well as from the positive control (CMW 2113-T) (Fig. 2). No amplification occurred for the isolates that lacked visual signs of virus transfer, indicating that the hypovirus was not transferred to these isolates. In all, the hypovirus was transmitted to 14 South African *C. cubensis* isolates residing in 11 VC groups from the fungal population of 23 VC groups (32).

Virus transfer to conidia. Six weeks after inoculation of *Eucalyptus* spp. branch pieces with CMW 2113-T, pycnidia of the inoculated fungus were evident on the surface of the bark. Examination of the fruiting structures with light microscopy confirmed the presence of conidia in these pycnidia. The single conidial cultures obtained did not show the characteristic bright yellow-orange pigment typical of transfected isolates grown on MEA. RT-PCR tests on the nucleic acid isolated from these single conidial isolates resulted in no amplification, further confirming that these isolates do not contain the virus. This suggests that the virus is not transmitted to conidia derived from infected *C. cubensis*.



Fig. 2. Confirmation of hypovirus transmission in different *Cryphonectria cubensis* isolates by reverse transcription polymerase chain reaction (RT-PCR). Agarose (1.5%) gel stained with ethidium bromide showing RT-PCR products that verified the presence of double-stranded RNA. Lanes 1 and 13, molecular weight marker with 100-bp ladder; lanes 2 and 3, control isolates that had no visual pigment transfer, and no RT-PCR product was observed; lanes 4 to 11, examples of 600-bp RT-PCR product obtained from isolates selected; and lane 12, transfected isolate (CMW 2113-T) served as positive control for RT-PCR detection.

DISCUSSION

The results presented here extend the report by Chen et al. (7,9) of successful hypovirus transfection of *C. cubensis*. The previous study involved transfection of a poorly characterized Florida *C. cubensis* isolate obtained from the American Type Culture Collection (ATCC 64159) and did not examine the effect of hypovirus infection on *C. cubensis* virulence due to limited access to appropriate *Eucalyptus* spp. host material. The present study takes advantage of a South African *C. cubensis* isolate, for which extensive pathogenicity data on *Eucalyptus* spp. clones exists (S. W. van Heerden, unpublished data), and the availability of a collection of fungal isolates that represent the VC groups currently identified in the South African *C. cubensis* population (32).

As was observed for the Florida *C. cubensis* isolate by Chen et al. (9), hypovirus infection of South African isolate CMW 2113 by transfection, and other South African isolates by anastomosis, resulted in the production of a bright yellow-orange pigment, reduced radial growth, irregular colony margins with hyphae penetrating into the growth medium, reduced aerial mycelia, and the absence of fruiting structures on synthetic medium. Thus, the symptoms reported by Chen et al. (7,9) are not unique to the original infected Florida *C. cubensis* isolate.

The finding that hypovirus infection significantly reduces virulence of South African isolate CMW 2113 on *Eucalyptus* spp. established a primary requirement in the evaluation of hypovirus-mediated hypovirulence as a potential biocontrol strategy for *Cryphonectria* canker in South Africa. This possibility was supported further by the related finding that the virus was readily transmitted from infected CMW 2113-T by hyphal anastomosis to *C. cubensis* isolates representing nearly half of the VC groups identified in the South African *C. cubensis* population (Table 1). This result suggests that the *C. cubensis* *vic* system may not pose virus transmission barriers to the extent observed for *C. parasitica* (3). However, given the limited information available for virus transmission by *C. cubensis* isolates, interpretation of these results must be approached with some caution. Although there is a clear negative correlation between the frequency of virus transmission and the number of *vic* genes that differ between fungal isolates in the hypovirus-*C. parasitica* system (24), examples of *C. parasitica* isolates with broad conversion capacities have been reported (23,26). Thus, it is conceivable that *C. cubensis* CMW 2113-T may be unique in its ability to convert isolates representing a broad range of VC groups. It is also possible that the intensity or kinetics of the incompatibility reaction is sufficiently different for *C. cubensis* and *C. parasitica* that conversion is less stringently controlled for the former. Irrespective of the mechanism, the high degree of conversion is encouraging for biocontrol potential and warrants additional study. In contrast to the apparent ease with which horizontal hypovirus transmission proceeds in *C. cubensis*, vertical transmission through conidia appears to be blocked, confirming results reported by Chen et al. (7) for the transfected Florida *C. cubensis* isolate.

A range of strategies has been employed for biological control of chestnut blight with hypovirus-mediated hypovirulence. For example, beginning in 1974, the French Government undertook aggressive treatment of French chestnut orchards by annually treating new cankers with mixtures of compatible hypovirulent *C. parasitica* placed in closely spaced holes at the canker margins (17,25,31). Treatment of forest ecosystems has relied more on natural hypovirus spread after limited introductions of hypovirulent isolates (3,28). Given the failure of hypovirus transmission through *C. cubensis* conidia, it would appear that an aggressive treatment protocol would be most appropriate for treatment of *Cryphonectria* canker disease in South African *Eucalyptus* spp. plantations. However, the low profit margins associated with plantation-grown *Eucalyptus* tree production would make such treatments uneconomical. In this regard, transgenic hypovirulent *C.*

cubensis isolates that contain both a chromosomally integrated hypovirus cDNA and the cDNA-derived cytoplasmic viral RNA might provide a more economical approach. By virtue of the integrated viral cDNA, hypoviruses are expected to be transmitted from transgenic hypovirulent strains to all asexual spores and to approximately 50% of the ascospore progeny derived from a sexual cross (8,11). Additional improvements can be envisioned with infectious cDNA clones of mild hypovirus isolates in order to balance the effects of hypovirus infection on reduced virulence and on ecological fitness, as recently discussed for *C. parasitica*, following the development of infectious cDNA clone of hypovirus CHV1-Euro7 (10).

ACKNOWLEDGMENTS

We acknowledge the financial support of the South African forestry industry to the Tree Pathology Cooperative Programme and of the National Research Foundation, South Africa.

LITERATURE CITED

1. Alfenas, A. C., Jeng, R., and Hubbes, M. 1983. Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. Eur. J. For. Pathol. 13:179-205.
2. Anagnostakis, S. L. 1977. Vegetative incompatibility in *Endothia parasitica*. Exp. Mycol. 71:306-316.
3. Anagnostakis, S. L. 1982. Biological control of chestnut blight. Science 215:466-472.
4. Anagnostakis, S. L. 1982. Genetic analysis of *Endothia parasitica*: Linkage data for four single genes and three vegetative compatibility types. Genetics 102:25-28.
5. Anagnostakis, S. L., and Day, P. R. 1979. Hypovirulence conversion in *Endothia parasitica*. Phytopathology 69:1226-1229.
6. Biraghi, A. 1950. Caratteri di resistenza in *Castanea sativa* nei confronti di *Endothia parasitica*. Boll. Stn. Patol. Veg. Roma 7:161-171.
7. Chen, B., Chen, C.-H., Bowman, B. H., and Nuss, D. L. 1996. Phenotypic changes associated with wild-type and mutant hypovirus RNA transfection of plant pathogenic fungus phylogenetically related to *Cryphonectria parasitica*. Phytopathology 86:301-310.
8. Chen, B., Choi, G. H., and Nuss, D. L. 1993. Mitotic stability and nuclear inheritance of integrated viral cDNA in engineered hypovirulent strains of the chestnut blight fungus. EMBO J. 12:2991-2998.
9. Chen, B., Choi, G. H., and Nuss, D. L. 1994. Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. Science 264:1762-1764.
10. Chen, B., and Nuss, D. L. 1999. Infectious cDNA clone of hypovirus CHV1-Euro7: A comparative virology approach to investigate virus-mediated hypovirulence of the chestnut blight fungus *Cryphonectria parasitica*. J. Virol. 73:985-992.
11. Choi, G. H., and Nuss, D. L. 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science 257:800-803.
12. Day, P. R., Dodds, J. A., Elliston, J. E., Jaynes, R. A., and Anagnostakis, S. L. 1977. Double-stranded RNA in *Endothia parasitica*. Phytopathology 67:1393-1396.
13. Elliston, J. E. 1979. Pathogenicity and sporulation in normal and diseased strains of *Endothia parasitica* in American chestnut. Pages: 95-100 in: Proc. Am. Chestnut Symp. W. L. MacDonald, F. C. Cech, J. Luchok, and H. C. Smith, eds. West Virginia University Press, Morgantown.
14. Elliston, J. E. 1985. Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. Phytopathology 75:151-158.
15. Florence, E. J. M., Sharma, J. K., and Mohanan, C. 1986. Stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. Kerala For. Res. Inst. Sci. Pap. 66:384-387.
16. Fulbright, D. W., Weidlich, W. H., Haufler, K. Z., Thomas, C. S., and Paul, C. P. 1983. Chestnut blight and recovering of American chestnut trees in Michigan. Can. J. Bot. 61:3164-3171.
17. Grente, J., and Berthelot-Sauret, S. 1978. Biological control of chestnut blight in France. Pages 30-34 in: Proc. Am. Chestnut Symp. W. L. MacDonald, F. C. Cech, J. Luchok, and H. C. Smith, eds. West Virginia University Press, Morgantown.
18. Havir, E. A., and Anagnostakis, S. L. 1983. Oxalate production by virulent but not hypovirulent strains of *Endothia parasitica*. Physiol. Plant Pathol. 23:369-376.
19. Hodges, C. S., Geary, T. F., and Cordell, C. E. 1979. The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. Plant Dis. Rep. 63:216-220.

20. Huber, D. H., and Fulbright, D. W. 1994. Preliminary investigations on the effect of individual *vic* genes upon the transmission of dsRNA in *Cryphonectria parasitica*. Pages 15-19 in: Proc. Int. Chestnut Conf. M. L. Double and W. L. MacDonald, eds. West Virginia University Press, Morgantown.
21. Huber, D. H., and Fulbright, D. W. 1995. Heterokaryon formation under nonselective conditions and its restriction by vegetative incompatibility genes in *Cryphonectria parasitica*. (Abstr.) *Phytopathology* 85(suppl.): S1201.
22. Hillman, B. I., Shapira, R., and Nuss, D. L. 1990. Hypovirulence-associated suppression of host functions in *Cryphonectria parasitica* can be partially relieved by high light intensity. *Phytopathology* 80:950-956.
23. Kuhlman, E. G., Bhattacharyya, H., Nash, B. L., Double, M. L., and MacDonald, W. L. 1984. Identifying hypovirulent isolates of *Cryphonectria parasitica* with broad conversion capacity. *Phytopathology* 74: 676-682.
24. Liu, Y.-C., and Milgroom, M. G. 1996. Correlation between hypovirus transmission and the number of vegetative incompatibility (*vic*) genes different among isolates from a natural population of *Cryphonectria parasitica*. *Phytopathology* 86:79-86.
25. MacDonald, W. L., and Fulbright, D. W. 1991. Biological control of chestnut blight: Use and limitation of transmissible hypovirulence. *Plant Dis.* 75:656-661.
26. Maresi, G., Giovannetti, L., Ventura, S., and Turchetti, T. 1995. Transmission of hypovirulence agents among some *Cryphonectria parasitica* strains from Italy. *Eur. J. Pathol.* 25:191-196.
27. Nuss, D. L. 1996. Using hypoviruses to probe and perturb signal transduction processes underlying fungal pathogenesis. *Plant Cell* 8:1845-1853.
28. Nuss, D. L. 2000. Hypovirulence and chestnut blight: from the field to the laboratory and back. Pages 149-170 in: *Fungal Pathology*. J. W. Kronstad, ed. Kluwer Academic Publishers, Dordrecht, the Netherlands.
29. Preisig, O., Wingfield, B. D., and Wingfield, M. J. 1998. Coinfection of a fungal pathogen by two distinct double-stranded RNA viruses. *Virology* 252:399-406.
30. Sharma, J. K., Mohanan, C., and Florence, E. J. M. 1985. The occurrence of *Cryphonectria* canker of *Eucalyptus* in Kerala, India. *Ann. Appl. Biol.* 106:265-279.
31. Van Alfen, N. K. 1982. Biology and potential for disease control of hypovirulence of *Endothia parasitica*. *Annu. Rev. Phytopathol.* 20: 349-362.
32. Van Heerden, S. W., and Wingfield, M. J. 2001. Genetic diversity of *Cryphonectria cubensis* isolates in South Africa. *Mycol. Res.* 105:94-99.