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Article abstract

Cytoplasmically-transmissible viral double-stranded RNAs of the genus *Hypovirus* cause reduced virulence (hypovirulence) in the chestnut blight fungus *Cryphonectria parasitica*. Biological control of this fungal disease is done by inoculating selected artificial hypovirulent strains of *C. parasitica* in the wounds of attacked chestnut trees and is followed by testing the transmissibility of dsRNA to *C. parasitica* isolates affecting these trees. Here we present a modified protocol of isolation and detection of dsRNA. The proposed procedure requires smaller amounts of fungal material for dsRNA detection and uses less reagents, thus resulting in appreciable cost savings.

Modified small-scale batch procedure for isolation of dsRNA from *Cryphonectria parasitica*

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Cytoplasmically-transmissible viral double-stranded RNAs of the genus *Hypovirus* cause reduced virulence (hypovirulence) in the chestnut blight fungus *Cryphonectria parasitica*. Biological control of this fungal disease is done by inoculating selected artificial hypovirulent strains of *C. parasitica* in the wounds of attacked chestnut trees and is followed by testing the transmissibility of dsRNA to *C. parasitica* isolates affecting these trees. Here we present a modified protocol of isolation and detection of dsRNA. The proposed procedure requires smaller amounts of fungal material for dsRNA detection and uses less reagents, thus resulting in appreciable cost savings.

Key words: *Cryphonectria parasitica*, dsRNA isolation protocol.

[Procédure modifiée d'isolement à petite échelle d'ARNdb de *Cryphonectria parasitica*]

Les ARN double brin (ARNdb) viraux du genre *Hypovirus* à transmission cytoplasmique réduisent la virulence (hypovirulence) du *Cryphonectria parasitica*, le champignon responsable de la brûlure du châtaigner. La lutte biologique contre cette maladie fongique se fait par l'inoculation, dans des blessures de châtaigniers atteints, de souches choisies de *C. parasitica* artificiellement hypovirulentes, suivie de l'analyse de la transmissibilité des ARNdb aux isolats de *C. parasitica* qui affectent ces arbres. Nous présentons ici un protocole modifié d'isolement et de détection d'ARNdb. La procédure proposée requiert une plus petite quantité de matériel fongique pour la détection d'ARNdb et demande moins de réactifs; il en découle donc des économies appréciables.

Mots clés : *Cryphonectria parasitica*, protocole d'isolement d'ARN double brin.

Cryphonectria parasitica (Murrill) Barr, an ascomycete and the causal agent of chestnut blight, virtually eliminated American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) in the US at the beginning of the last century, and later also hit chestnut-growing areas in Europe and in other parts of the world. Fortunately, transmissible hypovirulence, a viral disease in the pathogen population, has been associated with the decline of the chestnut blight epidemic that was observed in many regions of Europe after 1951, including the Carpathian basin (reviewed by Heiniger and Rigling 1994). Many of the viruses that infect and cause hypovirulence in *C. parasitica* belong to the Hypoviridae family (Hillman *et al.* 1995). Members of this family have no true capsid but contain a single large double-stranded (ds) RNA encapsulated in pleomorphic vesicles. Morris and Dodds (1979) were the first to publish a simple and rapid method for isolation and screening of dsRNA from virus-infected fungal tissues and plants. Although this method has been successfully utilized

so far, several modifications to the procedure have been suggested (Allemann *et al.* 1999; Allen *et al.* 2003; Hillman *et al.* 1990; Peever *et al.* 1997; Rigling *et al.* 1989).

Here we present a modified protocol that further improves dsRNA extraction efficiency and makes possible its detection using smaller amounts of fungal material and at reduced financial costs.

The virulent and hypovirulent strains of *C. parasitica* were grown on 3% Malt agar media in Petri dishes (60 mm diam) at 25°C for 8 d. The mycelium was harvested by scraping the surface with a scalpel (0.04-0.1 g) and was thereafter frozen in liquid nitrogen, grounded to a fine powder using a mortar and pestle, and transferred into 1.5 mL microfuge tubes containing 500 µL of 2X STE buffer (1X STE: 0.1 mol L⁻¹ NaCl, 0.05 mol L⁻¹ Tris, pH 8.0; 1 mmol L⁻¹ EDTA) supplemented with 2% SDS, 2 mmol L⁻¹ dithiothreitol to extract the cellular nucleic acid. The samples were incubated for 5 min at room temperature (RT) and

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inverted several times. Before shaking at 300 rpm for 40 min at RT, 500 μ L of a phenol/chloroform/isoamyl alcohol (25:24:1 vol) mixture had been added to each tube containing the samples. After centrifugation (11 000 X g, 15 min at 10°C), the aqueous phase was transferred into a clean 1.5 mL microfuge tube and the ethanol content was adjusted to 17%. In another 1.5 mL microfuge tube, 0.05 g Whatman cellulose powder CF 11 was saturated with 1X STE containing 17% ethanol, shortly spun (4 000 X g, 1 min at RT), and subsequently the redundant buffer was removed and replaced with a sample containing 17% ethanol. During the 10 min incubation period of the sample on the multi-bio rotator, dsRNA was bound to cellulose in the presence of 17% ethanol. Next, the tube was spun (4 000 X g, 2 min at RT), the liquid phase was removed, and the cellulose was washed with 1 mL 1X STE and 17% ethanol. Following centrifugation (4 000 X g, 2 min at RT), the washing buffer was decanted and the dsRNA was released from cellulose in 400 μ L of 1X STE for 10 min. After a short centrifugation (4 000 X g, 2 min at RT), the liquid phase was transferred into clean microtubes and dsRNA was precipitated in the presence of 1/10 vol of 3 mol L⁻¹ sodium acetate (pH 5.2) and 2 vol of 96% ethanol overnight at -80°C. After centrifugation (11 000 X g, 15 min at 4°C), nucleic acids were washed with 70% ethanol (70% ethanol was prepared by dilution with RNAase-free water). The nucleic acids were shortly dried and dissolved in 8 μ L of RNAase-free water. To remove contaminated DNA that might be bound to the cellulose, 1 μ L of 10X DNase I reaction buffer and 1 U DNase Amp Grade (Invitrogen) were added to the tubes containing the samples and incubated for 15 min at RT. DNase I was inactivated by the addition of 1 μ L of 25 mmol L⁻¹ EDTA solution to the reaction mixture. Subsequently, the dsRNA was made visible under UV light following agarose gel electrophoresis in 0.8% agarose gel containing ethidium bromide (0.5 μ g mL⁻¹) at 90 V for 40 min.

This modified small-scale batch method allows rapid, efficient isolation and analyses of dsRNA from small amounts (0.04-0.1 g) of fungal mycelia of *C. parasitica*. (Fig. 1). Morris and Dodds (1979) used chromatographic cellulose powder for dsRNA isolation and reported its detection from approximately 1.0 g of fungal tissue by gel electrophoresis. We have successfully reduced the amount of fungal mycelium 25 times (to 0.04 g), and concomitantly the amount of extraction buffer, phenol/chloroform mixture and cellulose could also be decreased. Similarly, as described in the present study, Allemann *et al.* (1999) isolated and detected dsRNA from 40 mg of mycelia of *C. parasitica* in 2 mL Eppendorf tubes after modification of the protocols described by Morris and Dodds (1979) and Rigling *et al.* (1989). However, their procedure requires a 24 h long lyophilization and the grounding of mycelia to a fine powder using a Mikro-Dismembrator II (B. Braun, Melsungen, Germany). In our protocol, pulverization of fungal tissues in liquid nitrogen before nucleic acid extraction significantly reduced the duration of the method and contributed to increase the efficiency of nucleic acids extraction.

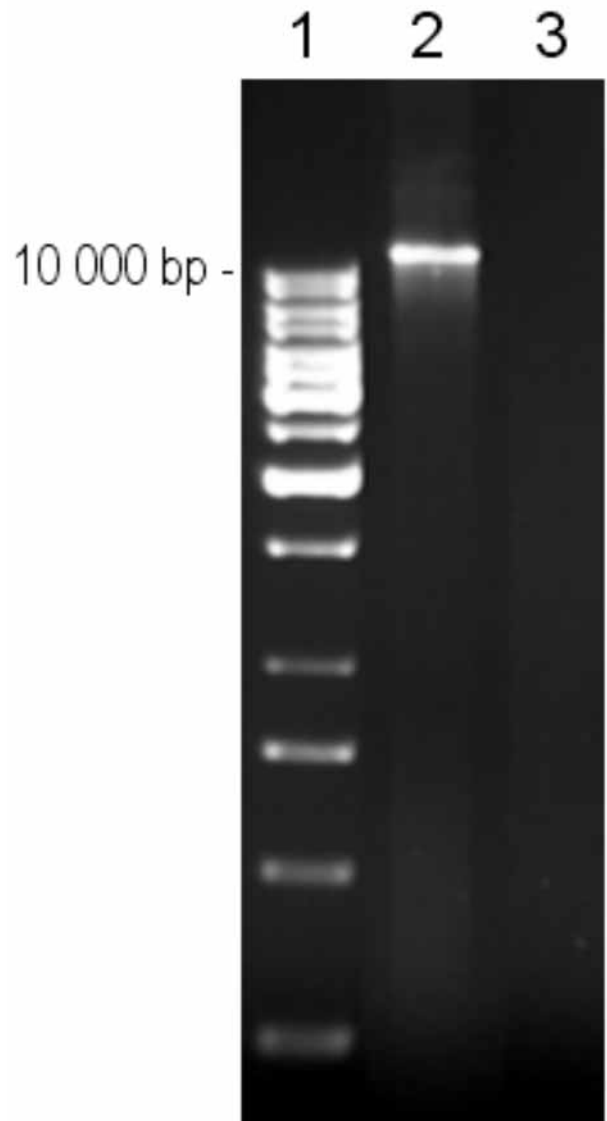


Figure 1. Agarose gel analyses of dsRNA recovered from mycelia of *C. parasitica* virulent and hypovirulent isolates. Lane 1, 1 kb DNA ladder Fermentas; Lane 2, dsRNA extracted from hypovirulent isolates; Lane 3, no dsRNA was extracted from virulent isolates.

The dsRNA isolation protocol described here was successfully used for dsRNA detection in *C. parasitica* isolates taken from cankers of chestnut trees treated with Slovak converted hypovirulent strains of *C. parasitica*. Biological control of chestnut blight was conducted on infected trees from the Stivnicko-krupinska region of Slovakia, and its efficiency was more than 30% (Juhásová *et al.* 2005), which is considered very good for controlling this disease. Except for the presence of dsRNA, the hypovirulent isolates of this fungus were also phenotypically different from their virulent counterparts. The virulent isolates were mainly reddish-yellow and the hypovirulent isolates had little or no pigment. In addition, they grew more slowly than normal virulent strains at 25°C on PDA media in the laboratory.

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