Comparison of Multiplex Real-Time PCR and Ergosterol Assays in Quantifying *Heterobasidion annosum* in Planta

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Abstract

A quantitative multiplex real-time PCR procedure was developed to monitor the dynamics in Norway spruce-*Heterobasidion annosum* pathosystem. The assay reliably detected down to 1 pg of *H. annosum* DNA and 1 ng of host DNA in multiplex conditions. As a comparative method for quantifying fungal colonization, we applied the ergosterol assay. There was a very high correlation between the results obtained with the two methods, this strengthening the credibility of both assays. The advantages and disadvantages of these assays are discussed.

Keywords: *Heterobasidion*; Norway spruce; infection; quantification

INTRODUCTION

Accurate and timely diagnoses of plant diseases are extremely important so that appropriate control measures and eradication procedures can be carried out quickly. Disease symptoms often aid with making decisions, but a definitive diagnosis requires unambiguous pathogen identification. Real-time polymerase chain reaction (PCR) is the most recent development in quantitative diagnostic methods and promises to be very useful in quantifying infection agents in their natural substrates. The most frequently employed application of this technique utilizes TaqMan® chemistry in conjunction with a sequence detection system (SDS). The fluorogenic TaqMan® probe, labeled on opposite ends with a reporter dye and a quencher dye, anneals between the PCR primers. During the extension phase of the PCR, the nuclease activity of Taq DNA polymerase cleaves annealed probe molecules. Release of the reporter dye results in intense fluorescent signal that is measured by the SDS during each cycle of the PCR process. The signal is proportional to the amount of product generated and this in turn is proportional to the amount of template present at the start of PCR. Real-time PCR has several advantages compared with classical PCR. First, it combines the sensitivity of PCR with the specificity of nucleic acid hybridization. Second, there is no need for agarose gels and the subsequent southern blot hybridization steps that are necessary to confirm the identity of the PCR products. Third, up to four different fluorescent dyes can be incorporated in a single reaction; this allows the simultaneous detection of several pathogens from a single sample. Finally, many samples can be assayed simultaneously (up to 96 with the ABI Prism 7700 SDS), and the assays can be completed within 3 h.

TaqMan chemistry has contributed to the development of extremely specific, sensitive and accurate assays to quantify pathogen infection in both crop plants (e.g. FREDERICK *et al*. 2002) and trees (WINTON *et al*. 2002). The basidiomycete *Heterobasidion annosum* is a major root rot pathogen of conifers and breeding programmes targeted to disease resistance would be most beneficial in combating the pathogen. In order to screen host clones for differential resistance accurate quantification methods for pathogen colonization rates are essential. The aim of the study was to develop a real-time PCR assay for quantifying *H. annosum* colonization in Norway spruce (*Picea abies*) and to compare the assay with an ergosterol-based method.

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MATERIALS AND METHODS

Tissue cultures from two 30-year-old Norway spruce clones, 053 and 589, were used as host material in the testing. The clones were chosen on the basis of previous field experiments where they had displayed differential disease resistance (weak clone, 053; strong clone, 589). Tissue cultures were inoculated with a heterokaryotic S type strain of *H. annosum* and samples were taken for real-time PCR and ergosterol assay at days 1, 3, 6 and 9 after inoculation. The fungal primer/probe set was designed for a laccase gene (fwd. primer 5'-CCAGAAAGTAGACAATTATTGGATTCG-3', rev. primer 5'-GAGTTGCGGCCATTATCGA-3', probe 5'-AGCGCCCAACACAGTACCCCCG-3'). For the host, a polyubiquitin gene was utilized (fwd. primer 5'-TGGTCGTACTCTGGCCGATTATA-3' and the rev. primer 5'-ACACCTAGCGGCACACAGTTAA-3', probe 5'-TGCTCCGTCTCCGTGGTGGCT-3'). Real-time PCR was performed with the ABI PRISM 7700 and data analysed with the SDS 1.7a software Package (Applied Biosystems). The ergosterol assay was performed according to MARTIN *et al.* (1990).

RESULTS AND DISCUSSION

A standard curve based on the critical threshold values and the amount of template was generated from known host/pathogen DNA concentrations in multiplex PCR. Host and fungal DNA were reproducibly detected over the concentration ranges used, 0.9–25 ng and 0.001–25 ng, respectively (Figure 1). To obtain the pathogen/total DNA ratio in inoculated samples, the amounts of host and pathogen DNA were calculated using the respective standard curves. Both the real-time PCR and ergosterol assay indicated that the weak clone was hosting more fungal DNA over the incubation time (data not shown). The rank correlation between real-time PCR and ergosterol assay was strong (0.987).

The high correlation between the assay methods strengthens their credibility. The real-time PCR is, however, considerably more sensitive than the ergosterol assay; due to the low infection rate at day 1, the latter did not detect any ergosterol in some samples whereas real-time PCR can detect levels considerably lower than those now observed at day 1. Another great advantage of real-time PCR is that it allows the monitoring of both partners of a pathosystem. One problem with real-time PCR is the fact that it does not, unlike ergosterol assay, distinguish between the necromass and living biomass, as PCR can amplify a target from dead cells as long as the target sequence is intact. Therefore a more complete picture is obtained when the technique is used in combination with the ergosterol assay.

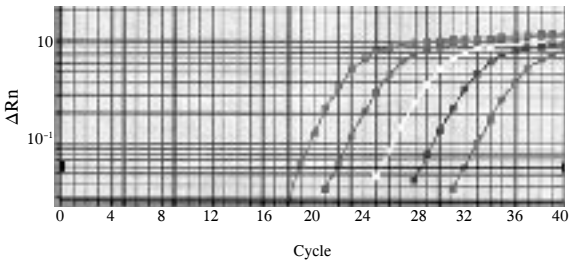

Figure 1. Laccase-gene based real-time PCR (TaqMan) quantification of *H. annosum* DNA in a multiplex situation [the fungal DNA was mixed with a known concentration of Norway spruce DNA, which was simultaneously monitored with the polyubiquitin set (data not shown)]. The curves from left to right: 10, 1, 0.1, 0.01 and 0.001 ng DNA of *H. annosum*

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