Bavarian State Research Center for Agriculture

Institute for Crop Science and Plant Breeding [1]
Institute for Plant Protection [2]

Real-time PCR based Diagnostics and Meristem Culture – Essential Tools for Healthy Hops

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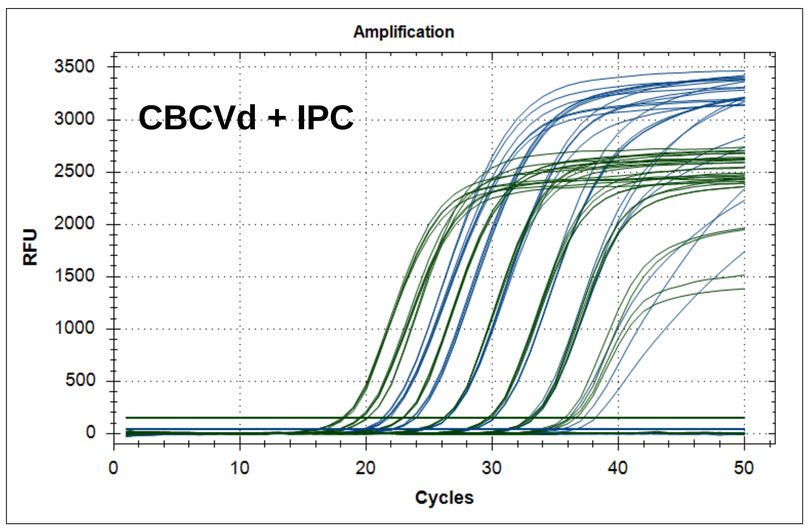
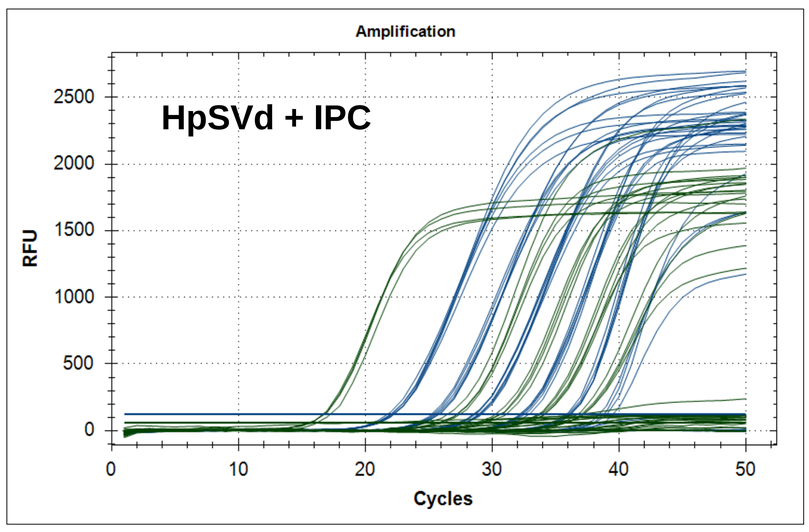
Introduction

With the occurrence of lethal forms of the *Verticillium* wilt (*Verticillium nonalfalfae*) in German hop production and the detection of two highly deleterious viroids – *Hop stunt viroid* (HpSVd) alone or in combination with *Citrus bark cracking viroid* (CBCVd) in commercial hop fields abroad - the focus in our research was to establish and optimize powerful diagnostic tools to detect these pathogens in hop plants. Since there are no chemicals available to directly fight these pathogens a crucial part in the management and control of these diseases is the identification and eradication of infected plants as well as the production of healthy planting material for the hop and brewing industry.

TaqMan® real-time RT (reverse transcriptase)-PCR to detect HpSVd and CBCVd

RNA is extracted from hop leaves using the RNeasy Plant Mini Kit (QIAGEN). For real-time RT-PCR the Bioline SensiFast Probe No-ROX One-Step Kit is applied. All samples are tested in a duplex real-time RT-PCR for one viroid (HpSVd or CBCVd) and a nad5-mRNA based internal positive control (IPC) confirming a fully functional PCR run (1). Primers and probe for detection of HpSVd are according to (2). For CBCVd detection primers and probe were designed comparing 32 genomic sequences (NCBI database) from different isolates of different hosts and one additional sequence from a hop isolate. CBCVd primers and probe showed a 100% homology with the hop sequence and the greatest possible similarity with respect to the other sequences. In default of CBCVd-infected material testing for inclusivity (detection of different hop isolates) is still missing. Exclusivity was checked on the basis of other viroids (ASSVd, CEVd, PLMVd, PSTVd, CSVd, CLVd, HpLVd, PLMVd,ToCDVd, TPMVd). HpSVd primers and probe showed a 100% exclusivity. With the CBCVd primers and probe only a weak cross reaction of CEVd was observed, but so far this viroid was not detected in hops. For determination of the relative analytical sensitivity serial dilutions of RNA in water were carried out. Both viroids were reproducibly detected up to and including the dilution 10^{-5}.

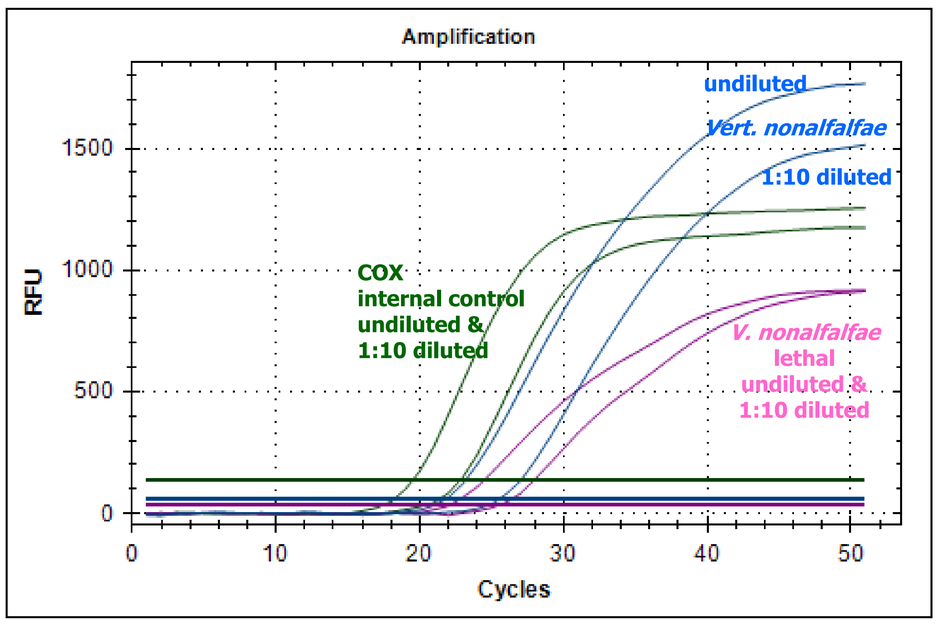




Duplex real-time RT-PCR detection of CBCVd (left, blue curves), HpSVd (right, blue curves) and IPC (green): ten-fold dilutions of RNA in water from undiluted to 10^{-5} were tested. Amplification plots resulting from increasing FAM-fluorescence of CBCVd and HpSVd specific probes and increasing HEX-fluorescence of the IPC-specific probe are shown. At the highest dilution (10^{-5}) Cq-values were less consistent with detection rates below 100%. Negative and water controls gave no signals. IPC gave reliable results, also in practice with hundreds of hop samples tested.

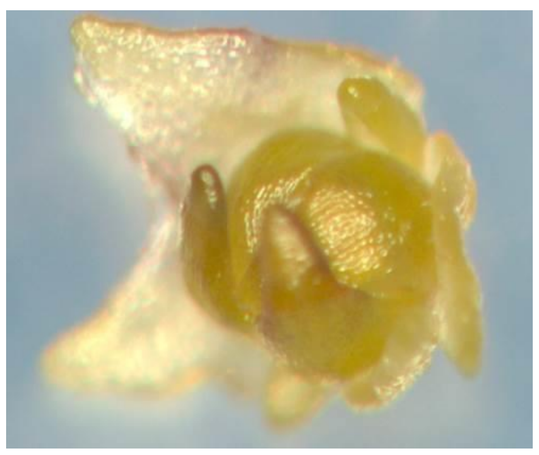
In planta multiplex TaqMan® real-time PCR to detect *Verticillium nonalfalfae*

Core pieces from the base of hop bines are used to test for *Verticillium*. DNA is isolated with the Invisorb® Spin Plant Mini Kit (Invitek Molecular) and tested using SensiFAST™ Probe No-Rox Kit (Bioline) in a real-time multiplex PCR assay according to (3) with the following changes: a triplex TaqMan® real-time PCR is conducted enabling to simultaneously detect *Verticillium nonalfalfae (FAM labeled probe, blue curve)* in general and identify the lethal form of *V. nonalfalfae* (Cy5, red) in one run. An internal COX-based positive control (4; HEX, green) in each run excludes false negative results due to failed DNA extractions or PCR inhibitors and has already proven full reliability when testing hundreds of hop plants from different varieties in practice. Each sample extract is tested undiluted and 1:10 diluted. Healthy *Verticillium*-free plants do not show amplification signals of FAM and Cy5. Analytical specificity of primer pairs and probes for *Verticillium nonalfalfae* also discerning mild and lethal strains was confirmed by (3) and Seefelder (unpublished). Further validation is in progress.



Meristem culture

With the aim to cure infected hop plants the meristem culture in combination with heat treatment was optimized so that healthy plants could be regenerated from the meristematic shoot tips of *Verticillium* and virus infected hops. Efforts to eliminate viroids were only rarely successful – with no HpSVd or CBCVd infected hops available plants with HpLVd were used in all investigations.



Shoot tips of infected hop plants grown in the greenhouse are cut and meristems (0.3-0.5mm) are excised from shoot tips pretreated with heat for seven days. Regeneration of meristematic tissue starts on semi-solid Murashige-Skoog (MS) medium for 3 weeks. Regeneration continues on a semi-solid MS medium with BAP between 0.5 and 1mg/l. For the liquid culture phase in RITA™ vessels MS based regeneration medium is used without agar. Meristem-derived plantlets are cloned using the node culture technique. Cloned plantlets are tested for *Verticillium* and viruses in order to confirm the elimination of these pathogens. Healthy plants are transplanted into earth and used as planting material.

References: 1 Botermans M,. van de Vossenberg B.T.L.H., Verhoeven J.T.J, Roenhorst J.W., Hooftman M, Dekter R, Meekes E.T.M. 2013. J. of Virological Methods 187,43ff.
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