EARLY DETECTION PROJECT – MAKE A GTD INFECTION VISIBLE WITHOUT DISEASE SYMPTOMS

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Abstract:
Context and purpose of the study - The presence of grapevine trunk diseases (GTDs) related pathogens leads to severe economic losses in wine-growing regions all over the world. GTDs cause foliar discoloration, stunted growth, decline, sectorial and/or central necrosis of the trunk wood, and dieback, while the quality and the quantity of the grapes and therefore the wine production is reduced. The disease management is challenging for vine-growers since the responsible fungi colonize wood tissues (and are therefore inaccessible for conventional fungicides) and the related symptoms occur mostly after a long period of latency. The aims of this project were first to distinguish between healthy and infected plants before the symptoms appear and second to document the efficacy of BASF ´s Tessior®-System for wound protection under field conditions.
Material and methods - Long term field trials were established between 2014 and 2015 in Germany, France, Greece, and Italy, where each year the pruning wounds are treated with Tessior®. In order to increase the infection pressure, some of the vineyards are artificially inoculated with spores of *Phaeomoniellachlamydospora* and *Botryosphaeriaceae* species. The presence of *P. chlamydospora* – a pathogen causing esca-disease – and *Botryosphaeriaceae* species – causing Botryosphaeria dieback – in grapevines was determined with an optimized protocol. Samples were collected by drilling a 5 mm diameter hole in the spurs below a pruning wound which was closed then with a wound sealant. The wood chips were lyophilized and afterwards homogenized using TissueLyser II (Qiagen). Total genomic DNA was extracted from the grapevine samples and quantitative Real-Time PCR using TaqMan probes was performed.
Results - This protocol has been proved to be fast and accurate to quantify the DNA amount of GTDs related pathogens in grapevine wood. Furthermore, the efficacy of Tessior® wound protectant has been verified showing significant reduction of infection with *P. chlamydospora* and *Botryosphaeriaceae* species.

Keywords: Grapevine, *Phaeomoniellachlamydospora*, *Botryosphaeriaceae*, quantitative Real-Time PCR, TaqMan, Tessior®

1. Introduction

Grapevine trunk diseases (GTDs) are currently the most destructive and fast rising diseases of grapevine plants. The related symptoms vary from foliar discoloration, stunted growth, decline, sectorial and/or central necrosis of the trunk wood to dieback, while the quality and the quantity of the grapes and therefore the wine production is reduced causing huge economic losses in wine-growing regions all over the word. The disease management is challenging for vine-growers since the first symptoms occur mostly after a long period of latency, when the wood is already compromised. For this reason, it is important to establish a method which is accurate to detect early contamination in the plants.

So far, traditional isolation is a technique, which is poorly used to detect fungi in plant tissues because other pathogens can outgrow the trunk pathogens leading to false negative results. Moreover, identification of the fungi by their morphological aspects is not sufficient and often, additional analyses are needed using molecular tools. Quantitative Real-Time PCR (qPCR) has been used as one approach to detect and quantify GTD pathogens such as *Phaeomoniellachlamydospora* (PCH) and

Phaeoacremoniumaleophilum (Martín et al. 2012, Pouzoulet et al. 2013), *Eutypalata* (Moisy et al. 2017, Pouzoulet et al. 2017), and the *Diplodiaseriata*-complex (Pouzoulet et al. 2017) using either SYBR green or TaqMan chemistry. These studies present the effectiveness of this method, nevertheless mostly grafted grapevines and cuttings were used as plant material for the experiments. Pouzoulet et al. (2017) collected samples from naturally and artificially infected grapevine spurs and analysed them by qPCR using SYBR green master mix and a primer pair to amplify the β-tubulin gene of three different *Diplodia*-species. However, at least 21 different *Botryosphaeriaceae* species (BOT) can infect grapevine plants worldwide (Úrbez-Torres, 2011), and a recent paper considered this fact when the primers have been designed to detect these fungi from environment (Billones-Baaijens et al. 2018).

BASF developed an innovative wound protectant product, which is highly effective in reducing new infections of GTDs via pruning wounds. It combines physical and chemical components in its formulation: the physical activity is ensured by a polymer that hardens after being sprayed on the wound surface, while the chemical activity is ensured by two BASF broad spectrum fungicides: pyraclostrobin and boscalid.

The objectives of the Early Detection Project were to develop an efficient technique to obtain wood samples from grapevine plants, elaborate a DNA extraction protocol,establish a qPCR method to detect PCH and BOT in grapevine samples, and finally approve the efficacy of the BASF wound protectant Tessior® under field conditions.

2. Material and methods

Verification of the method – In 2018,five grapevine trunks were removed from a vineyard in Forst (Germany), which is monitored plant by plant according to GTD related symptoms since 2016. Based on the severity of symptoms (mainly esca-associated "tiger-stipes"), these vines were classified from 0 (symptomless plant) to 4 (dead plant). In our laboratory, wood chips were collected from different points of the trunks using a 5 mm diameter drill bit. The amount of fungal DNA in the samples was determined by qPCR as described below.

Field conditions – Long-term field trials were established between 2014 and 2015 in Germany, France, Greece, Italy, and Spain. Treatment with Tessior® was performed each year after pruning, and in order to increase the infection pressure, the pruning wounds were inoculated with spores of PCH and BOT in some vineyards starting this procedure after two or three years of the planting.

Sample collection – Two sample collection methods were evaluated according to the plants' habitus. In 2018, wood chips were received from pruned spurs using a 5 mm diameter drill bit. The chips were collected on an aluminum foil, placed in a plastic bag and then conserved at -20 ^{o}C. To sample young and weak vines, 2 cm from the end of a pruned cane were cut. Afterwards, the hole or wound was closed with a wound sealant to prevent infections. Wood samples were lyophilized for 24 h, then homogenized using TissueLyser II (Qiagen).

DNA extraction, quantity and quality control – DNA extraction protocol was optimized for wood tissues using NucleoSpin® Plant II (Macherey-Nagel) kitwith the SDS-based buffer PL2. DNA concentration of the samples was measured with Qubit™ 4 Fluorometer (Invitrogen). In order to make sure that the DNA had appropriate quality for the further analysis, the grapevine 18S rRNA gene was amplified.

Primer design and qPCR – Primers and probe were designed to detect and quantify PCH with TaqMan chemistry. In order to detect BOT species, previously published (Billones-Baaijens et al. 2018) primers were used with a new TaqMan probe. qPCR was performed with iTaq™ Universal Probes Supermix (Bio-Rad Laboratories, Inc.) using a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc.). Data were analysed with Bio-Rad CFX Manager™ software (Version 3.1).

Absolute quantification using the standard curve method – DNA was extracted from pure cultures of PCH (strain CBS 101571) and BOT (strain DLR BOT41, kindly provided by Dr. Andreas Kortekamp) with DNeasy Plant Mini Kit (Qiagen). Standard curves were generated using 10-fold dilution series of both fungal DNAs plotting the cycle quantification values (Cq) versus the dilution factor in a base-10 semi-

logarithmic graph. These plots were then used to extrapolate the concentration of the target fungi in the DNA template.

3. Results and discussion

Verification of the method – DNA samples extracted from grapevine plants collected in Forst were analysed for the amount of PCH and BOT DNA by qPCR using TaqMan probes. The grapevine plants for these experiments were chosen based on their symptoms, which meant esca-disease associated "tiger-striped" leaves. Compering these symptoms with the results obtained by qPCR a clear correlation was observed: the more symptomatic the plant was, the higher was the amount of PCH DNA measured in the samples. Great amount of BOT DNA was only detected in the dead plant. Based on these samples, different infection levels were defined according to the amount of fungal DNA in 1 ng total DNA: more than 100,000 fg – dead tissue, 10,000-100,000 fg – abundant infection, 1,000-10,000 fg – moderate infection, below 1,000 fg – presence of the fungus.

Evaluation of the vineyard of Merville – The vineyard was established in 2015, then each year a treatment with Tessior® was performed after pruning. The grapevines had not been inoculated before, infection could occur due to natural inoculum sources in the vineyard. Wood chips were received from the spurs pruned in 2017. After total DNA extraction, the amount of PCH and BOT DNA was quantified in the samples by qPCR. Difference of the DNA quantity of PCH was observed between the untreated and Tessior®-treated samples (Fig. 1A); however, the positive samples presented low amount of fungal DNA. Thirteen of the twenty analysed untreated samples showed infection with PCH, and four of twenty Tessior®-treated ones. The number of Tessior®-treated samples infected with BOT was also reduced compared to the untreated samples (Fig. 1B). Regarding the results of PCH and BOT the effect of Tessior® is obvious: the number of infected plants was reduced by 55-70 %, and the intensity of colonization was lower in positive samples collected from Tessior® plots than in positive samples from untreated plots.

Evaluation of the vineyard of Florence – Vines were planted in 2014, treated each year after pruning with Tessior® and in a separate plot with a *Trichoderma*-based wound protection product. Plots were inoculated with spores of PCH each year since 2016. According to the plants' weak habitus, samples were collected by cutting the end of a cane pruned in 2017. The results verified our expectations: the number of PCH and BOT infected plants which were treated with Tessior® was reduced by 100 % and 78 %, compared to the untreated uninoculated and inoculated plants (Fig. 2). Interestingly, there was no difference between the numbers of untreated and uninoculated (naturally infected) or PCH-inoculated samples, but variation could be observed in the amount of the detected PCH DNA. BOT infection was detected in nine untreated uninoculated samples, but only in three of the PCH inoculated plants. Two Tessior®-treated samples were found to be infected with BOT and seven of the *Trichoderma* product-treated ones.

4. Conclusions

Sample collection method was developed as drilling wood chips from grapevine plants with a 5 mm diameter drill bit. An alternative method, as cutting the end of the pruned canes, was also taken into consideration regarding weak plants. DNA extraction protocol was optimized to grapevine wood tissues using NucleoSpin® Plant II (Macherey-Nagel) kit and qPCR technique to detect and quantify PCH and BOT in grapevine samples was elaborated using TaqMan chemistry. Furthermore, infection levels of PCH and BOT were compared in grapevine samples of untreated and Tessior®-treated blocks, and the efficacy of Tessior® wound protectant has been also verified under field conditions in Merville and Florence. We provided further evidence that the qPCR method using TaqMan probes is a fast and accurate technique to quantify the DNA amount of PCH and BOT in grapevine wood and our investigations into this project are still ongoing to analyse samples collected from more vineyards in Europe.

5. Acknowledgments

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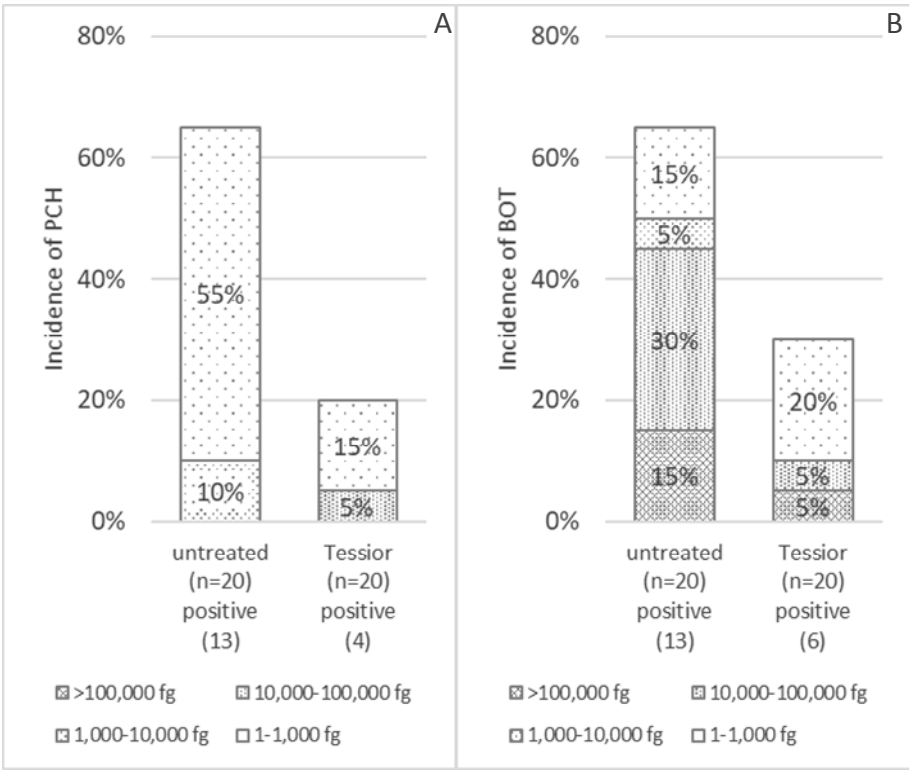

Figure 1: Frequencies of detection of *Phaeomoniellachlamydospora* (PCH) **(A)** and *Botryosphaeria* spp. (BOT) **(B)** in wood samples. Samples were collected from untreated and Tessior®-treated grapevine plants in Merville, France, in 2018. The amount of fungal DNA per ng of total DNA was determined by qPCR in fg.

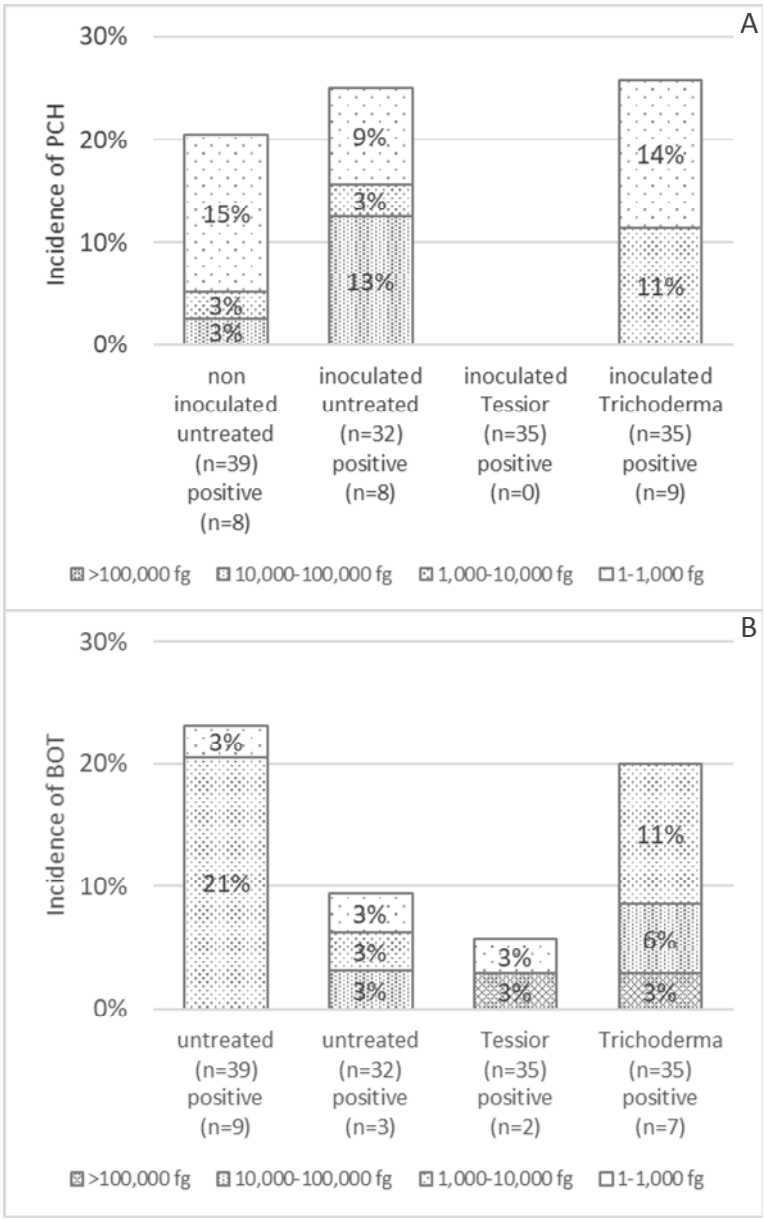

Figure 2: Frequencies of detection of *Phaeomoniellachlamydospora* (PCH) **(A)** and *Botryosphaeria* spp. (BOT) **(B)** in wood samples. Samples were collected from untreated, Tessior®-, and *Trichoderma*-based product-treated grapevine plants in Florence, Italy, in 2018. Artificial inoculation was performed after treatment with *Phaeomoniellachlamydospora* (PCH) spores in 2017. The amount of fungal DNA per ng of total DNA was determined by qPCR in fg.