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Grapevine trunk diseases in German viticulture IV. Spreading of spores of *Phaeomoniella chlamydospora* in Esca-affected vineyards

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Summary

The anamorphic fungus *Phaeomoniella chlamydospora* (*Pch*), related to Grapevine trunk diseases (GTDs) such as "Petri disease" and Esca, was originally restricted to the Mediterranean area but can now be found worldwide. GTDs are the most destructive diseases in vineyards causing high losses every year. As there are no effective fungicides available it is important to understand the epidemiology of this fungus. To investigate the occurrence and distribution of *Pch* in the field, spore traps were placed in two selected vineyards located at the Julius Kühn-Institut (JKI) in Siebeldingen, Germany, for three consecutive years. Plots were planted with cultivars 'Chardonnay' and the fungus resistant cultivar 'Phoenix'. Analysis of the traps was performed by a specifically developed nested-PCR approach. As a result it was proven that *Pch* is present in the vineyards throughout the whole year, including wintertime. The occurrence of *Pch* conidia during the winter months is a central issue as the annual pruning of vines is done in the winter season and pruning wounds are supposed to be the main entry point for this pathogen. During the three year survey also symptom appearance, both chronic and apoplectic, on leaves as well as weather conditions have been recorded. Symptoms were evident in both vineyards; however, no clear correlation was obtained between symptoms and spore flight. High temperatures combined with low humidity may have a negative impact on spore dispersal, while cold temperatures such as in wintertime have no negative effect on the appearance of spores.

K e y w o r d s : grapevine trunk diseases; nested-PCR; *Phaeomoniella chlamydospora*; spore traps; viticulture.

Introduction

Esca and "Petri disease", a pre-form of Esca, can be found worldwide and have become great dangers to German vineyards during the last twenty years. Esca is supposed to be a complex disease caused by at least three different wood-inhabiting fungi: the anamorphic *Phaeomoniella chlamydospora (Pch)* (Crous and Gams 2000), the ascomycete *Phaeoacremonium aleophilum (Pal;* renamed as *P. minimum,* see Crous *et al.* 1996, Gramaje *et al*. 2015), and the basidiomycete *Fomitiporia mediterranea (Fmed)* (Fischer 2002). Additional fungal pathogens are discussed as being involved in the disease complex (Bertsch *et al.* 2013, Fischer *et al.* 2016). However, the pathogenic significance of these additional pathogens is not fully resolved, yet.

Pch and *Pal* are supposed to act as primary pathogens (Larignon and Dubos 1997, Retief *et al.* 2006) causing the early form of Esca, the "Petri disease" (Surico 2001). Only after a plant is additionally infected by *Fmed*, "Esca-proper" develops. The disease not only damages berries causing symptoms of leather berries and black measles, but also causes tigerstripe-patterns on leaves leading to low rates of photosynthesis and lower yields. Furthermore, symptoms caused by *Pch* and *Pal* can also be found in the trunk of vines, with "gummosis" and brown wood streaking being the most prominent (for an overview, see Mugnai *et al*. 1999). Infestation with *Fmed* then leads to white rot, which weakens the plants further leading to a slow "chronic" decline over the years. However, the resulting symptoms in the wood are not necessarily visible from the outside and foliar symptoms do not have to be strong or appear in consecutive years (Surico *et al*. 2006). A second form of Esca symptomatology is the acute form called "apoplexy". In this form the plants show sudden wilting and usually die within weeks (Mugnai *et al.* 1999).

For all the fungi mentioned above wounds in the bark and especially pruning wounds are supposed to be the main point of entrance. In the field the infection of wounds is most likely initiated by airborne spores (Eskalen *et al.* 2003; for German vineyards, Fischer and Kassemeyer 2003, Fischer 2012), while contaminated pruning tools are considered negligible under these circumstances.

As there are no curative treatments available (Bertsch *et al.* 2013, Gramaje *et al.* 2018) and since the appearance of Esca-symptoms is highly complex and discontinuous, it is vital for any protective measure to know about the spread and the distribution of these fungi in the vineyard. Infection by *Pch* alone already may lead to a decline of young vines (Whiteman *et al*. 2003); the species also has been found to be the most virulent one when isolated from dead vines (Fourie and Halleen 2001, Whiteman *et al*. 2003). So it was the aim of this study to investigate the appear-

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ance of *Pch* conidia in selected vineyards, comprising a traditional and a fungus-resistant cultivar, of the Palatinate winegrowing area for a period of three consecutive years (2013-2015). Special attention was on the winter season from November to February when winter pruning is performed and pathogens may access the wood through pruning wounds. The presence of *Pch* conidia in the air was monitored by the use of spore traps changed on a weekly basis essentially following the procedure as described by Eskalen and Gubler (2001). However, analysis of the traps was based on a new nested-PCR approach specifically developed for this project, which should allow a fast, specific and sensitive detection of *Pch*.

Besides weather data such as humidity and temperature, also a monitoring of the occurrence of chronic and acute Esca was performed over a period of four consecutive years (2012-2015). In search for possible interactions, weather data and symptomatology should be compared with the seasonal appearance of *Pch* in the traps.

Material and Methods

Vineyards: Two vineyards at the JKI in Siebeldingen were chosen for the survey. The first cultivar 'Chardonnay', comprises 750 vines planted in 30 rows à 25 plants in north-south direction and was established in 2008. The second, 'Phoenix', includes 1,530 vines planted in 17 rows with 90 plants each in east-west direction and was established in 1995. Both vineyards had shown foliar symptoms in different severity levels in 2012. In the 'Chardonnay' plantation 0.3 % of the plants were affected, while 4.1 % in the 'Phoenix' plantation exhibited symptoms.

Monitoring foliar symptoms: Monitoring of the vineyards for visible symptoms was done for four consecutive years from 2012 to 2015 from the beginning of June until harvest of the grapes, usually by the end of September. Both vineyards were analyzed for the occurrence of tigerstripe-patterns as form of "chronic Esca" and for apoplexy as form of "acute Esca".

Spore traps: Monitoring of the vineyards using spore traps started in April 2013 and ended in December 2015. Microscope slides coated on both sides with petroleum jelly (Balea, Karlsruhe, Germany) were used as spore traps (Eskalen and Gubler 2001). In a first step at the beginning of the survey in 2013 one trap each was placed near the stem heads of two 'Chardonnay' vines (Fig.1 left), both of which had shown symptoms in 2012. A third trap was placed between the respective trunks at the level of the grape bunches. In the 'Phoenix' vineyard two traps were placed at stem heads of infected vines. In a second step, initiated in 2014 and continued in 2015 in both plots an extra spore trap was added, placed in the high wire in the canopy of vines (Fig. 1 right).

Analysis of spore traps: During the sampling periods all spore traps were changed on a weekly basis. Traps were rinsed with 15 mL of sterile water. Water was filtered (Ms Scientific, Berlin, Germany) using a 5 µm pore size filter to remove larger spores and particles followed by a 0.45 µm pore size filter to trap *Pch* conidia

Fig. 1: Spore trap at stem head in 'Chardonnay' (left) and in the canopy in 'Phoenix' (right), May 2015.

(modified after Eskalen and Gubler 2001). From both filters DNA was isolated using the InnuPrep Plant DNA Kit (Analytik Jena, Jena, Germany).

Molecular detection of *Pch*: For detection of *Pch* a nested-PCR was performed with DNA isolated from the filters. The first PCR was performed to detect asco- and basidiomycetes using the primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White *et al.* 1990). PCR was performed using KAPA Hifi Hotstart Polymerase (Kapa Biosystems, Wilmington, USA) with the following parameters: 5 min denaturation at 95 °C, 25 cycles with 20 sec denaturation at 98 °C, 15 sec annealing at 53 °C, 20 sec elongation at 72 °C and a final elongation with 1 min at 72 °C. The second PCR round was performed using the *Pch*-specific primers PCH5 (5'-AATCTAGAGAGACTTCTGCAACAAAACAATAG-3') and PCH3 (5'-AACTCGAGGTGACGTCTGAACGGTTCCATC-3') and 1 µL of the first round PCR as a template. The second PCR was also performed using KAPA Hifi Hotstart Polymerase with the following parameters: 5 min denaturation at 95 °C, 25 cycles with 20 sec denaturation at 98 °C, 15 sec annealing at 63 °C, 15 sec elongation at 72 °C and a final elongation with 1 min at 72 °C. PCR products were separated using 2 % agarose gels and documented using a QUANTUM ST5 gel documentation system (Vilber Lourmat, Eberhardzell, Germany). For sensitivity tests of the nested-PCR, DNA concentrations were measured using a NanoDrop 2000c (Peqlab, Erlangen, Germany).

Results

Monitoring: In the 'Chardonnay' vineyard, comprising 750 vines, only two vines showed a tigerstripe-pattern in the first year of monitoring (2012). In 2013 and 2014 no foliar symptoms were visible and in 2015, two vines, but different from those in 2012, had tigerstripes (Table).

The number of externally affected vines was very different in the 'Phoenix' plantation (Table). In 2012, 109 out of 1,530 plants showed a tigerstripe-pattern and besides three apoplectic plants were found (Fig. 2). In 2013, 125 vines showed tigerstripes and 17 became apoplectic.

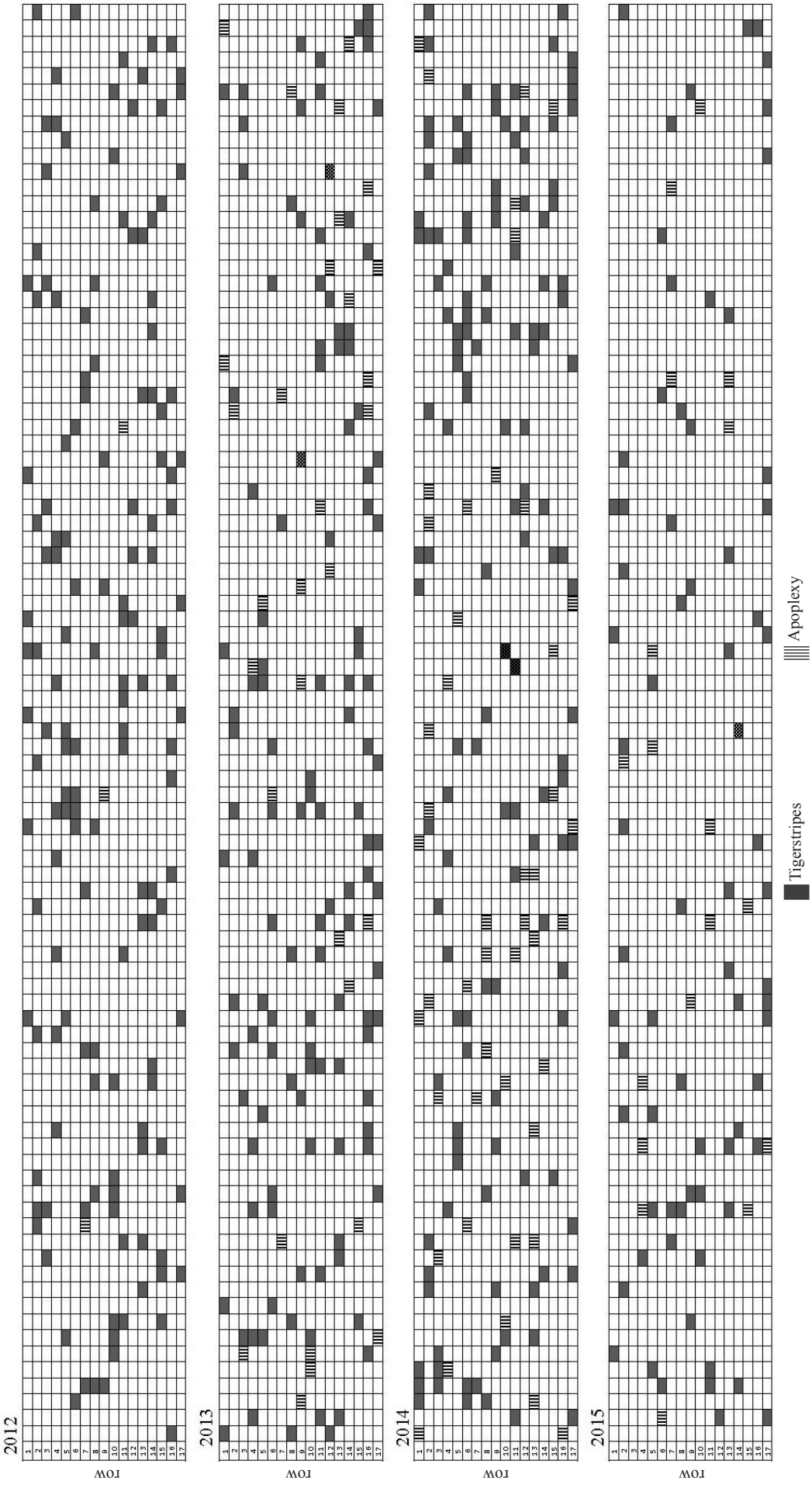

Fig. 2: Appearance of tigerstripe symptoms and apoplexy in the vineyard 'Phoenix' at the end of September in 2012, 2013, 2014 and 2015. Seventeen tows with 90 vines each.

Table

Number of vines with tigerstripe-pattern and number of apoplectic vines in vineyards of 'Phoenix' and 'Chardonnay' in the years 2012-2015

		2012	2013	2014	2015
Phoenix	tigerstripes	109	125	126	70
	apoplexy	3	17	60	14
Chardonnay	tigerstripes	2	0	0	2
	apoplexy	0	0	0	0

In 2014, the number of affected vines with tigerstripe-pattern remained essentially unchanged; apoplectic plants however increased to 60. In 2015, only 14 trunks became apoplectic and 70 showed tigerstripes. Throughout the survey, no symptoms appeared before July in both vineyards (data not shown). In the heavily infested 'Phoenix' plot, it was noticeable that not a single plant showed symptoms in all four consecutive years; only seven plants showed foliar symptoms in three consecutive years and 59 plants showed foliar symptoms in two successive years (Fig. 2). The distribution of symptoms was more or less random over the years. However, the southeastern part of the plot in general seemed more affected (see right hand area in Fig. 2).

Proof of *Pch* by a nested-PCR approach: Nested-PCR using primers ITS1-F und ITS 4 in the first round, and the specific primers PCH5 and PCH3 in the second round resulted in an amplicon size of 373 bp (Fig. 3). Sensitivity of the reaction was tested on extracted DNA derived from pure cultures of two selected *Pch* strains, CKV and KS356. With 1 ng of DNA as a starting concentration in round one, a tens steps dilution series showed that proof of *Pch* was possible as low as 0.1 pg, even though bands were only faintly visible at this concentration (Fig. 3). Results were reproducible and valid for both strains of *Pch* (Fig. 3).

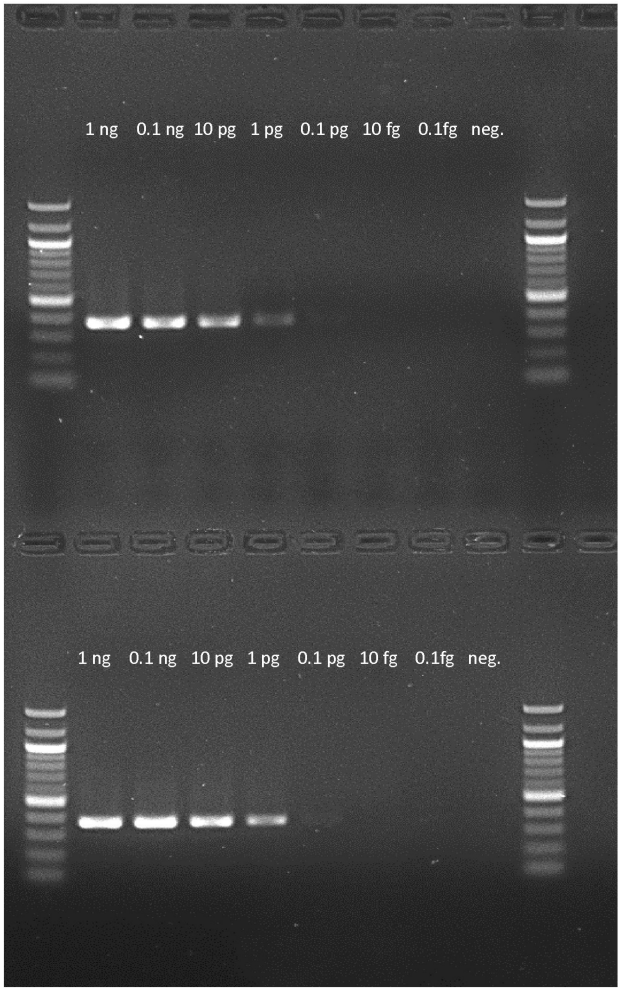

Fig. 3: Sensitivity of the nested PCR approach for demonstration of *Phaeomoniella chlamydospora*. Based on pure cultures of strains CKV (above) and KS356 (below) a DNA-concentration as low as 0.1 pg could be demonstrated. Size of specific amplicon is 373 bp.

Pch in spore traps of the trunk area: In 2013, the analysis of the traps started in week 15 (Fig. 4). For both vineyards, *Pch* was first detected in the middle of May and then could be found continuously until the beginning of September (calendar weeks 21 to 36). The detection period was similar in both vineyards. After week 36, the fungus was undetectable in both vineyards until the end of the monitoring, in late November 2013. In 2014, with a sampling period from week 5 to week 50, the first proof of *Pch* in vineyard 'Chardonnay' was at the end of January, while in 'Phoenix' it was at the beginning of February (Fig. 5). In both vineyards, spores of *Pch* were found until December with a distinct gap in August, *i.e.* from week 31 to week 35.

In 2015, with the sampling period starting in week 2 and ending in week 50, *Pch* was detectable in both vineyards from the end of January to the end of December

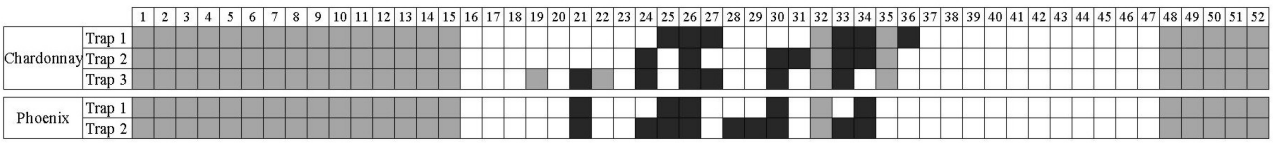

Fig. 4: Detection of *Pch* in the trunk area of the vineyards 'Chardonnay' and 'Phoenix' in 2013 using spore traps (dark grey: positive testing; white: negative testing; light grey: no data available).

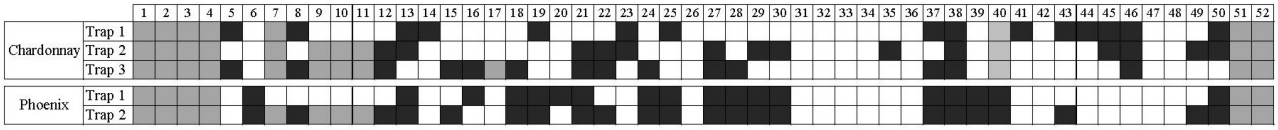

Fig. 5: Detection of *Pch* in the trunk area of the vineyards 'Chardonnay' and 'Phoenix' in 2014 using spore traps (dark grey: positive testing; white: negative testing; light grey: no data available).

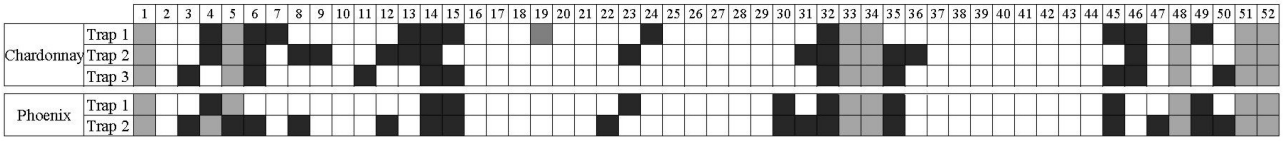

Fig. 6: Detection of *Pch* in the trunk area of the vineyards 'Chardonnay' and 'Phoenix' in 2015 using spore traps (dark grey: positive testing; white: negative testing; light grey: no data available).

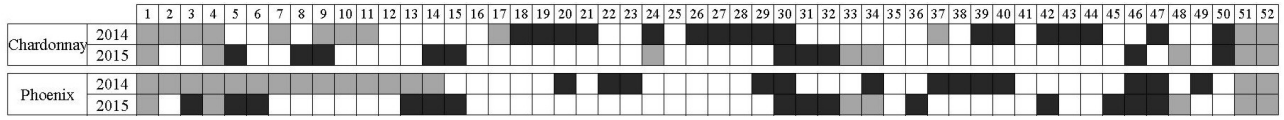

Fig. 7: Detection of *Pch* in the canopies of vineyards 'Chardonnay' and 'Phoenix' in 2014 and 2015 using spore traps (dark grey: positive testing; white: negative testing; light grey: no data available).

(Fig. 6). There were two major gaps in the detection period: the first one from week 16 to week 30, with single detections in weeks 22 to week 24, and the second from week 37 to week 44.

Pch in spore traps of the canopy area: In 2014, first evidence of *Pch* in the canopy was by the beginning of May (week 18) in 'Chardonnay', while in 'Phoenix' it was first demonstrated in the middle of May, week 20 (Fig. 7). In both plots, *Pch* was evident until the end of the year.

In 2015, first proof of *Pch* in the 'Phoenix' plot was by the end of January, and by the beginning of February in 'Chardonnay'. It remained detectable in both plots until the end of the year. As with trunk related traps, the traps in the canopy showed detection gaps from week 31 to week 35 in 2014 and two gaps from week 16 to week 29 and from week 37 to week 44 in 2015 (see Figs 5 and 6). Overall, no difference was found in the appearance of *Pch* between the canopy and the trunk area.

Discussion

Appearance of Esca symptoms in the vineyards: The divergence in the external expression of Esca between the two vineyards is mainly assignable to the respective age of vineyards, which in 'Chardonnay' has been planted in 2008 and in 'Phoenix' in 1995. Usually, foliar symptoms will not appear before approximately the 5th year of the vines and the number of infected vines increases with the age of plants (Mugnai *et al.* 1999). Furthermore, it is well known that the expression of foliar symptoms is discontinuous and can vary in its intensity in consecutive years (Mugnai *et al.* 1999, Surico *et al.* 2006).

As for 'Chardonnay', susceptibility against GTDs is considered lower when compared with 'Cabernet Sauvignon' or 'Trebbiano' (Andreini *et al*. 2014), while results were inconclusive in other studies (for instance, Bruez *et al*. 2013). With 'Phoenix', this is the first report of this cultivar in relation to Esca symptoms, and additional data are needed for a more definite statement.

According to Fischer (2006) and Marchi *et al.* (2006) the expression of foliar symptoms cannot be strictly related to the infection of the trunks with Esca related pathogens as the vines do not necessarily show immediate foliar symptoms after an infection, a phenomenon which was described by Marchi as "hidden esca". Therefore, it can be assumed that literally all vines in the 'Phoenix' plot are infected by pathogens but at the same time not all of them are showing the symptoms. To a less degree, this might also apply to the 'Chardonnay' plot. It is well known that vines both in their scion, and, above all, in their rootstock part may be infected already during the propagation process (Ridgway *et al.* 2002, Retief *et al*. 2006, Aroca *et al*. 2010; for Germany: Fischer 2019). However, if this alone will be necessarily leading to the formation of external symptoms remains unclear. As becomes evident by the spatial distribution of wood symptoms in adult vines, infection pressure more likely is related to pruning wounds. That is, symptoms both in the wood and, subsequently, on leaves and berries, are more likely due to ongoing infection events in the field (Fischer 2019). Inoculum source may be located both in the vineyard and, to a degree unknown, outside, with host plants different from *Vitis vinifera* (Fischer *et al*. 2016, Gierl and Fischer 2017).

The analysis of the spore traps combined with the discontinuity of the symptoms suggest that a four year monitoring of foliar symptoms may be not fully sufficient to evaluate the infection ratio of a vineyard. This conclusion was also drawn by Surico *et al.* (2006) in a previous study where a survey period of at least five years is recommended. While no correlation is possible between the proof of airborne *Pch* spores and the expression of external symptoms, the existence of viable spores (Eskalen and Gubler 2001) over the year indicates that i) this is an important mode of distribution, and ii) that spores occur all over the year, also covering the period of pruning measures.

Analyzing the spore traps using a nested-PCR approach: In previous studies trapped spores of *Pch* were detected by rinsing of the filters, plating the water on PDA medium and counting the germinated conidia (Eskalen and Gubler 2001, Eskalen *et al.* 2007). In both surveys only the 0.45 µm pore filter was analyzed; the 5 µm pore filter was primarily used to remove unwanted fungi and particles.

In comparison, screening of the filters using a nested-PCR approach is much faster and more sensitive. To get reliable results the approach via culturing takes 2-4 weeks according to van Niekerk *et al*. (2010), while nested-PCR results are available within 24 h. As a fundamental prob-

lem of the counting method, the spores of *Pch* can be easily overgrown by other fungi leading to a "false negative" result (van Niekerk *et al.* 2010). Therefore, it is conceivable that *Pch* was also trapped with the 5 µm filter in previous studies, but had remained unidentified with the methods used. In our studies we have been able to detect *Pch* not only in the 0.45 µm filter, but in the 5 µm filter as well, which clearly underlines the necessity of analyzing both filters. It has to be mentioned that the sensitivity of the nested-PCR does have certain limits, which in a previous study have been reported to be 10 fg of DNA of *Pch* in mixtures with foreign DNA (Retief *et al*. 2005). This particular amount of DNA also could be proven in our study, however was based on dilutions in water. As we do not know about the exact DNA content of *Pch* nuclei, the minimum number of conidia to be potentially demonstrated by our method remains unknown. Therefore, it is possible that not all of the trapped conidia were documented in our study. As a result, our data represent minimum numbers only, with the pathogen possibly more widespread over time than expected. Furthermore, while the PCR shows the occurrence of conidia, no statement about their vitality and their infectious potential is possible. While a nested-PCR does not allow an exact quantification statement, this could be provided by methods like qPCR. This could then be supported by counting of *Pch* based colonies in culture, as demonstrated by Eskalen and Gubler (2001).

Detection of *Pch* in vineyards: In contrast to former statements that *Pch* spores can be found only during the warm (summer) periods as shown for Italy (Quaglia *et al.* 2009) and South Africa (van Niekerk *et al.* 2010), the fungus in our study was essentially detected during the whole year. It was found even in the winter periods where sub-zero temperatures were measured like week 5 to week 8 and week 50 to week 52 in 2014 or weeks 1 to 9 and 48 to 50 in 2015 (weather data derived from the weather station of the DLR (www.dlr.rlp.de) located at the JKI). Detection during cold periods has also been described by Larignon and Dubos (2000), where spores were documented throughout the year in vineyards at Naujan-et-Postiac, France. Furthermore, pycnidia of *Pch* were found to form in cracks in the bark of vines and are thought to release conidia all year round (Edwards and Pascoe 2001). The detection of *Pch* conidia during the winter months is crucial insofar that the pruning is done in this particular time of the year, with the produced wounds being highly susceptible for minimum one month (Michelon *et al.* 2006) and still susceptible after four months (Elena and Luque 2016).

With the available data at hand, a definite evaluation of the relations between climatic conditions and the occurrence of conidia is hardly possible and only some regional tendencies are apparent. It has been proposed that the occurrence of conidia may be related to rain fall as they are increasingly detected after such weather events (Larignon and Dubos 2000, Eskalen and Gubler 2001), but this could not be confirmed during our study. However, other parameters such as temperature and humidity also might have some impact: in our study this would explain the observed detection gaps in 2014 and 2015 (Figs. 5 and 6) as those periods were unusually hot and dry with temperature peaks up to 40 °C. Even though there had been some slight rainfalls during these periods no conidia could be detected. A similar effect was observed in Italy, where no conidia could be detected during the hottest periods of the year (Quaglia *et al.* 2009). A low temperature limit for the occurrence of conidia at 12 °C as suggested by Larignon and Dubos (2000) in French vineyards was not confirmed in our study as the conidia could be found even during sub-zero temperature periods.

Not surprisingly, no correlation was found between the proof of conidia and the occurrence of foliar symptoms in our two vineyards. Numerous parameters, such as cultivar, age of vineyards, training system (and others) need to be carefully considered to properly assess frequency and intensity of external symptoms of Esca.

Conclusions

In the present study, covering a period from 2013 through 2015, airborne conidia of *Pch* were demonstrated in spore traps. Results in 2014 and 2015 for the first time in German vineyards show the existence of conidia throughout the year. An all year occurrence of *Pch* has already been reported for California, with climatic conditions very different though. In contrast to previous studies we were not able to positively link the occurrence of conidia to rainfall. Heat and lacking humidity however may have a negative impact on the appearance of conidia. During winter time and early spring, the availability of conidia in the air is concurrent with the annual pruning measures and in this way represents a serious source of infection.

Analyzing the spore traps *via* a nested-PCR approach allows for a more sensitive and faster detection of *Pch*; this approach was successfully applied in our survey. It has to be kept in mind however that the presented method does not provide accurate data with respect to quantification and vitality of airborne conidia.

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