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Use of a real-time PCR method to quantify the primary infection of *Plasmopara viticola* in commercial vineyards

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Abstract

Grapevine downy mildew (GDM) caused by *Plasmopara viticola* is a recurrent disease of wine grapes in the Ningxia Hui Autonomous Region (Ningxia) of northwestern China. However, the primary infectious pathogen in this region has not been thoroughly investigated. In this study, a multiplex real-time PCR assay was utilized to quantify *P. viticola* in soil, leaf residues, and asymptomatic leaf samples from ten commercial vineyards in two consecutive years to better understand the epidemiological significance of overwintering primary inoculum and its inoculum potential before the appearance of the first visual GDM symptoms. The DNA primers and multiplex real-time PCR assays that had been established exhibited specificity towards *P. viticola* within the test samples. The majority of the asymptomatic leaves (60%), leaf residues (80%), and soil samples (100%) tested positive for *P. viticola*. In addition, the amount of primary inoculum of *P. viticola* was found to be lower in soil than in leaf residues. The area under the disease progress curve in terms of the molecular disease index (AUDPC-MDI) was used to evaluate the overall latent *P. viticola* infection in asymptomatic leaves. Asymptomatic leaves were found to have different levels of *P. viticola* infection, and high AUDPC-MDIs correlated with a high AUDPC in terms of disease index (AUDPC-DI), with a significant correlation relationship between them ($P < 0.01$). Additionally, a well-correlated relationship was observed between the disease progress in the previous year and the MDIs of leaf residues and asymptomatic leaves in the following year, as well as the AUDPC-DI (Spearman's correlation coefficient $\rho = 0.643$, 0.498, and 0.595, respectively) ($P < 0.01$). These findings provide valuable information for quantifying the primary infection of *P. viticola* in commercial vineyards.

Keywords Grapevine downy mildew, Multiplex real-time PCR, Primary, Latent, Infections, Molecular disease index

Background

Grapevine (*Vitis vinifera* L.) is the second most important fruit crop in China in terms of cultivation area and production (Wang et al. 2004). The Ningxia Hui Autonomous Region (Ningxia; 37° 43′–39° 5′ N, 105° 46′–106° 28′ E) is located in the arid desert region of Northwest China and is known as the golden zone of wine grapes production in the world. The climatic conditions in Ningxia, such as solar radiation, temperature variability between day and night, and annual rainfall, are favorable for grapevine cultivation (Wang et al. 2020). In addition, wine grape varieties have become appealing because of the recent development in the regional economy,

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bringing considerable economic benefits to Ningxia. Nevertheless, grapevines are affected by numerous insect pests and diseases throughout their growth cycle, representing a constant threat to grape production and potentially resulting in significant yield and economic losses (Wang et al. 2004; Yang et al. 2020; Ammour et al. 2021).

Grapevine downy mildew (GDM) is one of the most destructive grapevine diseases worldwide (Wong et al. 2001; Rumbou et al. 2004) and has become the most severe disease limiting wine production in China (Yang et al. 2020). *Plasmopara viticola* [(Berk. & M. A. Curtis) Berl. & De Toni] is an obligate oomycete pathogen that causes GDM. In its life cycle, *P. viticola* produces two reproductive forms, sexual and asexual, which are responsible for primary and secondary infections, respectively (Wong et al. 2001; Rossi et al. 2011). Primary infections of *P. viticola* are caused by oospores that overwinter in infected leaf debris above the soil surface (Wong et al. 2001; Rossi and Caffi 2007; Gessler et al. 2011; Rossi et al. 2011). During the winter, oospores reach morphological maturity but are prevented from germinating due to dormancy; when dormancy is broken, oospores are considered physiologically mature and capable of germinating under favorable environmental conditions and form macrosporangia (Kennelly et al. 2007; Rossi and Caffi 2007; Ammour et al. 2020; Brischetto et al. 2020). The primary infections of *P. viticola* end with the formation of sporangia containing zoospores (Rossi et al. 2008). The sporangia trigger secondary infections of *P. viticola*, in which the released zoospores are transported to the leaf surface by rain splash and produce hyphae that colonize the host tissue (Burruano et al. 2000; Salinari et al. 2006; Rossi et al. 2013). GDM symptoms do not appear immediately after the completion of the secondary infections. After a latent period, the infected host tissues may exhibit GDM symptoms depending on the region's environmental conditions or host susceptibility. Many studies have shown that *P. viticola* oospores are the only source of primary infection and were long thought to only play a role in triggering the epidemic in the early grapevine season; the subsequent explosive increase in the disease was attributed to asexual multiplication (Rumbou et al. 2004; Gobbin et al. 2006; Rossi et al. 2013; Ammout et al. 2020). Thus, during the life cycle of *P. viticola*, primary infections are determinants of the occurrence of the GDM epidemic, while secondary infections are critical to disease progression.

To our knowledge, most GDM studies in China have been focused on the asexual reproductive stage (Yin et al. 2014), but little is known about the sexual stage of *P. viticola*. Furthermore, the epidemiological role of overwintering primary inoculum in commercial vineyards and the effect of inoculum potential on GDM epidemics are particularly unknown. As a result, quantitative detection of inoculum from seemingly healthy plant materials using a molecular detection method is needed, as well as investigation of the primary infection source and the impact of latent *P. viticola* infections on GDM epidemics. It helps predict GDM occurrences and epidemics in the following year and lays the groundwork for the precise prevention and control of GDM in vineyards.

Traditional assessment methods are incapable of quantifying *P. viticola* in infected samples, especially those with low pathogen concentrations. Thus, a molecular method with high specificity and sensitivity for pathogen quantification is required (Luo et al. 2007, 2020; Ammour et al. 2020). The real-time PCR assay is a reliable tool to predict potential epidemics and detect specific fungal species in mixed populations (Gindro et al. 2014; Chu et al. 2019; Ammour et al. 2020) and has been used extensively to quantify the inoculum in other pathosystems (Valsesia et al. 2005; Luo et al. 2007, 2019, 2020; Miguel et al. 2011 Babu et al. 2015). Recently, a real-time PCR assay has been used to efficiently identify oomycetes and quantify infection levels in soil, air, and plant tissues. For instance, Osawa et al. (2021) quantified *Phytophthora infestans* DNA from soil and found a positive correlation between the quantity of *P. infestans* DNA and inoculum potential. Carisse et al. (2021) employed a real-time PCR assay to quantify *P. viticola* clades *riparia* and *asetivalis* simultaneously and monitored the airborne sporangia thereof over multiple years in experimental and commercial vineyards. For *P. viticola*, Valsesia et al. (2005) developed a multiplex real-time PCR method for the relative quantification of *P. viticola* DNA directly from *V. vinifera* leaves, and Ammour et al. (2020) used a similar assay to quantify the amount of *P. viticola* DNA in diseased, senescent leaves. Although previous studies have primarily used real-time PCR to detect *P. viticola* in symptomatic leaves, it has not been used to detect *P. viticola* in soils, leaf residues, and asymptomatic leaves.

Therefore, we implemented the DNA primers previously formulated by Valsesia et al. (2005), along with the multiplex real-time PCR assay method published in the literature to achieve the following objectives: (i) quantify the current epidemiological significance of overwintering *P. viticola* oospores and the source of primary *P. viticola* infections in Ningxia commercial vineyards; (ii) quantify the primary inoculum potential and the levels of latent infection before the GDM epidemic; and (iii) discuss the relationship between the quantity of overwintering inoculum in soil or leaf residue and *P. viticola* in asymptomatic leaves detected by real-time PCR and the impact of latent *P. viticola* infection levels on the GDM epidemic.

Results

Development of standard curves

In the real-time PCR assay, the primer sets Giop-F/Giop-R and Res-F/Res-R were used to amplify 42 bp region from ITS1/5.8S (*P. viticola*) and 42 bp region from the resveratrol synthase I gene (*V. vinifera*), and the TaqMan probes (VIC [*P. viticola*] and FAM [*V. vinifera*]) were used for the simultaneous detection of these amplicons. The assay was sensitive and specific, as demonstrated by the high R^2 and E, which were good indicators of robust and reproducible results. In the first assay, the real-time PCR procedure was able to detect as little as 1 oospore/g soil of *P. viticola* oospore DNA, with a Ct value less than 36 (Fig. 1a). The DNA-based standard curve showed a significant negative correlation between the log of the known concentration of *P. viticola* oospore DNA and the Ct value, with an R^2 of 0.9775 and an E of 102% ($P < 0.001$) (Fig. 1a).

In the second assay, two standard curves were constructed by plotting the known concentrations of *P. viticola* and *V. vinifera* DNA against the Ct value obtained from real-time PCR (Fig. 1b, c). The linear regression between Ct and log values was significant ($P < 0.001$). The standard curve for purified *P. viticola* DNA had an E of 95% and R^2 of 0.9931 (Fig. 1b), with a minimum detectable target concentration of 0.1 pg/μL. The standard

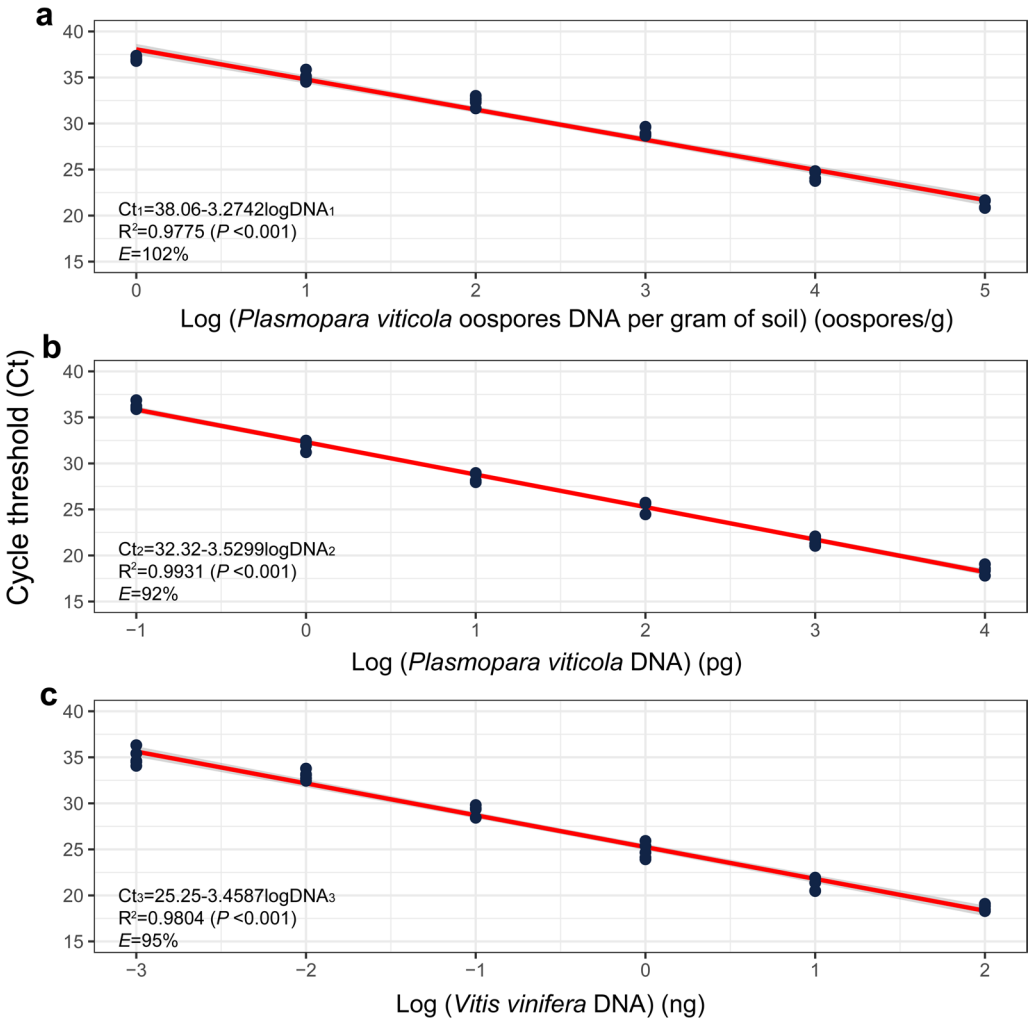

Fig. 1 Standard curve from quantitative multiplex real-time PCR by plotting the threshold cycle (Ct) versus the log-transformed DNA concentrations. **a** Relationship between fungal biomass introduced in soil and fungal DNA quantified by real-time PCR for *Plasmopara viticola* oospores. **b**, **c** a tenfold dilution series of *P. viticola* DNA (10^1–10^{-4} ng/μL) (**b**) and *Vitis vinifera* DNA (10^2–10^{-3} ng/μL) (**c**) was analyzed in triplicate by real-time PCR. The linear relationship between log-transformed DNA and the cycle threshold (Ct) values was determined for both *P. viticola* (**b**) and *V. vinifera* (**c**). Each data point represents five replications per concentration level for each test. The amplification efficiency was calculated as $\mathbf{E} = 10^{\left(\frac{1}{-\text{slope}}\right)} - 1$

curves for purified *V. vinifera* DNA had an E of 92% and R^2 of 0.9804 (Fig. 1c), with a minimum detectable target concentration of 10 pg/μL. No amplification was observed in the control samples, and all concentrations significantly differed from each other as determined by ANOVA and Tukey's test with five independent replicates ($P < 0.001$) (Table 1).

Quantification of *P. viticola* DNA in soil, leaf residues, and asymptomatic leaf samples

The relative quantification of *P. viticola* DNA in all test samples was estimated using a real-time PCR assay. All soil samples were found to carry *P. viticola*, while 80% (36 out of 45 in 2018) and 84% (42 out of 50 in 2019) of the leaf residue samples were found to carry the pathogen. To detect potential *P. viticola* in asymptomatic leaf samples, 211 out of 360 DNA samples tested positive for *P. viticola* in 2018, while 145 out of 150 DNA samples tested positive for *P. viticola* in 2019.

In the 2018 experiment, the quantity of *P. viticola* oospores in positive soil samples ranged from 156 to 872 oospores/g soil, with the lowest amount detected in the Yuma vineyard and the highest in the Hedong vineyard (Fig. 2a). Among the nine vineyards, soil samples from one vineyard had less than 200 oospores/g soil, soil samples from five vineyards had between 200 and 500 oospores/g soil, and soil samples from three vineyards had more than 500 oospores/g soil (Fig. 2a). Similarly, the quantity of *P. viticola* oospores in positive leaf residue samples ranged from 151 to 24,744 oospores/g, with the lowest amount detected in the Yuanrun vineyard and highest in the Huibin vineyard (Fig. 2a). Except for those from the Yuanrun vineyard, the amount of *P. viticola* DNA in leaf residue samples was higher than that in soil samples (Fig. 2a). The AUDPC-*P. viticola* DNA in asymptomatic leaves was highest in the Hedong vineyard and lowest in the Huibin vineyard, with corresponding mean values of 9532.84 and 214.84 pg, respectively (Fig. 2a). Of the nine vineyards, only samples from one vineyard had less than 1000 pg, five vineyards had between 1000 and 5000 pg and three vineyards had more than 5000 pg (Fig. 2a). The number of *P. viticola* oospores in soil did not differ significantly ($P = 0.12$) among the nine vineyards, whereas there were significant differences in the number of *P. viticola* oospores in the leaf residues ($P = 0.010$) and AUDPC-*P. viticola* DNA of asymptomatic leaves ($P = 0.011$) (Table 2).

In the 2019 experiment, the quantity of *P. viticola* oospores in positive soil samples ranged from 93 to 1035 oospores/g soil, with the lowest amount detected in the Nandatan vineyard and the highest in the Hedongvineyard (Fig. 2b). Among the ten vineyards, soil samples from four vineyards had less than 200 oospores/g soil, soil samples from three vineyards had between 200 and 500 oospores/g soil, and soil samples from three vineyards had more than 500 oospores/g soil (Fig. 2b). Similarly, the quantity of *P. viticola* oospores in positive leaf residue samples ranged from 50 to 28,931 oospores/g, with the lowest amount detected in the Hedong vineyard and highest in the Xixiawang vineyard (Fig. 2b). Except for those of the Hedong and Lanyi vineyards, the amount of *P. viticola* DNA in the leaf residues was higher than that in the soil (Fig. 2b). The AUDPC-*P. viticola* DNA in asymptomatic leaves was highest in the Yuanrun vineyard and lowest in the Huibin vineyard, with corresponding mean values of 3469.65 pg and 75.31 pg, respectively (Fig. 2b). Of the ten vineyards, samples from six vineyards had less than 1000 pg, and samples from four vineyards had between 1000 and 5000 pg *P. viticola* DNA (Fig. 2b). While ANOVA showed no significant difference

Table 1 Summary of commercial vineyards in Shizuishan, Yinchuan, and Wuzhong cities, Ningxia, China, along with the vineyard name, soil type, grapevine variety, and location information

Code	District	Vineyard name	Soil type[a]	Grapevine variety	Latitude	Longitude	Altitude (m)
1	Dawukou District, Shizuishan City	Hedong	Sandy loam	Pinot noir	38° 58′ 47″	106° 18′ 24″	1100
2	Helan District, Yinchuan City	Yuanrun	Sandy loam	Cabernet sauvignon	38° 43′ 59″	106° 7′ 26″	1124
3	Xixia District, Yinchuan City	Lanyi	Sandy loam	Cabernet sauvignon	38° 38′ 56″	106° 3′ 45″	1140
4		Yuanlinchang	Sandy loam	Cabernet sauvignon	38° 37′ 45″	106° 8′ 9″	1140
5		Zhihuiyanshi	Sandy loam	Cabernet sauvignon	38° 34′ 53″	106° 0′ 48″	1190
6	Yongning County, Yinchuan City	Huibin	Sandy loam	Cabernet sauvignon	38° 17′ 11″	106° 3′ 30″	1120
7		Lilan	Sandy loam	Cabernet sauvignon	38° 16′ 22″	105° 57′ 24″	1200
8		Xixiawang	Sandy loam	Cabernet sauvignon	38° 15′ 24″	106° 3′ 38″	1150
9		Nandatan	Sandy loam	Merlot	38° 13′ 38″	106° 0′ 33″	1150
10	Qingtongxia County, Wuzhong City	Yuma	Sandy loam	Cabernet sauvignon	38° 4′ 48″	105° 54′ 58″	1170

[a] The soil type of all assessed vineyards is a combination of sandy and loamy soils. Soil total salt content ≤ 1.5 g/kg, pH ≤ 8.5, rich in organic matter, sediment content < 50 mg/L

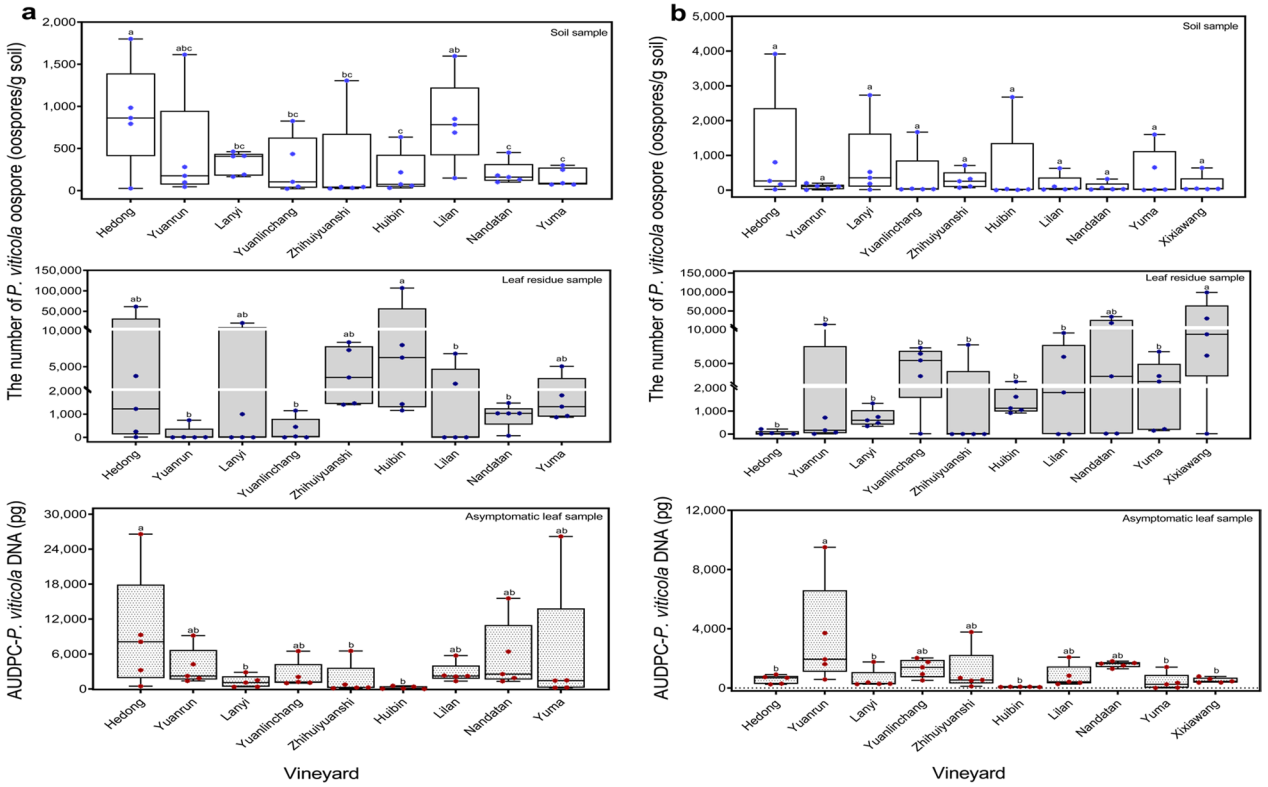

Fig. 2 Quantification of *Plasmopara viticola* in soil, leaf residues, and asymptomatic leaves collected from commercial vineyards in 2 consecutive years using multiplex real-time PCR assay. **a**, **b** Box plots showing the number of *P. viticola* oospores in soil and leaf residues and the area under the disease progress curve of *P. viticola* DNA in asymptomatic leaves from nine vineyards in 2018 (**a**) and ten vineyards in 2019 (**b**) prior to the appearance of visible symptoms of downy mildew. The boxplot corresponds to the numerical changes at five sampling points in each vineyard in 2018 (**a**) and 2019 (**b**). The thick line in the boxes is the median. The lowest value in each box represents the minimum detected value, and the top part of each box represents the maximum detected value. ANOVA with the Kruskal–Wallis nonparametric test was applied to determine the difference in the number of *P. viticola* oospores and AUDPC-*P. viticola* DNA among vineyards. The same letter on the graph indicates no significant difference for each vineyard group at $P < 0.05$

Table 2 An analysis of variance (ANOVA) was performed using the Kruskal–Wallis nonparametric test to compare each parameter across multiple commercial vineyards during 2018 and 2019

Parameter	2018 (n = 45)			2019 (n = 50)		
	df[a]	Kruskal–Wallis *H* test	*P* value[b]	df[a]	Kruskal–Wallis *H* test	*P* value[b]
The number of *P. viticola* oospores in soil	8	12.763	0.12	9	8.531	0.482
The number of *P. viticola* oospores in leaf residues	8	19.961	0.010*	9	16.717	0.053
MDI of leaf residues	8	14.566	0.068	9	21.994	0.009**
AUDPC-*P. viticola* DNA of asymptomatic leaf	8	19.852	0.011*	9	28.036	0.001**
AUDPC-MDI of asymptomatic leaf	8	22.061	0.005**	9	22.855	0.007**
AUDPC-DI	8	35.499	0.000**	9	38.854	0.000**

[a] *df* Degree of freedom

[b] *$P < 0.05$, **$P < 0.01$

in the number of *P. viticola* oospores in soil ($P = 0.482$) and less variation ($P = 0.053$) in the leaf residues among ten vineyards, there was a significant difference in AUDPC-*P. viticola* DNA of asymptomatic leaves among the ten vineyards ($P = 0.001$) (Table 2).

Assessment of latent infection in the leaf residues and asymptomatic leaves, and disease progression in commercial vineyards

The MDI values were used to assess the latent *P. viticola* infection levels in the leaf residues and asymptomatic

leaves in each vineyard. The mean MDIs of leaf residues in the Hedong vineyard were higher than those of the leaf residues in the other vineyards, with a corresponding mean value of 27.75, and the mean AUDPC-MDIs of asymptomatic leaves in each vineyard ranged from 18.51 to 475.57, with the Lanyi vineyard having the highest and Huibin vineyard having the lowest in 2018 (Fig. 3a). In 2019, the mean MDIs of the leaf residues in the Lanyi and Nandatan vineyards were higher, with corresponding mean values of 54.58 and 42.45, respectively, and the mean AUDPC-MDIs of asymptomatic leaves in each vineyard ranged from 100.57 to 498.31, with the Lanyi vineyard having the highest mean AUDPC-MDI and the Zhihuiyuanshi, Hedong, and Huibin vineyards having the lowest, with mean values of 0.101, 0.102, and 0.109, respectively (Fig. 3b). The AUDPC-DI data were used as a reference to assess the disease progression of GDM. The Lanyi vineyard had the highest AUDPC-DIs in both 2018 and 2019, with mean values of 739.589 and 1510.756, respectively, while the lowest AUDPC-DIs were in the Zhihuiyuanshi vineyard in 2018 and the Huibin vineyard in 2019, with mean AUDPC-DIs of 11.978 and 21.156, respectively (Fig. 3a, b). In general, the AUDPC-DIs were higher in 2019 than in 2018, except for those of the Huibin and Yuma vineyards. A comparison of AUDPC-DIs of three *V. vinifera* varieties, cv. Cabernet sauvignon, Pinot noir, and Merlot showed that Cabernet sauvignon was more sensitive to *P. viticola*, while grapes in different vineyards showed different sensitivities to *P. viticola*. Pinot noir and Merlot were among the most susceptible varieties in the target vineyard, with a medium resistance to *P. viticola* (Fig. 3).

According to the ANOVA results, there was less variation in the MDI of leaf residues ($P=0.068$) in 2018, but a significant difference was observed in 2019 ($P=0.009$). Furthermore, there were significant differences in the AUDPC-MDI of asymptomatic leaves ($P=0.005$) and AUDPC-DI ($P=0.000$) among the 9 vineyards in 2018 and in the AUDPC-MDI of asymptomatic leaves ($P=0.007$) and AUDPC-DI ($P=0.000$) among the 10

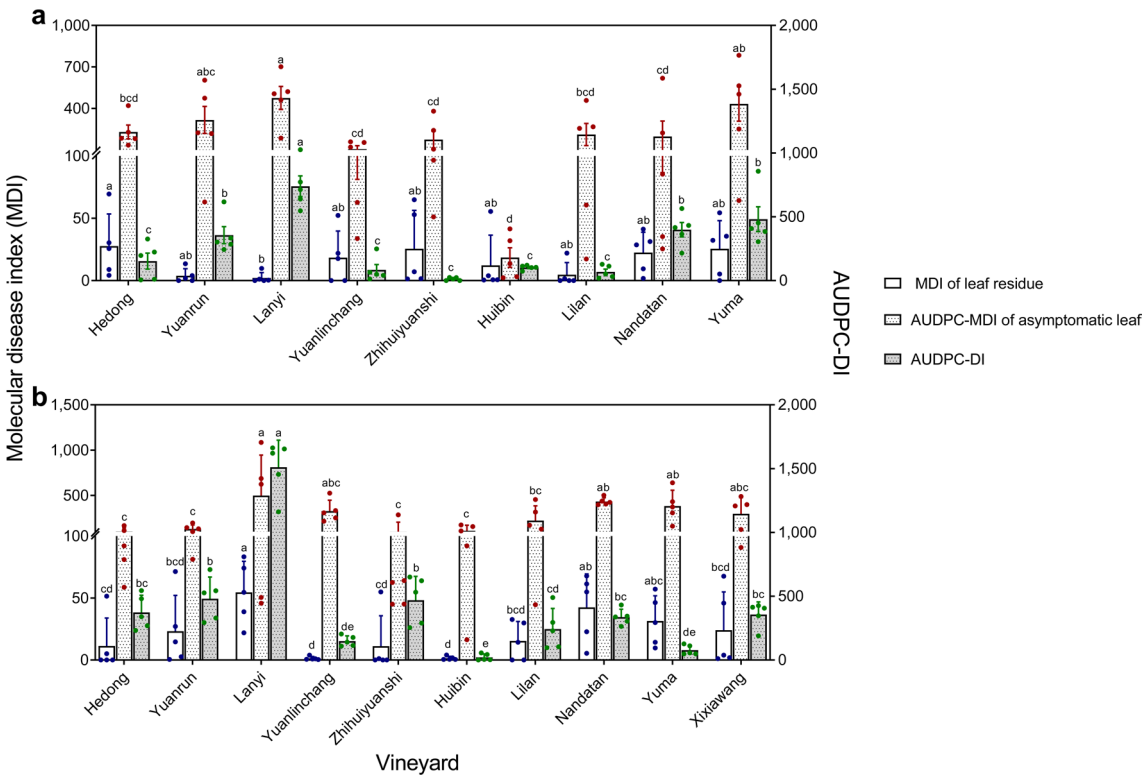

Fig. 3 Assessment of latent infection of *Plasmopara viticola* in leaf residues and asymptomatic leaves based on real-time PCR detection. The molecular disease index (MDI), calculated as the ratio of the amount of *P. viticola* DNA (ng) to the amount of *Vitis vinifera* DNA (ng), was used to quantify the levels of latent infection. **a**, **b** Different bars and vertical lines show the mean and standard deviation for each MDI of leaf residues, area under the disease progress curve of MDI (AUDPC-MDI) of asymptomatic leaves, and AUDPC of disease index (AUDPC-DI) from nine vineyards in 2018 (**a**) and ten vineyards in 2019 (**b**). Each numerical point represents an assessment point for each vineyard. ANOVA with the Kruskal–Wallis nonparametric test was applied to determine the difference in MDI of leaf residues, AUDPC-MDI of asymptomatic and AUDPC-DI among vineyards. Vertical bars represent the standard errors for the means. The same letter on the graph indicates no significant difference for each vineyard group at $P<0.05$

vineyards in 2019 (Table 2). In addition, for the three *V. vinifera* varieties in both years, significant differences were also observed in the AUDPC-MDI of asymptomatic leaves and AUDPC-DI (Fig. 3a, b).

Correlation relationship between the quantification of primary infections and disease progression

The results of Spearman's correlation analysis revealed the following relationships between parameters in 2018 and 2019. In 2018, vineyards with a high number of *P. viticola* oospores in soil, such as Hedong, Lilan, Lanyi, and Yuanrun, exhibited a high AUDPC-MDI of asymptomatic leaves, and a significant positive correlation was observed between the amount of *P. viticola* DNA in soil and AUDPC-MDI of asymptomatic leaves ($\rho = 0.510$, $P = 0.045$) (Figs. 2, 3 and 4). Furthermore, vineyards with high AUDPC-MDIs, such as Lanyi, Yuma, Yuanrun, and Nandatan, had higher AUDPC-DIs, while vineyards with low AUDPC-MDIs, such as Hedong, Zhihuiyuanshi, Yuanlinchang, and Lilan, had lower AUDPC-DIs (Fig. 3a). A significant positive correlation was observed between AUDPC-MDIs of the asymptomatic leaves and AUDPC-DIs with $\rho = 0.834$, at $P = 0.002$. Moreover, significant linear regressions were found between the mean AUDPC-MDIs of asymptomatic leaves and the mean AUDPC-DIs with $R^2 = 0.696$ (data not shown). In 2019, the Hedong, Yuanrun, Lanyi, Nandatan, Xixiawang, and Yuma vineyards exhibited high MDIs of leaf residues and high AUDPC-MDIs of asymptomatic leaves. Additionally, vineyards with high AUDPC-MDIs, such as Yuanrun, Lanyi, and Xixiawang, had higher AUDPC-DIs, while vineyards with low AUDPC-MDIs, such as Yuanlinchang, Huibin, Lilan, and Yuma, had lower AUDPC-DIs (Fig. 3b). A striking positive correlation was found between the MDI of leaf residues and AUDPC-DIs ($\rho = 0.480$, $P = 0.000$) (Fig. 4).

The analysis of the correlation between the parameters in the two years revealed noteworthy results. Specifically, *P. viticola* DNA in soil and leaf residues in 2018 was significantly positively correlated with *P. viticola* DNA in the soil in 2019, with Spearman's correlation coefficients of 0.300 ($P = 0.035$) and 0.382 ($P = 0.010$), respectively. Furthermore, the AUDPC-DIs in 2018 were found to be significantly and positively correlated ($P < 0.001$) with the MDIs of leaf residues, AUDPC-MDIs of asymptomatic leaves, and AUDPC-DIs in 2019, with corresponding Spearman's correlation coefficients of 0.643 ($P = 0.000$), 0.498 ($P = 0.013$), and 0.595 ($P = 0.000$), respectively (Fig. 4).

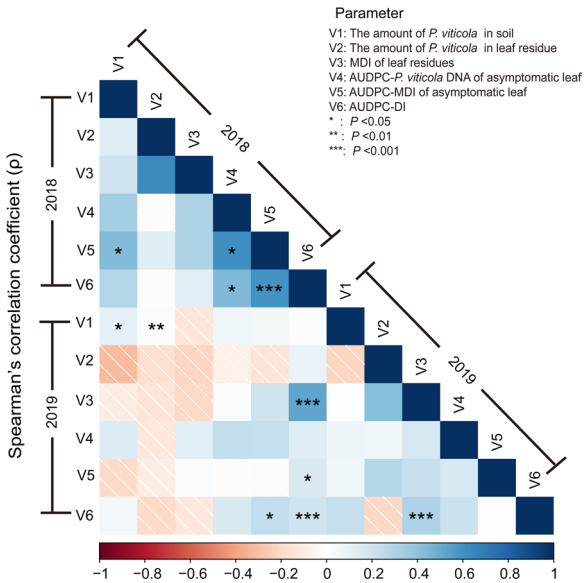

Fig. 4 A comprehensive analysis of Spearman's correlation coefficient between six parameters of the amount of *Plasmopara viticola* in soil (V1) and in leaf residues (V2), molecular disease index (MDI) values of leaf residues (V3), area under the disease progress curve (AUDPC) of *P. viticola* DNA of asymptomatic leaves (V4), AUDPC-MDI of asymptomatic leaves (V5), and AUDPC-DI of grape downy mildew (V6) in 2018 and 2019 was performed

Discussion

The purpose of this study was to molecularly quantify the presence of *P. viticola* in soil, leaf residue and asymptomatic leaf samples from naturally infected vineyards using a multiplex real-time PCR method. The field real-time PCR measurements showed that *P. viticola* was present in all soil samples and over 80% of leaf residue samples. However, the amount of *P. viticola* inoculum was found to be lower in the soil than in the leaf residue samples. Additionally, this study found that not all asymptomatic leaves carried *P. viticola* at early growth stages. However, as the grapevine developed and climate conditions changed, the presence of *P. viticola* increased in asymptomatic leaves 7–20 days prior to the appearance of symptoms. The amount of cumulative *P. viticola* in the asymptomatic leaves was related to the amount of primary inoculum in the assessed vineyards. The real-time PCR assay developed by Valsesia et al. (2005) was used in this study and proved effective in quantifying *P. viticola* obtained from various sources within commercial vineyards and accurately monitoring low levels of potential *P. viticola* infection at the early stage of disease development. This assay could provide valuable information on GDM epidemiology, and quantifying potential *P. viticola* levels in field samples is critical for accurate disease assessment, enabling the development of timely and effective control programs.

Rouzet and Jacquin (2003), Rossi and Caffi (2011), and Ammour (2020) described the dynamics of primary *P. viticola* infection in European vineyards, explaining

that *P. viticola* oospores can overwinter in leaf residues above ground or in the soil and are a source of the primary inoculum for GDM in the following growing season (Gobbin et al. 2005; Salinari et al. 2006; Caffi et al. 2009; Gessler et al. 2011; Rossi et al. 2013). However, this is not the case in the majority of vineyards in Western Australia, where it has been confirmed that oospores do not serve as the source of primary infection in vineyards (Killigrew et al. 2005). The situation is similar in China, where there is little known about the presence of primary inoculum (oospores) in commercial vineyards and its impact on GDM epidemics. Jin et al. (2015) found that overwintering oospores from leaf residues in the soil are the source of primary infections of *P. viticola* in the Xinjiang region. Zheng et al. (2021) discovered that the amount of primary inoculum of *P. viticola* in the Lilan vineyard in Ningxia is determined by the overwintering oospores present in the soil. Our study supports these findings and suggests that the primary inoculum of *P. viticola* in commercial vineyards in Ningxia is exclusively from oospores, with oospores from leaf residues being more competitive than those from the soil as a source of primary infection.

The relationship between sexual-asexual reproductive cycles and GDM was re-evaluated in this study, which evaluated the role of overwintering *P. viticola* oospores as inoculum for GDM epidemics in commercial vineyards. The results showed a good association between the number of *P. viticola* oospores and the amount of inoculum in asymptomatic leaves in 50% of vineyards. Vineyards with latent *P. viticola* infection often developed into diseased vineyards, and those with high levels of latent infection tended to have higher levels of disease progression. Furthermore, the amount of inoculum in leaf residues, asymptomatic leaves, and the AUDPC-DI in the following year was linked to the GDM epidemic in the previous year, highlighting the crucial role that overwintering *P. viticola* oospores play as inoculum in the development of GDM in Ningxia. This finding underscores the importance of early detection and management of GDM, which can aid in developing effective disease management strategies. However, the presence of a significant number of oospores does not always lead to disease symptoms, as latent *P. viticola* DNA infection was sometimes detected sporadically in some vineyards, such as in the case of the Huibin vineyard in 2018 and the Xixiawang vineyard in 2019 (Fig. 2). This could be due to the factors such as the timing and amount of primary inoculum release in the field, as well as the effects of climate change on host–pathogen interactions. This finding highlights the importance of effectively targeting these oospores to minimize disease progression and promote vineyard health. However, the limitations of this study suggest that environmental pressure has a more significant impact on the development of GDM epidemics than the primary inoculum.

The majority of the vineyards we assessed (e.g., Yuma, Yuanrun, Lanyi, and Lilan) were unmanaged, i.e., infected tissues (leaves, bunches, fruits, etc.) were left over on the ground after pruning. The overwintering oospores of *P. viticola* remain dormant during the winter but can be triggered to germinate by water and suitable temperatures in spring (Caffi et al. 2011; Gessler et al. 2011; Kennelly et al. 2007). If the field temperature is low and irrigation is not timely, oospores will accumulate in soil carrying leaf residues, thereby increasing the density of the oospore inoculum. However, the viability and germination of oospores in the soil environment are affected by temperature and precipitation. Cold and dry winters can cause some oospores to lose viability, while high temperatures, dry environments, and biocontrol agents (*Bacillus subtilis* and *Trichoderma hartianum* T39) can inhibit oospore germination (Dagonstin et al. 2006; Vecchione et al. 2005). At present, our study is unable to determine the persistence of nonviable or nongerminable oospores in soil/leaf residue. Despite this, previous research has indicated that oospores can remain viable for up to 65 months and that a limited number of viable oospores could trigger an epidemic during the growing season (Caffi et al. 2009, 2013). Therefore, understanding the seasonal prevalence of oospores in the soil environment will be necessary in the future, and this finding may provide a basis for managers to reconsider their management strategies.

Notably, the estimated indicators are based on real-time PCR standard curves established with known amounts of target DNA under controlled conditions, allowing quantification of pathogen DNA in host tissues and potentially avoiding several experimental errors. Furthermore, the real-time PCR system has a wide linear dynamic range, allowing for accurate quantification of the pathogen regardless of the stage of infection. Conversely, field parameters, such as the environment and host resistance, can lead to inaccurate and variable quantification. To avoid inhibitors/components affecting DNA quality, a new method was employed (Yuan et al. 2021), which is an effective treatment of soil and leaf residue samples that can quantify whether latent pathogen infections exist in samples is beneficial prior to DNA extraction and real-time PCR assays. Despite these efforts, the real-time PCR assay could not distinguish between viable or nonviable oospores even though their DNA was still detectable, which could bias the assessment of the inoculum potential in the vineyard. This merits consideration in GDM research since quantifying oospores in soil/leaf

residues is still challenging, and further improvements are necessary for accurate results.

In addition, the correlation between primary inoculum and disease progression may be affected by the resistance levels of the varieties and agricultural practices, which is necessary to consider in our study. The resistance of the grape varieties to GDM has an impact on the latent infection of *P. viticola* and GDM progression. Resistant varieties generally have lower levels of primary inoculum, leading to the disease not progressing as rapidly or severely. However, the resistance levels may impact the accuracy of the AUDPC-DI as a measure of disease severity, as they may influence the rate of disease progression. The three *V. vinifera* varieties, cv. Cabernet sauvignon, Pinot noir, and Merlot, which we assessed in this study, have been identified as susceptible in previous reports, but their susceptibility and performance in the field can vary. However, the susceptibility behavior of *P. viticola* in asymptomatic leaves after infection and prior to the appearance of disease symptoms is still unknown. Additionally, the effective application of fungicides within an IPM program can kill or control the growth of pathogens in plant tissues, limit the spread of the pathogen, reduce its population, and reduce the susceptibility of plants to infections and the level of pathogenicity within the tissues. In Ningxia, these practices, which are always applied after rainfall, can delay the onset of the first symptoms but cannot reduce the severity of an epidemic in the growing season. However, the effect of fungicides on primary infection, the disease epidemic, and the effect on the pathogenicity gene are still under study. At present, our findings can provide useful information to guide fungicide application and potentially improve the efficiency of GDM management in vineyards.

Conclusion

Real-time PCR assays could be useful as a diagnostic tool for detecting *P. viticola* and managing GDM in commercial vineyards. This method allowed for a preliminary determination of the primary infection source and latent infection level in commercial vineyards and the influence of overwintering *P. viticola* on the life cycle of GDM, providing a reference for the prediction and control of GDM epidemics in vineyards. Furthermore, the findings of this study provide agriculture advisory services and growers with knowledge of the primary inoculum potential and how those levels are related to GDM severity, as well as offer predictors for predicting disease epidemics in vineyards. Finally, combining molecular detection data with vineyard disease assessment data to identify potential disease hotspots in the vineyard and determine their development dynamics, accumulation of latent infection, and pathogen adaptation to environmental changes would aid in the establishment of a GDM early warning system for the Ningxia region and avoid unnecessary plant protection interventions.

Methods

Sampling and disease assessment

Sample collection

All the samples were collected from the major grapevine production areas of Yinchuan, Shizuishan, and Wuzhong cities in the Ningxia region of China (Table 1). Commercial vineyards were selected based on the same type of soil. In each vineyard, the plots with the most severe occurrence of GDM and vine spacing within and between rows of 1.0–3.0 m and 3.0–3.5 m, respectively, were selected. A total of 9 plots from 9 commercial vineyards were assessed in 2018, and 10 plots from 10 commercial vineyards were assessed in 2019 (the vineyards assessed in 2018 remained unchanged with the addition of the Xixiawang vineyard in 2019). Information on the vineyard plots is displayed in Table 1. All assessed vineyard plots were georeferenced using a hand-held global positioning system (GPS) (iPhone® utility Compass).

A five-point (Z-shaped) sampling method was employed in each vineyard plot. The interval between the five collection points per plot was determined based on the area of the plot and ranged from 10 to 20 m. Each collection point in the plot covered an area of 10 m^2, and four grapevines were assessed at each point. After the autumn harvest, grapevines were buried at a soil depth of 20–40 cm according to local management standards to protect them from frost damage. In early April, 200 g soil samples containing leaf residue were collected from a depth of 10 cm below the roots at each point after turning the soil. A total of 95 samples were collected (45 samples in 2018; 50 samples in 2019), with 1000 g of soil collected per vineyard plot. To facilitate the collection of asymptomatic leaves from the same collection point, each grapevine was marked with a plant tag that included the sampling date. Asymptomatic leaves (30 leaves per point) were collected every 7 days during the bloom period (BBCH 65) (Lorenz et al. 1995) until the appearance of 'oil spots'. Asymptomatic leaves were collected eight times in 2018 and three times in 2019, with a total of 360 asymptomatic leaf samples collected in 2018 and 150 asymptomatic leaf samples collected in 2019. All samples were collected at the same collection point in the same plot for two consecutive years.

All test samples were placed in polyethylene bags, refrigerated in a container, and brought back to the laboratory. Samples from soil and leaf residues were stored at 4°C, and asymptomatic leaves were stored at −20°C until DNA extraction.

All vineyards were managed by managers following an integrated pest management (IPM) program that included the application of herbicides, insecticides, long pruning, and drip irrigation. Fungicide (Bordeaux mixture) was applied by the field trial managers after growth stage BBCH 68 to ensure that GDM did not reduce yield.

Disease assessment

GDM disease assessment was performed weekly, starting from the first visual GDM symptom appearing at the end of the growing season (beginning of September). A total of fifteen assessments were performed each year. The disease assessment method is detailed in the paper authored by Yu et al. (2017). Briefly, 100 leaves were randomly assessed per marked point, and a total of 500 leaves were assessed per vineyard plot. Then, the number of diseased leaves and the disease severity were recorded, which was defined by disease grade classes 1 to 9 based on the percentage of leaf area with disease symptoms as follows: 0, no symptoms; 1, < 5.0%; 3, 5.1–25.0%; 5, 25.1–50.0%; 7, 50.1–75.0%; and 9, 75.1–100.0% (Yu et al. 2017). Finally, the disease index (DI) was calculated as follows: DI = ∑(no. of diseased leaves × disease grade per leaf) × 100/(total number of leaves × highest disease grade).

Sample preparation

Artificially inoculated soil

To extract *P. viticola* oospores, as described by Vercesi et al. (2000) and Ammour et al. (2020), diseased leaves with typical DM symptoms were collected, and fragments of the symptomatic leaves were soaked in a mixture of acetic acid–ethanol (1:3) overnight at room temperature. The oospores were examined under a light microscope after the soaked leaf fragments were rinsed in distilled water (DW). The fragments containing oospores were suspended in 15 mL of double-distilled water (ddH_2O) and filtered through two layers of medical gauze to remove debris. The oospore concentration was estimated using a hemocytometer, and the initial concentration was adjusted to 10^5 oospores/mL and then serially diluted tenfold. To establish a standard curve for quantifying oospore concentration in soil samples, 1 g of dried uninfected soil was inoculated with known amounts of oospore suspensions, with concentrations of 10^5, 10^4, 10^3, 10^2, 10, and 1.0 oospores per gram of soil (oospores/g soil). Each concentration was replicated five times and used for DNA extraction and real-time PCR assays.

Fresh *P. viticola* sporangia preparation

To obtain pure *P. viticola* DNA, diseased leaves (single spot) showing typical DM lesions with fresh sporulation on the abaxial side of the leaf were collected and then washed 3–4 times with sterile water to remove fungicides and *P. viticola* sporangia. Later, the leaves were incubated in an artificial climate box set at 25°C temperature, 95% relative humidity, and a 16 h/8 h light/dark photoperiod for 24–48 h. Subsequently, following the method of inoculation and propagation reported by Rumbolz et al. (2002), the newly grown sporangia were collected gently with a blade, suspended in a sterile centrifuge tube filled with 15 mL 70% ethanol and stored at −20°C until DNA extraction. As described above, the prepared sporangia suspension was centrifuged at 16,173 *g* for 15 min, and the aqueous phase was discarded. DNA extracted from sporangia was used as pure *P. viticola* DNA to construct the standard curve and as a positive control in the multiplex real-time PCR assay.

Naturally, infected soil and leaf residue sample preparation

The overwintering soil samples (including leaf residues) from the assessed vineyards were first weighed, homogenized, and air-dried at room temperature. Then, the samples were sieved three times with a mesh net (30–40 μm) to ensure complete separation of the soil and leaf residues. Finally, each sampling point yielded 3–5 g of leaf residues and more than 150 g of soil. Further isolation of leaf residues from soil was performed to improve the quality of the qPCR assay and remove inhibitors that would hinder PCR. Following an efficient soil purification (concentration) method that can concentrate large volume soil samples into small volumes for expected oospore enrichment (Yuan et al. 2021), 65 ml of 50% sucrose solution was used to purify 30 g of the soil sample, and a final 3 g of purified soil was obtained and used for DNA extraction.

DNA extraction

Genomic DNA was extracted from artificially inoculated *P. viticola* oospore-containing soil samples and naturally infected soil samples using the Qiagen DNeasy Power Soil Kit (NO. 12888-100) and from naturally infected leaf residue samples using the Qiagen DNeasy Plant Pro Kit (NO. 69204). The DNA was extracted from 250 mg of soil sample and 100 mg of leaf residue sample following the protocols provided by the manufacturer. Additionally, 100 mg each from healthy *V. vinifera* leaves, sporangia suspensions (without plant tissue), and asymptomatic leaves were transferred to 2 mL tubes containing a 2 mm diameter carbide bead per well. The samples were ground for 40 s at a frequency of 40/s using a FastPrep-24

homogenizer (LLXBIO, USA). Then, DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Chu et al. 2019). Three replicates of DNA extractions were performed for each soil, leaf residue, and asymptomatic leaf sample. The yield and purity of the pure oospore DNA and pure *P. viticola* DNA, pure *V. vinifera* DNA, and each test sample of DNA were estimated with a NanoDrop DS-11 spectrophotometer (Thermo Scientific, Wilmington, Delaware), and the DNA concentrations of the test samples were adjusted to 30 ng/μL. Finally, all DNA samples were stored at −20°C. All experiments were performed at the Plant Disease Epidemiology Laboratory of China Agriculture University (CAU).

Multiplex real-time PCR assay

Multiplex real-time PCR was performed using the TaqMan method, which utilized specific primer pairs and probes for both *V. vinifera* and *P. viticola*. All real-time PCR experiments were performed in 96-well plates using the QuantStudioTM 6 Flex Real-Time PCR System (Applied Biosystems), with *P. viticola* sporangia DNA and *V. vinifera* DNA as positive controls, and ddH_2O as a negative control. Real-time PCR reactions were prepared in a volume of 30 μL containing 15 μL of 2× T5 Fast qPCR Mix Probe (TSINGKE, China), 5 μL of template DNA, 0.9 μM of Giop-F/R, 0.25 μM of Giop-Vic, 0.12 μM μL of Res-F/R, 0.25 μM of Res-Fam, and ddH_2O to make up the volume. The thermal cycling conditions consisted of an initial denaturation for 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All samples were analyzed in three replicates to determine the cycle threshold (Ct).

DNA standard curves for real-time PCR

The detection of pure oospores in soil, pure *P. viticola* and pure *V. vinifera* DNA was performed by real-time PCR assays in duplex mode. As previously described, in the first assay, six different concentrations of *P. viticola* oospore DNA were extracted from 10^5 to 1.0 oospore/g soil, and five biological replications were analyzed. The results were then used to plot the known concentration of oospores against the threshold cycle (Ct) values to generate a standard regression curve for *P. viticola* oospores. In a second assay, six tenfold serial dilutions of *P. viticola* and *V. vinifera* DNA were adjusted to the following concentrations (5 replicates each): pure *P. viticola* DNA, 10^4, 10^3, 10^2, 10, 1, and 10^{-1} pg/μL; pure *V. vinifera* DNA, 10^2, 10, 1, 10^{-1}, 10^{-2}, and 10^{-3} ng/μL. The Ct values of *P. viticola* and *V. vinifera* were plotted against the logarithm of the initial DNA concentration to generate two standard curves, which allowed for the calculation of the amount of DNA. The coefficient of determination (R^2) and the amplification efficiency (E) were calculated using linear regression.

Data analysis

All statistical analyses and graphics were performed using Rstudio software (version 4.2.0). We conducted two types of analysis as follows:

First, for the unknown samples in each run, the relative quantification of target pathogens and the host was estimated using a standard curve derived from the linear regression of Ct and the log value of DNA. The standard regression lines from each plate, selected as reference curves, were used to convert the experimental Ct values to DNA amounts, i.e., $\mathrm{DNA} = 10^{[(\mathrm{Ct}-\mathrm{intercept})/\mathrm{slope}]}$. The mean number of oospores/g soil and the mean amount of *P. viticola* DNA in soil (picograms (pg)), leaf residues (pg), and asymptomatic leaves (pg) were calculated in triplicate reactions.

Second, the molecular disease index (MDI), as a relative value, reflects the level of latent pathogen infection in host tissues. Briefly, MDI(amount of *P. viticola* DNA (pg)/amount of *V. vinifera* DNA (ng) was calculated using the equation of the standard curve for the corresponding pathogen based on the Ct value from its reaction with the corresponding primers (Yan et al. 2011; Pan et al. 2016; Luo et al. 2019). A higher MDI indicates a high level of latent infection. The area under the disease progression curve (AUDPC) was used to describe the cumulative amount of *P. viticola* DNA and the cumulative MDI of asymptomatic leaves during the sampling, which reflected the overall latent infection of *P. viticola* in asymptomatic leaves. In addition, the AUDPC of the disease index (DI) values versus the corresponding assessment dates can be used as a standard measure to evaluate and compare the disease progression between vineyards and varieties. The AUDPC was calculated as follows (Yan et al. 2012):

$$\mathrm{AUDPC} = \sum_{i}^{n} \left[\left(\frac{X_i + X_{i+1}}{2} \right) (t_{i+1} + t_i) \right]$$

where X_i and X_{i+1} are the DNAs/MDIs/DIs at the ith and (i + 1)th disease assessments, respectively; t_i and t_{i+1} are days of the disease progression curve at the ith and (i + 1)th disease assessments (DNAs/MDIs/DIs), respectively; and n is the total number of disease assessments (DNAs/MDIs/DIs). The mean of the five sampling points was calculated using the "*plyr*" package implemented in R (Wickham 2011). The data for each parameter were statistically examined by analysis of variance (ANOVA) to detect any significant differences. A Kruskal–Wallis nonparametric test was used to compare whether any differences between vineyards were significant across two

years (Kruskal and Wallis 1952). All calculations were performed by using the '*pgirmess*' package in R.

Finally, before correlation analysis, all the data were transformed using the decimal logarithm function. Spearman's correlation analysis was performed using the '*corrplo*' package in R (Farawa 2014) and used to assess the relationship between the amount of *P. viticola* DNA in the soil, leaf residue, and asymptomatic leaf samples, as well as the MDI of leaf residue, AUDPC-MDI of asymptomatic leaf samples, and AUDPC-DI. The correlation coefficient (ρ) and *P* values were recorded to analyze their significance, and all tests were two-tailed, with $P < 0.05$, $P < 0.01$, and $P < 0.001$ as the level of statistical significance.

Abbreviations

GDM	Grapevine downy mildew
Ningxia	Ningxia Hui Autonomous Region
P. viticola	*Plasmopara viticola*
V. vinifera	*Vitis vinifera* L.
Real-time PCR	Real-time quantitative polymerase chain reaction
MDI	Molecular disease index
DI	Disease index
AUDPC	Area under the disease progress curve
AUDPC-MDI	Area under the disease progress curve in terms of the molecular disease index
AUDPC-DI	Area under the disease progress curve in terms of the disease index
ANOVA	Analysis of variance
E	Efficiency
pg	Picograms
ng	Nanograms
ρ	Spearman correlation coefficient

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Authors' contributions
LY, BC, and ZM designed the research. LY performed the research. CJ provided sampling help. BC, SQ, and KY provided methold for the research. LY, BC, and JD analyzed the data. LY and ZM wrote the manuscript. All authors read and approved the final manuscript.

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