Short Communication

Hyperparasitism on *Plasmopara viticola* by *Simplicillium lanosoniveum*

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Abstract

A previously unknown association between *Plasmopara viticola*, the causal pathogen of grape downy mildew, and the mycoparasite strain F2 is described in this paper. F2 could be consistently isolated from the mildew of diseased spots on grape leaves of Kyoho (*Vitis labrusca* × *V. vinifera* cv. Kyoho) infected with *P. viticola* but not from the surfaces of healthy grape leaves or within lesions of other diseases. It was observed that strain F2 was capable of coiling around sporangiophores and sporangia of *P. viticola* under a microscope, causing hyphae deformation, decreasing the pathogen growth, and reducing the number of sporangia. This fungal strain was identified as *Simplicillium lanosoniveum* on the basis of morphological characterizations and 28S rDNA sequencing. To our knowledge, this is the first report of *S. lanosoniveum* with hyperparasitic characteristics on *P. viticola*, and the hyperparasite has potential application in the control of grape downy mildew.

Keywords: grape downy mildew, hyperparasite, *Simplicillium lanosoniveum*

Grapevine downy mildew, caused by the obligate biotrophic oomycete *Plasmopara viticola*, is one of the most destructive diseases affecting viticulture (Fontaine et al. 2021). The pathogen has a complex life cycle, which includes sexual and asexual reproduction and polycyclic behaviors (Burruano 2000). Its oospores overwinter in soil and plant tissues, and produce a single macrosporangium, where the asexual spores (zoospores) are formed and severed as initial infection in spring under favorable weather conditions. As a polycyclic pathogen, *P. viticola* is able to undergo numerous infection cycles during a single grapevine growing season. Most of the cultivated *Vitis vinifera* varieties are highly susceptible to *P. viticola* (Toffolatti et al. 2016), and the pathogen can infect all green parts of the grapevine (Toffolatti et al. 2012), leaves and bunches in particular, causing early defoliation and necrosis of flowers and berries. Consequently, downy mildew can cause serious damage in vineyards, especially in humid climates (Pugliese et al. 2011).

Although agronomic practices represent a useful tool for disease management and the development of resistant varieties has made great progress (Bove et al. 2019; Özer et al. 2021), the chemical control of the pathogen is the most important measure to ensure an adequate yield (Du et al. 2013). However, frequent fungicide applications cause health and environmental problems and encourage the emergence of fungicide-resistant *P. viticola* populations. Until now, *P. viticola* has developed resistance to most of the fungicide classes currently used and constitutes one of the most important threats for grapevine production (Ghule et al. 2020; Giraud et al. 2013; Romanazzi et al. 2016;

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Santos et al. 2020). Therefore, it is necessary to search for alternatives to chemical treatments.

In this paper, a previously unknown association between *P. viticola* and the mycoparasite *Simplicillium lanosoneum* in China is described. While subculturing *P. viticola* four times on detached grapevine leaves in a moist chamber, abnormal spots of downy mildew with sparsely growing hyphae of *P. viticola* were consistently observed. Moreover, fungal hyphae showing different morphology intertwined around sporangiophores were also detected under the microscope but were absent on healthy leaf surfaces, and this fungal strain seemed to exhibit biotrophic and parasitic responses to *P. viticola*. The objective of the present study was to identify this fungal strain and to determine whether this fungus had hyperparasitic effects on *P. viticola*.

MATERIALS AND METHODS

Plant material and the pathogen

Leaves of 'Kyoho' grape (*Vitis labrusca* × *V. vinifera* cv. Kyoho) were collected from the vineyard of Hebei Agricultural University. *Plasmopara viticola* was collected from infected leaves of the same grape variety.

Discovery of the biocontrol agent

Field-grown grape leaves with typical symptoms of downy mildew were collected from the vineyard of Hebei Agricultural University, China, for propagation of *P. viticola*. After subculturing the oomycete four times on detached grapevine leaves in a moist chamber, abnormal spots of downy mildew with sparsely growing hyphae of *P. viticola* were consistently observed. A filamentous fungus was isolated from these atypical downy mildew lesions and purified fungal strains were obtained by the single spore purification technique. To determine whether the true fungus within *P. viticola* lesions differed from other species in proximity to *P. viticola*, further samples were taken from the edges of *P. viticola* lesions, symptomless grape leaf surfaces, lesions of brown spot, and senescing grape leaves.

Verification of colonization

To verify colonization of *P. viticola*, the filamentous fungus was used in co-inoculation bioassays performed in vivo on foliage of the grapevine variety Kyoho.

P. viticola was isolated from leaves of the Kyoho grape. Suspensions of *P. viticola* sporangia were prepared for inoculations by collection from sporulating lesions on leaves and washing sporangia with distilled water prior to adjusting to 10⁶ sporangia/ml following repeated hemacytometer counts. A conidial suspension was prepared by scraping the mycelium of the filamentous fungus from 2-week-old cultures grown on potato dextrose agar (PDA) at 25°C and vortex mixing the mycelia and conidia in distilled water. The suspension was filtered through a double layer of cheesecloth and adjusted to 10⁶ spores/ml following repeated counts using a hemacytometer. Microscopic examination suggested that suspensions consisted almost entirely of microconidia.

Fresh grape leaves were washed in sterile water, with the petiole wrapped in moist cotton wool, and placed abaxial side upward in a Petri dish with moist gauze to maintain high humidity. The abaxial sides of leaves were inoculated with a sporangia suspension of *P. viticola*. After 24 h, the leaves were sprayed with a conidial suspension of the filamentous fungus, and the leaves sprayed with distilled water were used as a control. Each treatment was replicated 5 times. Petri dishes with leaves were placed

in a growth chamber (12-h photoperiod with temperature of 20°C during the day and 18°C at night, 90% relative humidity) for 5 to 10 days until downy mildew symptoms were fully developed on the control leaves.

The in vitro interaction between the fungus and *P. viticola* was examined with a dissecting microscope, light microscope, and scanning electron microscope (SEM) (KYKY-2800B, KYKY Technology Co., China). For the SEM, samples were fixed by submersion in formalin acetic acid overnight at 4°C and dehydrated through an ethanol series (20, 40, 60, 75, 95, and 100% at 20 min per step) followed by absolute ethanol overnight. After dehydration, the material was critical-point dried (ethanol/CO₂), mounted on specimen stubs, and sputter-coated with gold palladium. Samples were examined with a KYKY-2800B SEM operated at 30 kV.

Morphological characterization

The isolated fungus was grown on PDA for 7 days at 25°C in darkness and its morphological characteristics on the PDA plates were examined. After 7 days, measurements were recorded from two crossed radii in each culture and averaged. Length and width of spores were measured under a compound microscope (Zeiss Axio imager), and the mean size of conidia was determined. Each experiment was repeated twice, with three replicates.

Molecular identification

Genomic DNA was extracted from the mycelium of 14-day cultures of the filamentous fungus using the hexadecyltrimethylammonium bromide method (Graham et al. 1994). Amplification of DNA by PCR was carried out using the fungal universal primer pairs NL₁ and NL₄ (Kurtzman and Robnett 1998), and PCR products were sequenced by Beijing Sunbiotech Co. The sequences obtained in this study were deposited in GenBank. Similarity checks were done on the NCBI website. The closely related sequences in NCBI were analyzed by BLASTn (<http://www.ncbi.nlm.nih.gov>) and sorted by maximum identity (Arnold et al. 2007). Alignment was done using MEGA 5.0 (Tamura et al. 2011).

RESULTS

Microscopy of the interaction between *P. viticola* and F2

The isolated and purified filamentous fungus was recorded as F2. Seven days after inoculation, lesions were examined visually until the number of lesions per leaf did not increase, on which the filamentous fungus F2 was growing and *P. viticola* was no longer sporulating, indicating that the growth of grape downy mildew had been restrained. Compared with fungi found elsewhere on the leaf surfaces, the mycelium of the filamentous fungus presented only on the mildew of *P. viticola*.

The fungal strain F2 showed extensive growth over the surface of *P. viticola* colonies 7 days after inoculation (Fig. 1A) but was not found on the control foliage (Fig. 1B). Closer examination with light and scanning electron microscopy showed growth of filamentous mycelium over sporangia and sporangiophores of *P. viticola* (Fig. 1C). The filamentous hyphae coiled around sporangia of *P. viticola* (Fig. 1D).

Morphological characterization

After 7 days of growth on PDA, the fungus F2 formed vigorous fluffy and white colonies with faint yellow pigmentation on the

reverse side of the culture, with a maximum colony diameter of 3.7 ± 0.1 cm. Morphological examination revealed long, narrow, single phialides arising from aerial hyphae and oval or ellipsoid conidia adhered in globose heads. Conidia were hyaline and oval with an average size of 2.5 ± 0.4 long and 1.8 ± 0.7 μm wide, appearing in globose heads on solitary phialide-like conidiophores (Fig. 2).

Identification of hyperparasite

The sequences at the 28S rDNA region were successfully amplified from fungus F2, and they were 556 bp in length (GenBank accession no. KX395184). The BLAST analysis showed that the DNA sequence of fungus F2 showed 99% identity to *Simplicillium lanosoniveum* (GenBank accession no. AF339554) (Fig. 3). By combining the morphological characteristics and molecular methods used to identify the hyperparasite, the filamentous fungus F2 isolated from sporangiophores of *P. viticola* was identified as *Simplicillium lanosoniveum*.

DISCUSSION

Although *S. lanosoniveum* was reported to be a causal agent of brown spot on *Salvinia auriculata* and *S. molesta* in Taiwan (Chen et al. 2008), the isolated *S. lanosoniveum* obtained in this study did not cause lesions or necrosis on grapevine. *S. lanosoniveum* was readily recovered from leaves infected with *P. viticola*, but the fungus was not found in association with lesions of other diseases of grapevine or on healthy leaf surfaces. The SEM observations revealed that the fungus was visible only on leaf surfaces when sporangiophores and sporangia of *P. viticola* were present.

FIGURE 1
Interactions between the fungus F2 and *Plasmopara viticola*. **A**, Downy mildew of *P. viticola* sprayed with F2 suspension (10^6 spores/ml). **B**, Downy mildew of *P. viticola* sprayed with distilled water. **C**, Hyphae of the fungus F2 coiled around a sporangiophore of *P. viticola* viewed by microscopy. **D**, Hyphae of the fungus F2 coiled around a sporangium of *P. viticola* viewed by scanning electron microscope (SEM) (KYKY-2800B, KYKY Technology Co., China).

Otherwise, it did not establish or colonize leaf tissue, and no lesions developed.

Some species of the genus *Simplicillium* have been reported to be mycoparasites and entomopathogens. The fungus *S. lanosoniveum* isolated from sori of soybean rust (*Phakopsora pachyrhizi*) on soybean was found as a mycoparasite on the soybean-rust pathogen *Phakopsora pachyrhizi* (Gauthier et al. 2014; Ward et al. 2011, 2012). *S. lanosoniveum* strains isolated in India show hyperparasitism on *Aecidium elaeagni-latifoliae*, which causes rust on *Elaeagnus latifolia* (Baiswar et al. 2014). *S. lanosoniveum* strains isolated from fruiting bodies generated on a pine cone in Korea have entomopathogenicity and can be used in biological control of insects (Lim et al. 2014). The

FIGURE 2
The fungus F2. **A**, Colony on potato dextrose agar (PDA) for 7 days. **B**, Reverse colony on PDA for 7 days. **C**, Solitary phialides bearing a single conidium per phialide of fungus F2 viewed by microscopy. C = conidia; and H = hypha.

FIGURE 3
The phylogenetic tree based on 28S rDNA gene sequences of fungus F2 and related taxa. Note: The scale bar corresponds to 0.01 substitution per nucleotide position. Numbers above branches indicate bootstrap values over 50%. The GenBank accession numbers for nucleotide sequence data are shown in the brackets. Alignment was done using MEGA 5.0.

entomopathogenic fungus *S. lanosoniveum* strain TYL001 isolated from the naturally diseased *Pseudaulacaspis pentagona* has high infectivity against *P. pentagona* (Wang et al. 2016). The fungus *S. lanosoniveum* DT06 isolated from the single-celled blue-green alga *Chroococcus* sp. can enhance cell growth and lipid synthesis of *Chlamydomonas reinhardtii* during co-culturing (Dong et al. 2018).

In this study, based on the morphological characteristics and molecular methods, the filamentous fungus F2 isolated from sporangiophores of *P. viticola* was identified as *Simplicillium lanosoniveum*. In our studies, *S. lanosoniveum* did not grow or establish on grape leaf surfaces until *P. viticola* erupted on co-inoculated leaves. When *S. lanosoniveum* was inoculated onto *P. viticola* lesions, structures were produced on the pathogen surface resembling penetration sites. We did not determine whether conidia of *S. lanosoniveum* germinated immediately upon inoculation onto leaf surfaces or after sporangiophores and sporangia of *P. viticola* were produced; however, *S. lanosoniveum* survived for 5 days following inoculation on leaf surfaces until sporangiophores and sporangia of *P. viticola* erupted. Based on these observations and experimental results, we concluded that infection by *P. viticola* is necessary for establishment of *S. lanosoniveum* on grape leaf surfaces. These observations suggest that the fungus *S. lanosoniveum* F2 is a mycoparasite of *P. viticola*.

To our knowledge, this is the first report of mycoparasitism of *P. viticola*, the pathogen causing grape downy mildew, by *S. lanosoniveum* in China. *S. lanosoniveum* has potential for use in managing this disease as a sustainable and ecofriendly alternative to fungicides.

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