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Pseudothecium development and ascospore discharge in *Venturia asperata* and *V. inaequalis*: relation to environmental triggers

Daniele PRODORUTTI[1,2,*], Valeria GUALANDRI[1,2], Vincent PHILION[3], Arne STENSVAND[4], Emanuela COLLER[1], Ilaria PERTOT[1,2]

[1] *Fondazione Edmund Mach, via Mach 1, 38098 San Michele all'Adige, TN, Italy*
[2] *Center Agriculture Food Environment, University of Trento, via Mach 1, 38098 San Michele all'Adige, TN, Italy*
[3] *Institut de Recherche et de Développement en Agroenvironnement, IRDA, Saint-Bruno-de-Montarville, Québec, Canada J3V 0G7*
[4] *Norwegian Institute of Bioeconomy Research (NIBIO), P.O. Box 115, NO-1431 Ås, Norway*
*Corresponding author. E-mail: daniele.prodorutti@fmach.it

Summary. *Venturia asperata* (*Ascomycetes*) was first described in 1975, as a saprotroph on overwintered apple leaf litter, and then, in 2007, as the cause of atypical apple scab symptoms on scab-resistant apple cultivars in southern France, and later in northern Italy and China. Information on *V. asperata* is limited. This study expanded knowledge by comparing development of pseudothecia and ascospore discharge in *V. asperata* and *V. inaequalis*. Leaf litters with pseudothecia of *V. asperata* or *V. inaequalis* were prepared, and a spore trap was placed above each litter. Over the 2-year study, pseudothecia of the two pathogens developed differently: *V. asperata* had delayed pseudothecium maturation and emptying in relation to degree day accumulation, compared to *V. inaequalis*. The ascospore release for *V. asperata* was also delayed, commencing and ending later than *V. inaequalis*. The delayed spore ejection and pseudothecium development of *V. asperata* compared to *V. inaequalis* may partly explain the late onset of symptoms in orchards during each growing season. These results have implications for plant protection strategies on scab-resistant apple cultivars, in particular under warm climates that occur in the Mediterranean region.

Keywords. Apple scab, disease management, epidemiology, light, resistant cultivars.

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ORCID:
DP: 0009-0004-1844-7596
VG: 0000-0002-3155-6066
VP: 0000-0002-9401-7916
AS: 0000-0002-8630-2434
EC: 0000-0003-1382-0318
IP: 0000-0002-8802-7448

INTRODUCTION

Use of apple cultivars resistant to scab, caused by *Venturia inaequalis* (Cke.) Wint., is increasing worldwide, with clear advantage in reductions in use of fungicides with potential negative impacts on human health and the environment (Didelot *et al*., 2016). However, reduced fungicide applications could create favourable conditions for other diseases, including fruit rots, sooty blotch, or Marssonina blotch, which all thrive in the absence of regular fungicide applications (Ellis *et al*., 1998; Boutry *et al*., 2023).

The main source of resistance to scab in apple cultivars for more than 50 years has been the *Rvi6* (=*Vf*) gene (Caffier *et al*., 2012, Gessler and Pertot, 2012; Didelot *et al*., 2016). An important drawback of monogenic host resistance is that it may be overcome, and virulent, resistance breaking strains of *V. inaequalis* have become dominant in several countries in Europe (Parisi *et al*., 1993, 2004; Gessler and Pertot, 2012; Patocchi *et al*., 2020). In 2007, atypical apple scab symptoms were observed for the first time in southern France, on fruit of cultivars carrying the resistance gene *Rvi6*. Initially, these symptoms were reported on the cultivar Ariane/Les Naturianes®, and in the following years on the cultivars Co-op 38/GoldRush® and Prima. Morphological and molecular analyses and pathogenicity tests showed that the causal agent of the scab-like symptoms in France was not *V. inaequalis* but was *Venturia asperata* Samuels & Sivan. (Caffier *et al*., 2012).

Venturia asperata (*Ascomycetes*) was first described in New Zealand by Samuels and Sivanesan (1975), and was later reported in Canada by Cortlett (1985). In both locations, *V. asperata* was reported as a saprotroph, growing on overwintered apple leaves on the ground, with no symptoms observed on fruit and leaves in growing seasons. In 2012, atypical apple scab symptoms were recorded in Italy (Cesena, Emilia-Romagna Region), on the fruit of cultivar CIVG198/Modì®, a resistant cultivar carrying the *Rvi6* gene (Turan *et al*., 2019). Symptoms were more severe at fruit harvest, and became established in the following years. Morphological and molecular analyses of isolates from conidia and ascospores confirmed that *V. asperata* was the causal agent of these atypical scab symptoms (Turan *et al*., 2019). In 2018, *V. asperata* was reported on the *Rvi6* gene resistant cultivar in a commercial orchard in the province of Trento, Italy (Gualandri *et al*., 2021; Prodorutti *et al.,* 2023). *Venturia asperata* was also reported in the nearby province South-Tyrol in 2019 (Öttl, 2021), and in other regions of northern Italy (Piemonte, Veneto, Friuli Venezia Giulia; Erschbamer, 2024). In 2018, *V. asperata* was reported from the Heilongjiang Province in China (Zhou *et al*., 2021).

Venturia spp. are hemibiotrophic fungal pathogens. They overwinter as saprophytes in host plant leaf litter on orchard soil, where pseudothecia mature in late winter and spring, producing ascospores that cause primary infections. Subcuticular mycelia develop in fruit and leaves, producing conidiophores and conidia that emerge through the cuticles. During this parasitic stage, *Venturia* spp. can only infect particular hosts, and *V. inaequalis* and *V. asperata* are the only *Venturia* spp. known to cause scab symptoms on apple (Caffier *et al*., 2012; Turan *et al*., 2019). Conidia and ascospores of *V. inaequalis* and *V. asperata* differ in shape and size and can be distinguished morphologically (Samuels and Sivanesan, 1975, Caffier *et al*., 2012; Turan *et al*., 2019).

Little information is available on the epidemiology of *V. asperata*. Caffier *et al*. (2012) carried out a single season trial comparing release of ascospores from *V. asperata* and *V. inaequalis*. The first ascospores of both fungi *V. inaequalis* and *V. asperata* were detected in March, but those of *V. inaequalis* were detected at the beginning of that month, and those of *V. asperata* at the end. However, both species had coincident peak release and depletion of ascospores. Pathogenicity tests in France and Italy revealed difficulties in reproducing symptoms on apple leaves and fruit following artificial inoculations of conidia of *V. asperata* (Caffier *et al*., 2012; Turan *et al*., 2019). The symptoms were weak and appeared later than those caused by *V. inaequalis*, and few or no conidia were recovered from lesions developed on fruit and leaves. This suggests that climatic conditions for infection and sporulation of *V. asperata* differ from those for *V. inaequalis* (Caffier *et al.,* 2012), or that *V. asperata* may be a weaker parasite than *V. inaequalis*.

Daylight has been shown to affect ascospore discharge of *V. inaequalis* (Brook, 1969, 1975; MacHardy and Gadoury, 1986; Gadoury *et al*., 1998; Rossi *et al.,* 2001; Stensvand *et al*., 2009), but no information is available for light effects on *V. asperata*. If rain started during night-time, and there was continuous leaf wetness for the next 24 hours, most ascospores of *V. inaequalis* (98%) were discharged between 7:00 and 18:00 (MacHardy and Gadoury, 1986). The peak of ascospore numbers was reached between 11:00 and noon, with a noticeable increase in ascospore discharge beginning at approx. 7:00, i.e., 2-3 hours after sunrise (MacHardy and Gadoury, 1986). Similar results were reported by Rossi *et al.* (2001) in northern Italy, where 93% of the ascospores of *V. inaequalis* were ejected during daylight, with most became airborne within the first two hours after sunrise.

Venturia asperata should be considered an emerging pathogen of apples, spreading in key apple-growing regions of Europe, with symptoms appearing on cultivars carrying the *Rvi6* gene. For this reason, there is a need for epidemiology on this pathogen, particularly to determine whether the conditions and timing for infections align with, or differ from, those of *V. inaequalis*. Increasing knowledge on the epidemiology of *V. asperata* also has practical implications for management of apple scab, because this may influence the timing of fungicide treatments for disease control. Future breeding programmes and selection of scab-resistant cultivars should also consider host susceptibility to *V. asperata*, alongside *V. inaequalis.*

The present study aimed to fill knowledge on the epidemiology of *V. asperata*, by comparing the development of pseudothecia and ascospore discharge with those of *V. inaequalis*. The objectives were to characterize: i) development of pseudothecia, and ii) ascospore discharge in relation to rainfall and degree-day accumulation; and iii) assess effects of daylight on ascospore discharge.

MATERIALS AND METHODS

Apple fruit and leaves with suspected symptoms of *V. asperata* infections were collected on 3 September 2018 from an organic commercial orchard of cultivar Modì, located in Romagnano municipality (Trento Province, Italy; coordinates 45.995053°N, 11.118136°E). Symptoms and fungal propagules on the host samples were described, and compared with those caused by *V. inaequalis,* on fruit and leaves collected from an organic commercial orchard of 'Golden Delicious' in the same general location (46.010059°N, 11.112870°E). The samples from fruit and leaves were then processed for molecular and morphological identification of the putative causal agent. To obtain monosporic isolates, fruit skin was cut from the margins of brownish suberose patches on fruit, or small pieces were cut from dusty patches on leaves, for these sample sources resembling symptoms and signs of *V. asperata*, and a drop of water was put on each host lesion. The drop was then transferred to, and plated on, potato dextrose agar (PDA; Oxoid) containing chloramphenicol (100 mg mL^{-1}; Sigma), and then incubated at 18°C. After 24 h, individually germinated conidia were picked up under stereomicroscope observation, and transferred to PDA + chloramphenicol, and incubated at 18°C.

After development of isolates, two monosporic cultures obtained from fruit and two from leaves were used in molecular analyses. Total genomic DNA was extracted from mycelia using Nucleospin Plant II (Macherey-Nagel), and the ITS region was amplified using the primer Vasp (5'-GTCTGAGAAACAAGTAATAG-3'), specific for *V. asperata* (Stehmann *et al*., 2001), in combination with ITS4 (White *et al*., 1990). After sequencing of the PCR products of the two isolates from fruit, a BLAST search was carried out in the NCBI database.

To confirm presence of *V. asperata* in the field, fruit and leaves from the same 'Modì' orchard were sampled in 2019 and 2020, from August to October each year, and assessed for molecular identification of this fungus.

To assess evolution of disease incidence, the 'Modì' apple orchard was monitored at the end of each growing season in 2019, 2020 and 2021. The proportions of diseased fruit and shoots were calculated by randomly checking symptoms on 500 fruit immediately before harvest, and on 50 shoots before start of leaf fall. Just before leaf fall, on 31 October 2019 and 5 November 2020, apple leaves with symptoms of *V. asperata* were collected in the 'Modì' orchard, from trees where no fungicides had been applied during the growing seasons. At the same time, apple leaves with symptoms of *V. inaequalis* were collected from untreated trees in the 'Golden Delicious' orchard.

Leaves from the two cultivars were immediately placed on the ground in two separated plots (each plot containing leaves of one cultivar), in San Michele all'Adige (Trento Province, Italy; 46.189922°N, 11.135227°E). A sample of the collected leaves from each sampled orchard ('Modì' or 'Golden Delicious') was observed under a light microscope to confirm presence of infections of, respectively, *V. asperata* or *V. inaequalis*, by assessing morphological characteristics of conidia and conidiophores (Figure 2). At an experimental site in San Michele all'Adige, the collected leaves were placed in two plots in a grass meadow. The plots each measured 2 × 2 m, and were 100 m apart from each other and at least 100 m away from any apple trees, to avoid cross-contamination (MacHardy, 1996). Additionally, a plot (2 × 1 m) of leaf litter was placed adjacent to each of the 2 × 2 m plots, and was used for monitoring of pseudothecia. To confirm that *V. asperata* pseudothecia developed in the leaf litter of 'Modì', in 2020 maturing pseudothecia were collected from leaves overwintered in the leaf litter, and were directly subjected to PCR with specific primers for *V. asperata*, using the methods described above.

The plots were prepared as described in Prodorutti *et al*. (2024). Grass was removed by light soil tillage, and a layer of leaves was placed on the soil above a white non-woven fabric (permeable polypropylene, Ortoclima, Tenax s.p.a.), which prevented earthworms from degrading the leaves. Each plot was then covered with a wire mesh (1 × 1 cm) to keep the leaves in place. The leaves (approx. 150 m^{-2}) were placed in a single layer, avoiding overlapping.

Development of pseudothecia of *V. asperata* and *V. inaequalis* was evaluated weekly, in 2020 and 2021, from the first week of March to mid-July, in order to cover the complete primary season for ascospore maturation and release. Each week, ten leaves were randomly selected from the leaf litter from each cultivar (Modì or Golden Delicious), and 60 pseudothecia were randomly harvested and observed under a light microscope. Pseudothecia were harvested from leaves previously soaked in water for 10 min, and were then crushed on glass microscope slides and classified in three groups ("immature",

"mature", or "empty") according to their stages of development. The immature pseudothecia included stages from pseudothecium initials to pseudothecia with most asci containing non-pigmented (immature) ascospores. For the "mature" group, most asci contained septate and pigmented (mature) ascospores. Pseudothecia were classified as empty if most of the asci were empty or aborted (Prodorutti *et al*., 2024). The percentages of immature, mature, or empty pesudothecia (out of 60) were calculated for each assessment.

On 27 February 2020 and 24 February 2021, a volumetric spore trap (Myco-trap, Paul Illi Mech.) was placed above each leaf litter, in the middle of each plot. During the entire primary season for ascospore maturation and release, ascospores of the two *Venturia* spp. (Figure 2) captured by the spore traps were counted on each rainy day (≥ 0.2 mm rain d^{-1}). A microscope tape was mounted on a 7-d rotating clock cylinder in each spore trap. The microscope tapes were cut in pieces representing single days, placed on glass slides, and ascospores were counted using a light microscope (200 × magnification). Ascospore counting on the tapes was carried out by assessing along three parallel horizontal lines of each glass slide (Mandrioli, 2000). The daily total number of counted ascospores (sum of the three horizontal lines per slide) for each spore trap was calculated, and was used to compute total seasonal ascospore ejection and percentage of the total numbers of ascospores trapped during each season.

To study the effect of daylight on the ascospore release of *V. asperata*, hourly counting of spores was carried out in the days when rain started before sunrise (https://www.timeanddate.com/sun/) and continued after sunrise. In these days, ascospore counting on each tape (at 400 × magnification) was carried out by assessing 24 vertical lines of the slide, corresponding to each hour of the day.

Weather data were recorded by a weather station (model TMF 500, Nesa s.r.l.), located at the San Michele all'Adige experiment site. Tree budbreak of the two cultivars (Modì or Golden Delicious) was assessed in the 2020 and 2021. Because budbreak of 'Modì' (27 February, 2020; 24 February, 2021) was earlier than for 'Golden Delicious' (3 March, 2020; 1 March, 2021), summation of degree days (DDs, base 0°C) from budbreak of cultivar 'Modì' to the last day of ascospore ejection and to the emptying of pseudothecia was used to compare data of the two *Venturia* species. Hours and related hourly data reported here refer to solar hour (Central European Time, CET). A leaf wetness (LW, min h^{-1}) sensor (Vantage Pro, Davis Instruments Corporation) was placed on the ground to assess hourly wetting of the leaf litter. Total solar irradiation (TSI, MJ m^{-2}) was recorded each hour, at 2 m above the ground.

'Tidyverse' packages (Wickham *et al*., 2019) of R language (version 4.4.1; R Core Team 2024) were used to handle data and generate plots. Cumulative proportions of empty pseudothecia of the two *Venturia* spp. were modeled as a function of scaled accumulated DDs since 'Modì' budbreak, using binomial regression. A mixed effect model (glmer, package lme4) was used, with sampling date as random effect. Different link functions were compared using the Akaike information criterion (AIC). The contribution of additional fixed effects (*Venturia* species, year, and their interactions) were assessed using likelihood ratio tests (LRTs). Scaled DDs were back transformed for interpretation.

The probability of observing ascospores on any given hour was modeled as a function of presence of light (TSI above zero) and LW at the ground level, using binomial regression. Similarly, hourly ascospore ejection intensity was modelled with count regression models using TSI. In all cases, the mixed effect model (glmer, package lme4) was used with sampling date as random effect.

RESULTS

Symptoms and signs on apple fruit were atypical apple scab spots, being less intense compared to scab lesions caused by *V. inaequalis* (Figure 1). The lesions on fruit developed first as small slightly grey spots, that slowly enlarged and then evolved into necrotic suberose spots, each surrounded with a light and smooth ring. Spots on fruit usually had less clear edges and were lighter (brownish) in appearance compared to those caused by *V. inaequalis* (grey to black; Figure 1). Leaf lesions caused by *V. asperata* were less distinct and more irregular compared to leaf lesions caused by *V. inaequalis*. Leaf lesions caused by *V. asperata* appeared only on abaxial leaf surfaces as velvety-grey spots, in contrast to those caused by *V. inaequalis* which were present on both surfaces of infected leaves (Figure 1). Observations with a light microscope showed that conidiophores and conidia, and pseudothecia and ascospores, collected from infected fruit and leaves in Romagnano, matched well with previous descriptions of *V. asperata* (Samuels and Sivanesan, 1975; Caffier *et al.,* 2012; Turan *et al*., 2019; Shen *et al*., 2020), that these structures differed from those of *V. inaequalis* (Figure 2). Conidiophores and conidia of *V. asperata* emerging on fruit or leaf epidermides were observed using stereo and light microscopes, developing from the margins of necrotic suber-

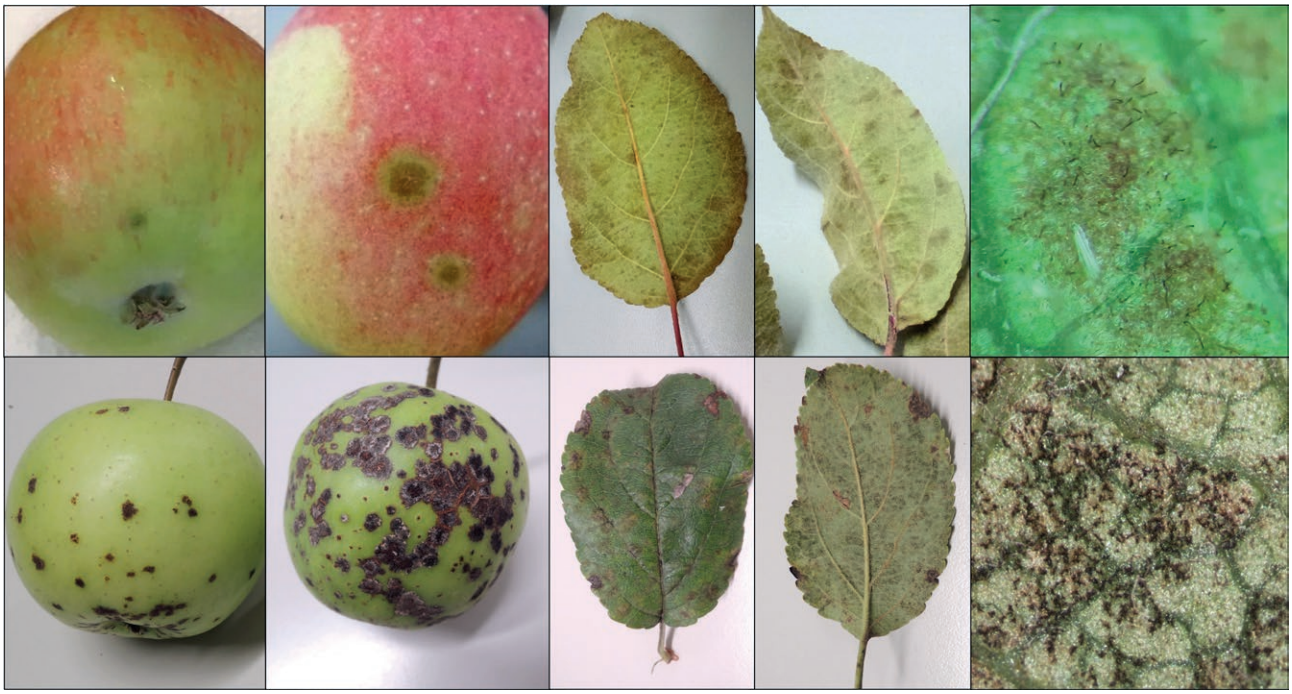

Figure 1. Fruit and leaves of apple cultivar Modì (top row) with (from left to right) sporulating lesions of *Venturia asperata* and leaf lesion detail, and of cultivar Golden Delicious (bottom row) with (left to right) sporulating lesions of *V. inaequalis* and leaf lesion detail.

ose spots on fruit or from spots on leaves (Figure 2). PCR with species-specific primers for *V. asperata* from monospore isolates obtained from fruit and leaves each gave the expected 450 bp product, and BLAST analyses of the sequences of the two isolates from fruit were 100% identical to the published sequence of *V. asperata* (KX156341). Consensus sequences of the two isolates were submitted and deposited in GenBank nucleotide database (accession nos. MT459450 and MT459451). The PCR analysis of pseudothecia collected on overwintered leaves of 'Modì' showed DNA amplification at 450 bp, and the PCR from symptomatic fruit and leaves samples collected in 2019 and 2020 confirmed establishment of *V. asperata* in the 'Modì' orchard.

Symptoms of *V. asperata* on apple fruit and leaves appeared later in the growing seasons than those caused by *V. inaequalis*. Symptoms caused by *V. asperata* on fruit occurred from the end of July to early August, and on leaves from September to October. The first symptoms of *V. inaequalis* infections on leaves appeared in April, and on fruit in May–June.

Monitoring of symptoms of *V. asperata* in the orchard showed increases in percentage of infected fruit at harvest (early September), from 1.2 to 9.4%, from 2019 to 2021 (Table 1). On 'Modì', only one to two spots per infected fruit were typically observed, and three to four or more spots were found on approx. 20% of symptomatic fruit. At the end of the growing seasons (late October to early November), high proportions of shoots were symptomatic (i.e., at least one leaf with scab per shoot), resulting in disease incidence of 40% in 2019, and 100% in 2020 and 2021 (Table 1). In the three years of monitoring, all fruit and shoots in the untreated trees of 'Golden Delicious' showed symptoms of *V. inaequalis* (100% incidence).

Table 1. Incidence of *Venturia asperata* symptoms on apple fruit and shoots during 2019, 2020 and 2021 in an orchard of cultivar Modì located in Romagnano (Italy).

Year	Day/month	Symptomatic fruit (%)[a]	Day/month	Symptomatic shoots (%)[b]
2019	06/09	1.2	23/10	40.0
2020	04/09	5.6	06/11	100.0
2021	03/09	9.4	22/10	100.0

[a] 500 fruit assessed.
[b] 50 shoots assessed.

Pseudothecia of the two *Venturia* spp. developed differently in the 2-year trial. Pseudothecia of *V. asperata* had delayed maturation and emptying of ascospores compared to pseudothecia of *V. inaequalis* (Figure 3, C and D). The periods of detection of mature *V. inaequalis* pseudothecia were similar in the 2 years (13 March to

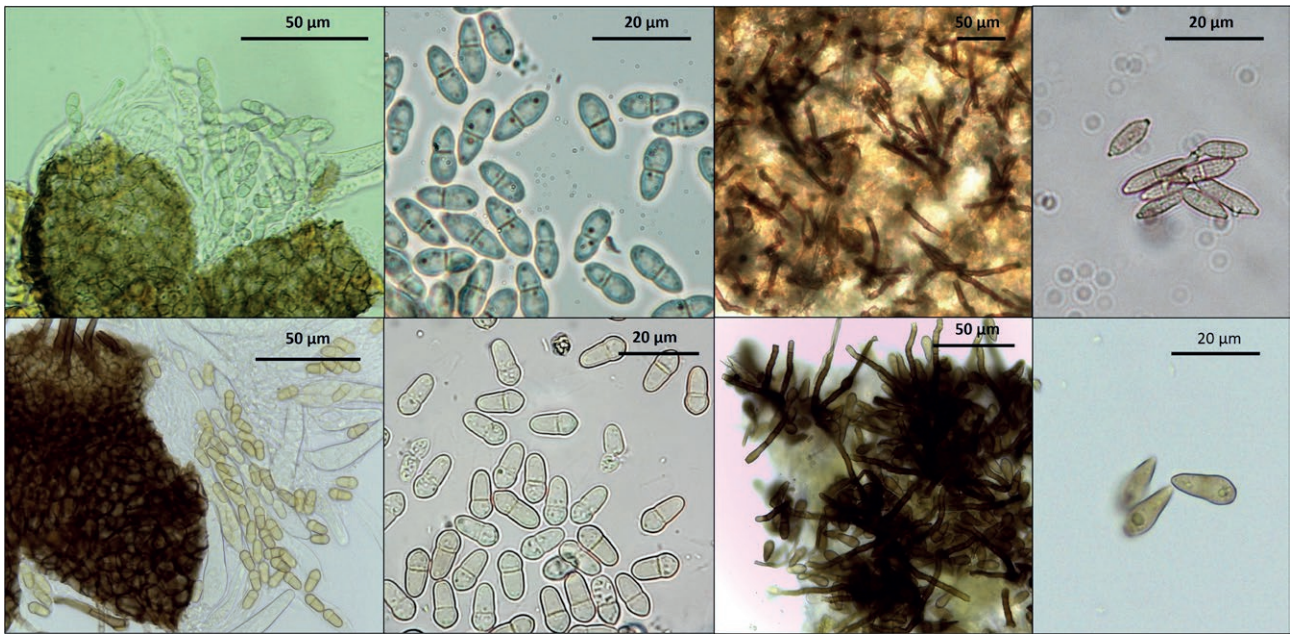

Figure 2. Pseudothecium with asci, ascospores, conidiophores, and conidia (left to right) of *Venturia asperata* (top) and *Venturia inaequalis* (bottom).

Table 2. Development of pseudothecia of *Venturia inaequalis* and *V. asperata* in San Michele all'Adige (Italy) in 2020 and 2021.

Year	Period with mature pseudothecia (day/month)		Peak of maturation (day/month)		DDs of 95% empty pseudothecia[a]	
	V. inaequalis	*V. asperata*	*V. inaequalis*	*V. asperata*	*V. inaequalis*	*V. asperata*
2020	13/03 to 29/05	08/05 to 26/06	24/04	22/05	1,181	1,744
2021	10/03 to 26/05	27/04 to 08/07	27/04	03/06	1,009	1,941

[a] Degree days (DDs, base temperature 0°C) from budbreak of cultivar Modì to 95% of the pseudothecia empty (data predicted by the probit model).

29 May in 2020; 10 March to 26 May in 2021). Mature pseudothecia of *V. asperata* were present from 8 May to 26 June in 2020, and from 27 April to 8 July in 2021 (Table 2). The peaks of proportions of mature pseudothecia of *V. inaequalis* were observed on 24 April (37% of mature pseudothecia) in 2020, and 27 April (32%) in 2021. *Venturia asperata* had greatest proportions of mature pseudothecia on 22 May (23%) in 2020, and on 3 June (15%) in 2021 (Table 2). These dates were approx. 1 month after equivalent dates for *V. inaequalis*.

The probit model gave overall better fits for pseudothecium emptying rate than logistic regression, and the probit model better represented data for *V. inaequalis* than for *V. asperata* (Figure 3, C and D). The triple interaction *Venturia* species × year × cumulative DDs was statistically significant (LRT = 9.7, df = 1, $P = 0.0019$), indicating that both the onset and rate of pseudothecium emptying were different for the two species for the two years. In both years, onset of emptying and the time of 95% empty pseudothecia predicted by the probit model were reached later for *V. asperata* than *V. inaequalis* (563 DDs later in 2020, 932 DDs later in 2021; Table 2). The difference in accumulated DDs between the two species when pseudothecia started emptying was similar in 2020 and 2021 (z = 0.8, $P = 0.85$). Rates of pseudothecium emptying were also similar for the two species in 2020 (z = 0.7, $P = 0.5$), but yearly variations in the rates of pseudothecium emptying were detected for *V. asperata* (z = 3.4, $P = 0.0008$), as the rate for *V. asperata* was slower in 2021, while a similar emptying rate was observed for *V. inaequalis* in 2020 and 2021 (z = 1.7, $P = 0.09$).

The periods of ascospore release were different for the two *Venturia* species, and were later for *V. asperata* than for *V. inaequalis* (Table 3; Figure 3, A and B). The

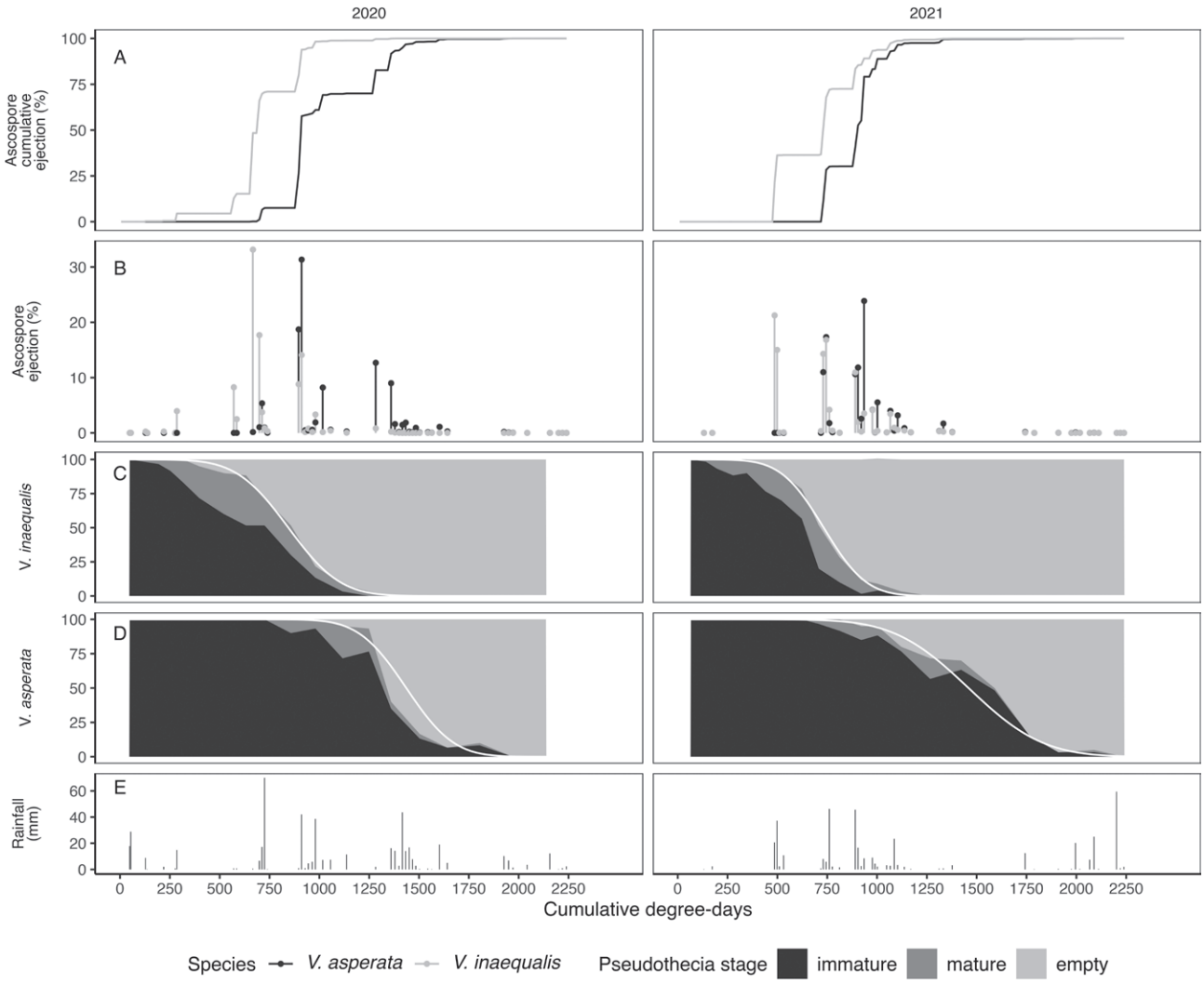

Figure 3. Degree-day accumulation (base temperature 0°C) since budbreak of apple cultivar Modì in 2020 and 2021 (San Michele all'Adige, Italy), in relation to the following. A, percentage of cumulated seasonal trapping of ascospores of *Venturia asperata* (black line) and *V. inaequalis* (grey line). B, percentages of the seasonal ascospores of *V. asperata* (black bars) and *V. inaequalis* (grey bars) released per day on days with rain (≥ 0.2 mm). C and D, percentages of immature, mature, and empty pseudothecia during the season for ascospore release of *V. inaequalis* (C) and *V. asperata* (D). Weekly assessments of 60 pseudothecia per species were evaluated in each year. Pseudothecia emptying was modelled using the probit model, represented by the white line. E, Daily rainfall (mm).

first ascospores of *V. asperata* were trapped at the end of April in both years, and continued until early July. Ascospore discharge of *V. inaequalis* commenced in mid-March and ended in early June in 2020, and commenced in early April and ended in the second half of June in 2021 (Table 3). Total numbers of ascospores released during entire seasons were greater in both years for *V. asperata* than for *V. inaequalis*. The difference was most noticeable in 2021, with *V. asperata* releasing almost three times the number of ascospores compared to *V. inaequalis* (Table 3). The peaks of ascospore ejection occurred later for *V. asperata*. In 2020, this was reached 26 April (at 667 DDs) for *V. inaequalis* and 11 May (at 911 DDs) for *V. asperata*, corresponding to 33.1% of the seasonal ascospore amount for *V. inaequalis* and 31.3% for *V. asperata* (Figure 3 B). In 2021, the peak of ascospore release for *V. inaequalis* was reached on 11 April (21% of the seasonal ascospores were trapped; 486 DDs), and that for *V. asperata* was on 15 May (24% of the seasonal ascospores were trapped, 935 DDs) (Figure 3 B). The time of 95% of trapped ascospores was reached earlier in *V. inaequalis* (435 DDs earlier in 2020 and 56 DDs earlier in 2021) than the equivalent periods for *V. asperata* (Figure 3 A; Table 3). The 95% DD accumula-

Table 3. Rainfall data, numbers of ascospores of *Venturia inaequalis* and *V. asperata* trapped, and degree days recorded, in San Michele all'Adige (Italy) in 2020 and 2021.

Year	Total rainfall (mm)[a]	Total numbers of ascospores[b]		Period of ascospore release (day/month)		DDs for 95% ascospore release[c]	
		V. inaequalis	*V. asperata*	*V. inaequalis*	*V. asperata*	*V. inaequalis*	*V. asperata*
2020	452.2	5,474	6,742	14/03 to 06/06	26/04 to 03/07	965	1,400
2021	333.2	9,823	28,350	11/04 to 23/06	28/04 to 08/07	1,048	1,104

[a] Rainfall was recorded from budbreak of cultivar Modì to the last day of ascospore ejection from *V. asperata*.
[b] Total number of ascospores collected by volumetric spore traps in the season.
[c] Degree days (DDs, base temperature 0°C) from budbreak of cultivar Modì to the time of 95% of seasonal ascospore trap count.

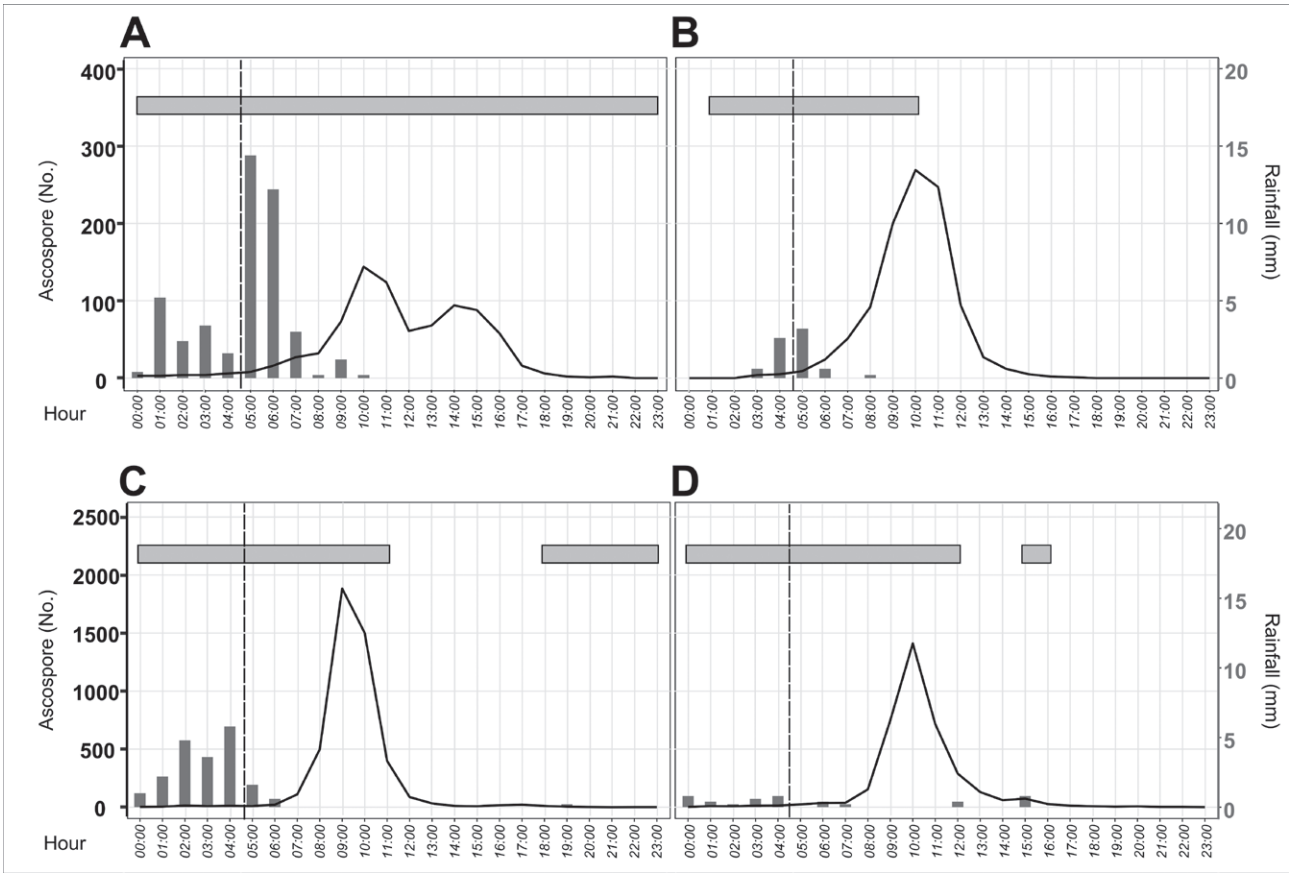

Figure 4. Hourly release of ascospores of *Venturia asperata* on four days when rainfall commenced before, and ended after sunrise. A) 15 May 2020, B) 17 May 2020, C) 12 May 2021, D) 25 May 2021. Sunrise occurred at 04:42 (Central European Time) on 15 May 2020, at 04:40 on 17 May 2020, at 04:46 on 12 May 2021 and at 04:32 on 25 May 2021. Vertical bars represent the hourly rainfall (mm), and horizontal bars indicate times when leaf wetness was recorded at ground surface. The solid line represents the hourly numbers of counted ascospores, and the vertical dashed lines indicate time of sunrise.

tions for empty pseudothecia and for ascospore ejection were similar in both years for *V. inaequalis,* and for *V. asperata* in 2020. However, in 2021 *V. asperata* reached 95% empty pseudothecia at 1,941 DDs compared to 1,104 DDs for 95% ascospore ejection (Table 2; Table 3). The last ascospores were trapped ca. 4 weeks later for *V. asperata* than *V. inaequalis* in 2020 and ca. 2 weeks later in 2021 (Table 3).

The total rainfall measured during the primary season was greater in 2020 (452 mm) than in 2021 (333 mm; Table 3). The rainfall conditions necessary to observe the potential diurnal periodicity of ascospore

release of *V. asperata* (rain started before and continued after sunrise) were fulfilled during 4 d of substantial ascospore trapping, i.e., on 15 and 17 May 2020, and 12 and 25 May 2021 (Figure 4). Sunrise occurred at 4:42 and 4:40 on 15 and 17 May 2020, and at 4:46 and 4:32 am on 12 and 25 May 2021. The patterns of ascospore release were similar in the four days, but total numbers of ascospores counted in 2020 were less than those counted in 2021. Very few ascospores were trapped from midnight to 4:00 am each day, and ascospore trapping began to increase after sunrise (from 5:00 to 7:00 am). From 7:00 to 9:00-10:00 am each day numbers of trapped ascospores rapidly increased, with exponential trends. Greatest numbers were detected at 10:00 am on 15 May and 17 May 2020, and 25 May 2021, and at 9:00 am on 12 May 2021. After these times, there were rapid declines in ascospore numbers, with values close to zero after 5:00-6:00 pm each day (Figure 4). In each of the four days, the TSI was above zero from 6:00 am to 8:00 pm. In these time intervals, the proportions of ascospores counted per 24 h ranged from 96.4% on 15 May 2020 to 98.9% on 25 May 2021.

Similar rainfall conditions were recorded in the four days when hourly counting of ascospores were carried out. Rain started during the night and continued until early-mid morning. Small rainfall amounts (0.2 – 0.8 mm h^{-1}), as occurred on 25 April 2021, induced relevant ascospore ejection. The LW measured at soil surface was recorded continuously through 24 h on 15 May 2020. In the other three events, LW was recorded for 2 to 5 h after rainfall ceased (Figure 4).

Both LW at the soil surface (LRT = 6.2, $P = 0.01$) and light (LRT = 14.4, $P < 0.001$) significantly increased probability of detecting ascospores in the spore traps. The odds ratio (OR) of observing ascospores during light hours, as opposed to dark hours, was 37 (z = 2.624, $P = 0.009$), while the OR for wet hours compared to dry hours was 16 (z = 2, $P = 0.045$). Hourly ascospore ejection intensity was over-dispersed, and was adequately modelled using a negative binomial distribution. Light had an exponential effect on the numbers of ascospores ejected, with an Incidence Rate Ratio (IRR) indicating that ejection was 38 times more intense during light hours compared to dark hours (z = 12, $P < 0.001$), and seven times more intense during wet hours compared to dry hours (z = 5.5, $P < 0.001$). Within the limited dataset, ascospore release intensity was better modelled as a function of light intensity, increasing linearly with the log of TSI (LRT = 38, df = 0). This relationship can be expressed as: Ejection intensity = $28 \times (\text{TSI} + 0.01)^{0.82}$, for TSI values ranging from 0 to 3.03 MJ m^{-2} during dry hours. For wet hours, ascospore release intensity was eight times greater than for dry hours. There was no interaction between the effect of TSI and wetness on ascospore release ($P = 0.96$).

DISCUSSION

In the present study, symptoms of *V. asperata* infections on apple fruit, and timing of their appearance, were similar to those previously described in France (Caffier *et al*., 2012), and in Italy (Turan *et al*., 2019). However, these symptoms were observed for the first time on leaves in a commercial orchard of the apple cultivar Modì. Caffier *et al*. (2012) were able to induce sporulating lesions of the pathogen on leaves of the apple cultivar Ariane, in controlled conditions and after artificial inoculations with conidia of *V. asperata*, and the symptoms they observed were weak and difficult to detect. In the present study, late appearance of leaf symptoms (September-October) was observed, with symptom intensity increasing towards leaf fall. Symptoms were confined to abaxial leaf surfaces and resembled late secondary infections of *V. inaequalis*, although lighter in appearance than for *V. inaequalis*. Because the symptoms of *V. asperata* on leaves are weak and often difficult to detect, confirmation through microscopic morphological analyses or molecular identifications are essential. The weak symptoms caused by *V. asperata*, observed both on fruit and leaves, could be due to fewer hyphae and conidia growing in each lesion, compared to *V. inaequalis* (Caffier *et al*., 2012; Turan *et al*., 2019).

Over the three years of observations, increases in incidence of symptoms and signs of *V. asperata* was observed in the monitored 'Modì' orchard, reaching almost 10% on the fruit in 2021. This increase over the 3 years may be partly due to gradual build-up of overwintering inoculum in infected leaf litter. The greater numbers of ascospores captured in 2021 compared to 2020, and the increasing incidence of disease, indicated increased overwintering of inoculum of the pathogen. High incidences of lesions caused by *V. asperata* on fruit of apple cultivars with *Rvi6* gene resistance to *V. inaequalis* were observed in southern France (up to 60%; Caffier *et al.,* 2012), and South Tyrol in Italy (up to 17.5% in 2023; Erschbamer, 2024). *Venturia asperata* has predominantly been reported in apple cultivars containing the *Rvi6* gene, mostly under organic management regimes. The limited use of fungicides, often only applied early in each growing season, probably facilitated outbreaks of *V. asperata* in the organic orchards with scab resistant cultivars. Little and unclear information is available about the presence of *V. asperata* on scab-sus-

ceptible (non-resistant to *V. inaequalis*) apple cultivars. Strict management of apple scab and greater competition of *V. inaequalis* in an orchard may lead to underestimation of the presence and spread of the less competitive *V. asperata* on these cultivars.

Pseudothecia of *V. asperata* had delayed maturation and ascospore emptying compared to *V. inaequalis*. The first mature pseudothecia were observed over a month and a half later than those of *V. inaequalis*, and the peak of maturation occurred about one month later for *V. asperata* than for *V. inaequalis*. The earlier onset of ascospore ejection for *V. inaequalis* in both years, and the slower rate of pseudothecium emptying for *V. asperata* in 2021, suggest that the two *Venturia* spp. have inherently different ejection patterns. As it was observed for the effect of irrigation on pseudothecium development (Prodorutti *et al*., 2024), probit regression gave a better fit for pseudothecium emptying rates, and better represented *V. inaequalis* than *V. asperata*, which is another indication that the two species have differences in seasonal development of their pseudothecia and ascospores.

In both years, the ascospore release for *V. asperata* was delayed compared to *V. inaequalis*, with initiation and peak of ascospore release occurring later in *V. asperata* than in *V. inaequalis*. In 2021, the first ascospores of *V. inaequalis* were detected almost a month later than in 2020, likely due to the prolonged dry period in March and early April of 2021. Extended dry periods delay pseudothecium maturation and extend ascospore release seasons of *V. inaequalis* (Stensvand *et al*., 2005), and this is also likely to be the case for *V. asperata*.

The less prominent difference in cease of ascospore release between the two fungi in 2021 than in 2020 may have been partly due to a more rapid breakdown of leaf litter of 'Modì' than 'Golden Delicious', although no records of leaf degradation were made in the present study.

Caffier *et al*. (2012) reported that the first ascospores of *V. asperata* were trapped approx. 20 d later than those of *V. inaequalis*, but that the peaks in ascospore release and end of ejection were observed at the same time for both species. The discrepancies between the present study results and those obtained in France may be due to the different methods used for leaf litter preparation. Caffier *et al*. (2012) placed leaves on ground covered by grass, so the leaves may have degraded more rapidly than in the present study experiments. Weekly development of pseudothecia of *V. asperata* had not been previously studied, and it was here shown that the periods of pseudothecium maturation and ascospore release overlapped.

Light triggered ascospore discharge of *V. asperata* in a manner similar reported for *V. inaequalis* (Brook, 1969; 1975; MacHardy and Gadoury, 1986; MacHardy, 1996; Gadoury *et al*., 1998; Rossi *et al*., 2001; Stensvand *et al*., 2009). As for *V. inaequalis*, increases in *V. asperata* ascospore discharge were observed during the first 2 to 3 h after sunrise, and even small amounts of rain (0.2 mm h^{-1}) were sufficient to induce substantial ascospore ejection. Similar to previous reports for *V. inaequalis* (MacHardy and Gadoury, 1986; Rossi *et al*., 2001), on average approx. 98% of the ascospores were released during daylight hours between 6:00 am to 8:00 pm. However, in the three days when LW was recorded until 10:00 to 12:00 am (2 to 5 h after the rain stopped), more rapid decreases in ascospore numbers were detected compared to 15 May 2020, when LW was recorded continuously for 24 h.

The OR and IRR values for ascospore ejection of *V. asperata* were both high and were similar, indicating the importance of light in triggering ascospore release. This suggests that the observed inhibition of release during dark conditions may have been proportional to numbers of ascospores primed for ejection, indicating a light-dependent regulatory mechanism of ascospore release, similar to that observed for *V. inaequalis*. The observations that ejection intensity was well-represented by the log of light intensity, and that no residual pattern was observed, indicate that the light effect rapidly saturates above a certain intensity. The substantial difference between the OR and IRR for the effect of wetness indicates that while ascospore ejection is much more likely during wet than dry periods, the total number of ejected ascospores is less influenced by wetness than by light. This implies that wet conditions are a primary trigger for ascospore release, but do not significantly increase the intensity of ejection. Again, this is similar to what has been observed for *V. inaequalis*.

CONCLUSIONS

Delayed pseudothecium development and ascospore ejection in *V. asperata* compared to *V. inaequalis*, may partly explain the late appearance of symptoms caused by *V. asperata* in apple orchards during the growing seasons. Further research is necessary to identify the optimal weather conditions for primary and secondary infections by *V. asperata* in susceptible apple tissues, and to define the latency period for this pathogen. The efficacy of fungicides commonly used for management of *V. inaequalis* should also be assessed for control of disease caused by *V. asperata*.

Future apple breeding programmes should consider resistance to emerging pathogens such as *V. asperata*,

and specific control strategies should be developed to address the delayed primary infections and late symptom onset caused by this pathogen. In Europe, *V. asperata* has been reported in northern Italy and southern France, areas with temperate climates. *Venturia asperata* probably has higher requirements of DD accumulation for maturation of pseudothecia compared to *V. inaequalis*. As climate change raises average temperatures, this could favour the spread and virulence of *V. asperata* in fruit-growing regions in Mediterranean areas and other areas across Europe and elsewhere, emphasizing that close monitoring of this pathogen in apple orchards is important.

AUTHOR CONTRIBUTIONS

The field experiments in this study were conducted and managed by DP, who oversaw the implementation and data collection. VG was responsible for conducting molecular analyses. VP carried out statistical analyses. DP, VG, VP, AS, EC and IP interpreted the experimental results, integrating field, molecular, and statistical data to draw conclusions. The manuscript was written by DP, with all the authors contributing to revision of the manuscript for intellectual content. All the authors read and approved the final manuscript of this paper.

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