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# *Venturia inaequalis* Can Initiate Sexual Reproduction Prior to Leaf-Fall: New Electron Microscopy Evidence

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## ABSTRACT

Apple scab, caused by *Venturia inaequalis*, is a major disease of cultivated apples (*Malus* × *domestica*) worldwide. Annually, there is one cycle of sexual reproduction (ascospores) in *V. inaequalis* between strains of two opposite mating types. Sexual reproduction mainly occurs on leaf litter over the winter, and mature ascospores readily infect young leaves the following spring. We do not yet know whether sexual reproduction is initiated before or after leaf-fall in autumn. The timing of sexual reproduction initiation can significantly impact the exchange of genetic information among pathogen strains. In this study, we used scanning electron microscopy (SEM) to determine the timing of sex initiation, in particular, whether sex can initiate between lesions on the same leaf prior to leaf-fall. SEM images showed that pseudothecial structures begin to develop before leaf-fall on leaves with either multiple lesions or a single lesion. Following an overwintering period under laboratory conditions, however, the pseudothecia on leaves with multiple lesions were larger and more mature than those on leaves with a single lesion, where initial pseudothecial structures were present but did not develop further. Thus, the results suggest that the initiation of sexual reproduction in *V. inaequalis* can occur prior to leaf-fall in autumn, limiting the opportunities for sexual recombination among strains from different leaves.

## 1 | Introduction

Apple scab is one of the most prevalent diseases of domesticated apple (*Malus* × *domestica*) worldwide. It has been characterised by the presence of dark scabby lesions on the surface of the fruit, causing huge yield losses of up to 70% (Belete and Boyraz 2017) because the scabbed fruit cannot be marketed (Belete and Boyraz 2017; Turechek 2004). Apple scab can also impact the growth and vigour of the tree because the infection can take hold in the leaves and woody tissues (forming wood scab) (Belete and Boyraz 2017; Rancāne et al. 2023). The causal agent of apple scab is the ascomycete fungus *Venturia inaequalis* (Bowen et al. 2011; MacHardy 1996). The fungus can reproduce both sexually and asexually, with environmental conditions often determining the development and severity of the disease (Turechek 2004). In temperate climates, the fungus usually overwinters as sexual fruiting bodies known as pseudothecia, which are a result of mating between two opposite mating types in the fallen leaf litter (Boone 1971; Keitt and Palmiter 1938; MacHardy 1996). Ascospores are then ejected from the pseudothecia as primary inoculum following a rain event the next spring and initiate primary infections. In warmer climates, the fungus is less likely to undergo the overwintering process in leaf litter as pseudothecia, but rather as asexual conidia on trees that make up the majority of primary inoculum in the spring (Boehm et al. 2003; Passey et al. 2017).

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The common management practices for scab include a combination of control measures, often in an integrated pest management (IPM) system (Passey and Xu 2019). Fungicide application is still the main management strategy and achieves satisfactory control in most growing seasons (Holb 2009; Passey and Xu 2019). However, a high frequency of fungicide applications is undesirable because of the potential pollution to water and soil (Wightwick et al. 2010). Moreover, the number of fungicides approved for use and the intensity of their use are ever decreasing. Orchard sanitation is often practised to reduce pathogen inoculum, particularly overwintering inoculum on fallen leaves. Leaf litter is often removed via leaf shredding, burning and burying, or by applying products such as urea to accelerate leaf degradation (Carisse and Dewdney 2002; Holb 2009).

Planting varieties with disease resistance is a key component of any disease management programme. Over the last two decades, apple breeding programmes worldwide have been reliant on a single major gene (*Vf*) for scab resistance. Unfortunately, resistance conferred by this gene has been broken down in many locations (Guérin and Le Cam 2004; Papp et al. 2020). Pyramiding resistance genes is proposed to overcome resistance breakdown (Bus et al. 2019). Another disease management strategy to exploit host resistance to scab is through mixed cultivar orchards, which have previously been successful in several other crops (Finckh et al. 2000; Mundt 2002). Mixing cultivars introduces genetic diversity in important agronomic traits, such as disease resistance, into the orchards, which can reduce scab development by reducing the probability of infectious inoculum reaching susceptible cultivars. Several field studies demonstrate the potential of using mixtures in reducing scab development (Didelot et al. 2007; Leroy et al. 2013; Parisi et al. 2013).

One of the main concerns about adopting mixed orchards for disease management is the emergence and subsequent rapid spread of pathogen races that could overcome resistance present in multiple mixture components, namely 'super races'. The likelihood of this happening is critically dependent on the mating system, among other factors. It is well documented in the literature that *V. inaequalis* sexually matures in fallen leaf litter and mature pseudothecia are found following a sufficient overwintering period. However, there is no experimental evidence of when the sexual mating process initiates relative to leaf-fall in autumn. If the mating can start before leaf-fall, then mating is most likely among strains with similar genetic backgrounds (with regard to scab resistance) compared with after leaf-fall. In the leaf litter, *V. inaequalis* enters a saprophytic phase whereby it lives and reproduces on the decaying organic matter. Because of this, it is generally assumed that mating can occur between strains from different leaves, provided that they are in contact for long enough periods of time to allow for initiation of sexual mating. Currently, in assessing the risk of emergence of super races, random mating among pathogen strains is often assumed. Previous studies have not observed pseudothecia prior to leaf-fall and have only imaged pseudothecia following the overwintering stage (Keitt and Palmiter 1938; Wilson 1928). Several research studies over the last two decades at East Malling, UK, have suggested that the probability of super races emerging from mixed orchards is far less than expected under the assumption of random mating. These studies have used isolate crossing, determination of spatiotemporal fungal genetic variation based on molecular markers and aggregation of single-nucleotide polymorphism (SNP) markers in sequences to infer that assortative mating is more likely than random mating (Barbara et al. 2008; Passey et al. 2016; Xu et al. 2013). One possible mechanism for assortative mating is that sexual mating could be initiated prior to leaf-fall. Of course, this mechanism does not exclude the possibility that sexual mating can also initiate after leaf-fall between strains from the same or different leaves.

The purpose of our study was to determine whether sexual mating in *V. inaequalis* can initiate among strains on the same leaf prior to leaf-fall in autumn.

Whilst previous studies have described the sexual mating phase of *V. inaequalis* with presumed data presented in diagrams (MacHardy 1996), there has not been conclusive visual proof of the formation of pseudothecia prior to leaf-fall or appropriate microscopic images of pseudothecia in vitro. The aim of this study was to use scanning electron microscopy (SEM) to determine whether *V. inaequalis* can initiate and form pseudothecia among strains on the same leaf, prior to leaf-fall in autumn. A previous study by James and Sutton (1982) extensively covered the developmental stages of pseudothecia following defoliation and overwintering. All the images were taken at a cross-section, whereas our images were taken from an aerial view with the purpose of identifying pseudothecia on the leaves prior to leaf detachment from the tree. To correctly identify mating structures on the leaf surface, in vitro images of known compatible or incompatible mating crosses were also obtained. To follow fungal development during winter in isolation, leaves with scab lesions were sampled from orchards at the time of leaf-fall and kept under low temperature (~4°C) in the laboratory. These leaves were imaged to confirm whether any immature pseudothecia observed prior to leaf-fall would mature.

## 2 | Materials and Methods

### 2.1 | In Vitro Culture (Mycelial Samples)

In vitro cultures of known compatible and incompatible strains that were kept in the same plate were included as a baseline for the experiment to allow visualisation of pseudothecial structures. By imaging both the compatible and incompatible mating crosses, we could then identify similar structures on leaves prior to leaf-fall as well as on overwintered leaves.

*Venturia inaequalis* isolates were obtained from each of two apple cultivars of differing susceptibility (*Malus*×*domestica* 'Gala' and *Malus floribunda*) in 2021 at NIAB East Malling. Mating crosses among these strains were set up on agar plates in spring 2022, following a published method (Barbara et al. 2008), and assessed for compatibility in winter 2022. A total of 130 ascospores (currently listed as 1–130) from one cross were obtained, grown in vitro and backcrossed with both parents in early spring 2023, with the mating compatibility assessed in autumn 2023. During the crossing period, a small piece of agar from the intersection between the two mating isolates was cut using a sterile scalpel. Each sample was placed in a 2 mL microcentrifuge tube and prepared for SEM in the same manner as

the leaf samples (see Section 2.4). The mating section was taken from crosses between progeny 13 or 20 and both parent isolates to ensure that both compatible and incompatible mating crosses were included for SEM analysis.

### 2.2 | Sampling Leaves Before Leaf-Fall in Autumn

For this experiment, two different timelines were adopted: (i) sampling leaves and immediate preparation for microscopy to find evidence of initiation of sexual reproduction before leaf-fall; and (ii) sampling leaves and then overwintering the sampled leaves for a few months in a laboratory to follow pseudothecial development.

For the first approach, we selected leaves with multiple discrete lesions to maximise the chance of finding leaf sections with the two opposite mating types present. We tested around 40 leaf samples to ensure we found leaves with isolates of compatible mating types. Leaves were collected from the cultivar Gala from a single orchard at NIAB East Malling, UK. Gala was chosen because it is highly susceptible to apple scab (Bus et al. 2011), and so we were more likely to find leaves containing isolates of opposing mating types.

We also included leaves sampled from a resistant apple genotype (TSR33T239) with no visual scab lesions, as well as visually clean Gala leaves. These were used as control samples to ensure that any structures found on scabbed leaves could be attributed to *V. inaequalis*, rather than to natural plant microscopic structures or other causes. We also sampled leaves with a single discrete visual lesion to confirm that pseudothecial structures would not develop without the presence of the opposite mating strain and to increase the likelihood that structures on multiple lesion leaves were pseudothecia.

Leaf discs (with no lesions, or a single or multiple scab lesions) were cut, using either a scalpel or 11 mm cork-borer, from the leaf samples immediately after sampling and were placed into 2 mL microcentrifuge tubes ready for SEM preparation.

### 2.3 | Overwintering Leaves

To investigate whether mature pseudothecial structures would develop, sections were cut from leaves using either a 11 mm cork-borer or a scalpel if the lesions were further apart. These sections had either a single discrete lesion or multiple scab lesions and were overwintered in a laboratory. The leaf sections were placed onto damp paper in clean Petri dishes and kept unsealed at 4°C for 3–4 months to simulate natural UK winter conditions. The leaves were moved to 9°C a few weeks before microscopical assessment to promote ascospore development.

### 2.4 | Sample Preparation for SEM

This protocol was amended from Alves et al. (2013). During the second fixative step, osmium tetroxide was replaced with 1% tannic acid for safety purposes. The preparation was effective for imaging the structures of *V. inaequalis* on leaves and on agar. Leaf discs were placed into 2 mL microcentrifuge tubes and covered with modified Karnovsky's fixative solution (2% paraformaldehyde; 2.5% glutaraldehyde; NaOH [1 M, 8 drops] and 0.2 M cacodylate buffer pH 7.2). The samples were refrigerated for a minimum of 24 h at 4°C. The samples were then washed three times with 0.05 M sodium cacodylate buffer for 10 min. They were post-fixed in 1% tannic acid solution and left at room temperature for 1.5–2 h. The samples were then washed three times in distilled water to remove the fixative solution. Following this, the samples were dehydrated in a series of acetone solutions (25%, 50%, 75%, 90%, 100%). They were washed in the first four solutions once and in 100% twice, each for 10 min. The samples were transferred to a freeze dryer and left overnight for maximum dehydration. Finally, the samples were mounted onto aluminium stubs with double stick carbon tape and then coated with gold in a sputter coater. These were either transferred immediately to the microscope or left in a desiccator until use.

### 2.5 | SEM

The morphology of *V. inaequalis* structures on both leaf discs and in vitro was examined using a VEGA TESCAN (www.tescan.com) (third or fourth Generation) scanning electron microscope equipped with secondary electron and backscattered electron detectors. The microscopy work was performed at Cranfield University.

## 3 | Results

### 3.1 | In Vitro Pseudothecial Structures

Images taken of mycelial samples (Figure 1) show the difference between an incompatible cross (Figure 1A–C) and a compatible cross (Figure 1D–F). Figure 1A–C show that initial pseudothecial structures may develop when two *V. inaequalis* isolates are present. However, if the mating types are the same (therefore incompatible for mating), the pseudothecia will not fully develop to produce asci and ascospores. Comparing this to the images of the compatible cross in Figure 1D–F, there is a significant difference in the size and development of the structures. The pseudothecia are much larger and more complex in the compatible cross than those in the incompatible cross. These crosses were all established at the same time (June 2023) and kept under the same conditions. These images were similar to images of sexual structures in other ascomycetes (Davey et al. 2009; Stone et al. 2008).

### 3.2 | Control Samples on Leaves

The clean control samples (Figure 2A,B) showed no evidence of *V. inaequalis* lesions. The clean control Gala leaf samples (Figure 2A) had a few hyphal/mycelial structures that might be from *V. inaequalis*; however, no conidial or pseudothecial structures were seen. The leaves taken from the resistant genotype (TSR33T239) (Figure 2B) did not have any typical scab symptoms, such as necrosis or hypersensitive response, and did not show any structures resembling *V. inaequalis* (pseudothecia or conidia). Non-leaf structures visible on these leaves appeared to

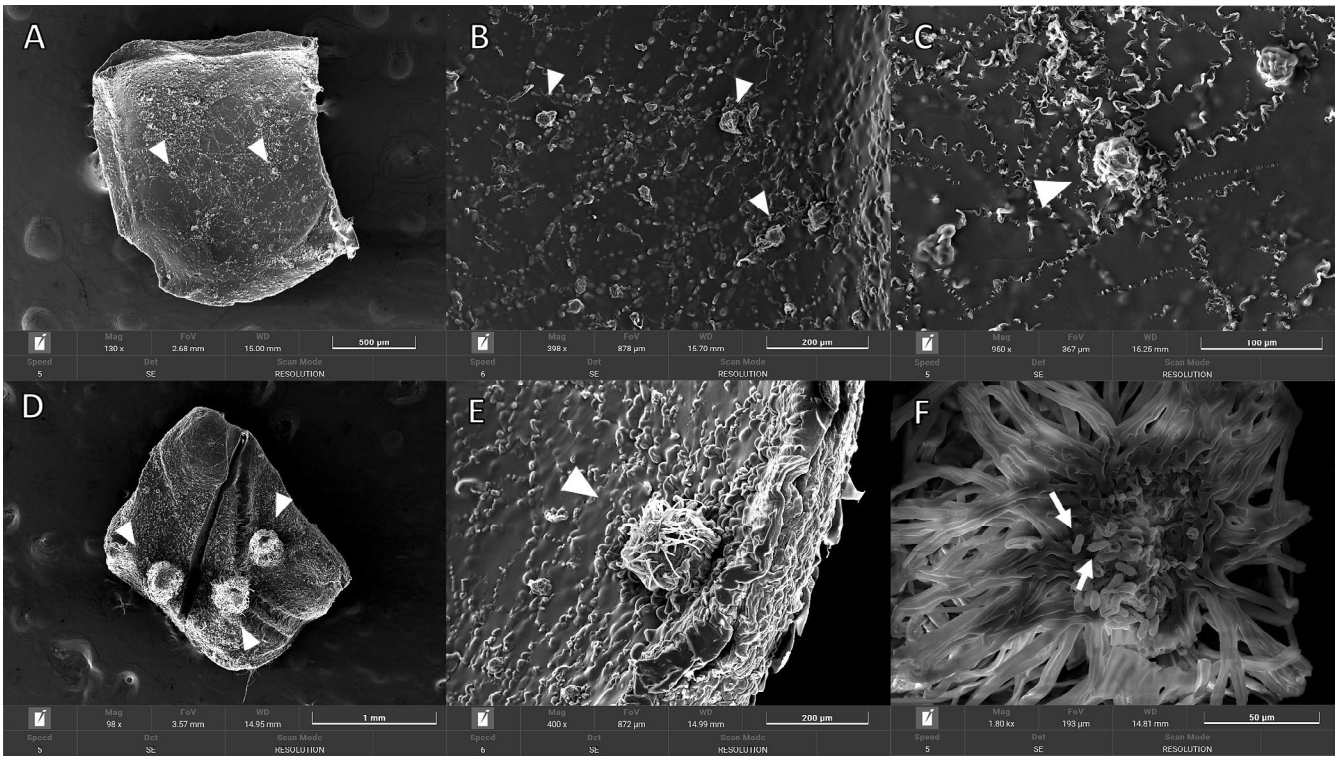


**FIGURE 1** | Scanning electron microscopy images taken of the mating region of a cross between isolates of *Venturia inaequalis* (Gala × *Malus floribunda*), originally collected from apple leaves, grown in vitro on agar plates. (A–C): An 'incompatible' cross, with small round structures, which are pseudothecial 'initial' structures. (D–F): More developed pseudothecial structures indicative of a compatible cross. Arrows indicate the pseudothecia on the images. Arrows in (F) indicate ascospores in the mature pseudothecia. Scale bars A, 500 μm; B, E, 200 μm; C, 100 μm; D, 1000 μm; F, 50 μm.

be from either debris, other microorganisms or organic materials. All the leaves were expected to have a visible community of microbes present on the leaf surface because they were taken from the field; therefore, it is unsurprising that mycelium from other fungi was present.

The leaf section with a single scab lesion (Figure 2C) did not show any development of pseudothecial structures but did show conidiophores, which are around 5–10 μm across and cluster together to form finger-like structures.

### 3.3 | Scab Structures on Leaves (Before Leaf-Fall)

In the samples of leaves with multiple lesions that were microscopically examined immediately after being taken from the orchard, several leaves had structures resembling early stage pseudothecia (Figure 2D–F). In all samples where pseudothecia were seen, they were protruding from a thicker section of mycelium and were growing upwards. The structures looked circular from the aerial view and more elongated from the side. We speculate that these structures shown are examples of initial unmatured pseudothecial structures that have protruded from the leaf's surface and show that sexual reproduction could be initiated prior to leaf-fall. As is the case with the in vitro samples, we could not confirm that these structures had occurred from a compatible mating event; however, we can speculate that if these structures begin to develop, then they could be the result of sexual mating in certain cases.

### 3.4 | Scab Structures on Leaf Sections Overwintered Under Controlled Conditions

For those leaves that were sampled prior to leaf-fall but overwintered for several months under controlled conditions in the laboratory, pseudothecial structures (~50–150 μm) developed on many leaves with multiple scab lesions (Figure 2G–I) as expected. In contrast, leaves with a single lesion showed only immature pseudothecial structures (~20 μm) (Figure 3), resembling the structures observed in vitro of the same size (Figure 1). Thus immature pseudothecia or pseudothecial initials can develop, irrespective of whether two strains are mating-compatible; however, they will not progress further developmentally and will not form ascospores for those non-compatible crosses (Keitt and Palmiter 1938; MacHardy 1996).

We could not confirm whether the exact samples from the pre-leaf-fall experiments would mature as they were fixed for microscopy. We did, however, use light microscopy to identify leaf samples with the same structure before leaf-fall, and these were overwintered for this secondary experiment (Figure S1).

## 4 | Discussion

This study investigated the nature of sexual reproduction within *V. inaequalis*, particularly the timing of initiation of mating crosses. The aim was to determine whether initiation of sexual

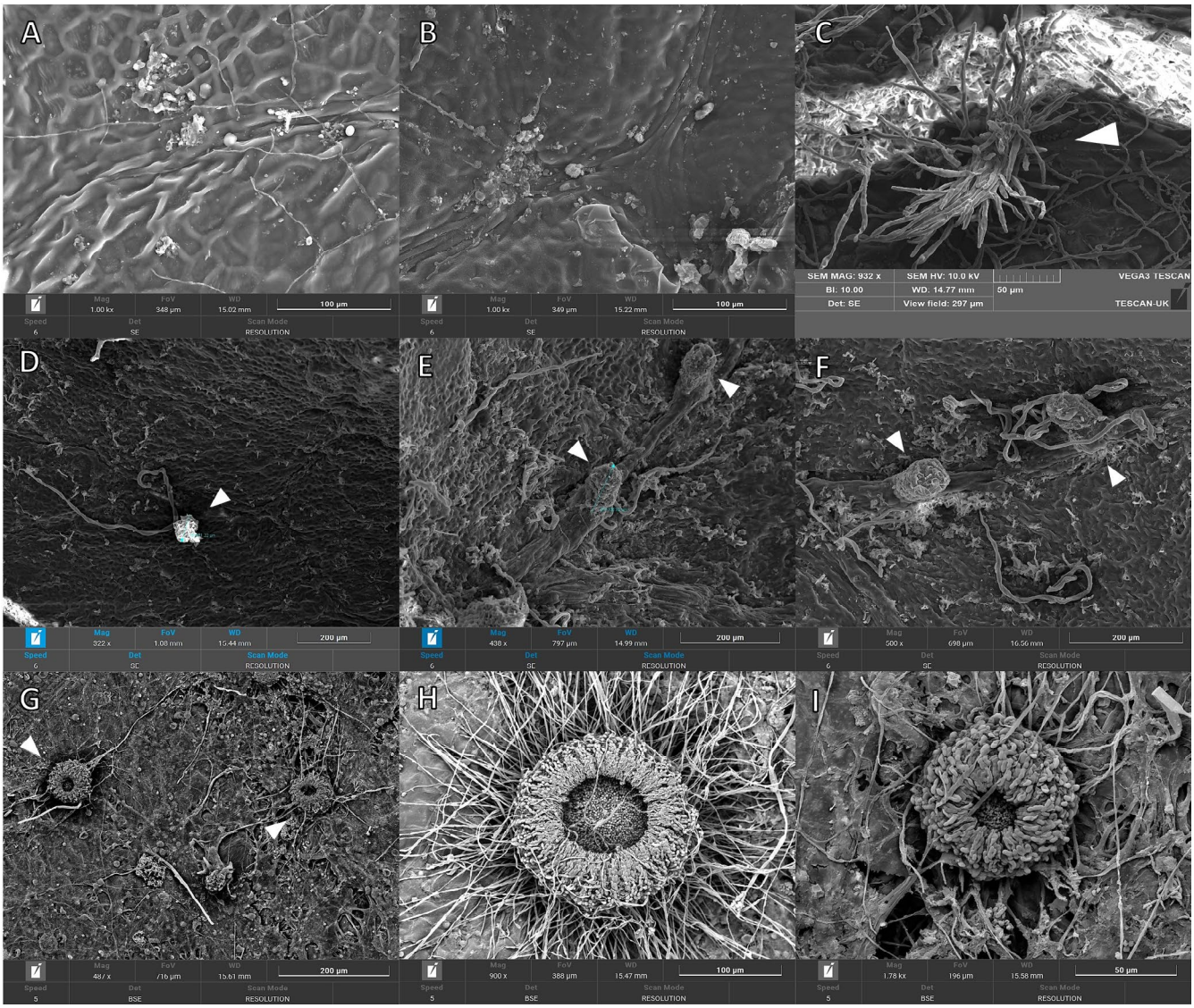


**FIGURE 2** | Scanning electron microscope images of *Venturia inaequalis* on susceptible (cv. Gala) and resistant (TSR33T239) apple leaves. (A) A 'clean' Gala leaf, with no visible scab lesions, but with a few mycelial structures. (B) A clean leaf taken from the scab resistant cultivar TSR33T239, which contains the *Rvi4* major resistance gene. (C) An example of an asexual conidial structure (arrow) found on leaves with single apple scab lesions. (D–F) Images of leaves taken from Gala apple trees before leaf-fall. Arrows indicate the presence of immature pseudothecial structures (arrows). (G–I) Images of sections from leaves with multiple lesions which were sampled prior to leaf-fall and overwintered for several months. These leaves had mature pseudothecia present, as indicated by arrows in (G). Scale bars A, B, H, 100 µm; C, I, 50 µm; D, E, F, G, 200 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

reproduction can occur before leaf-fall in autumn because, if so, mating is more likely to occur between isolates on the same leaf.

Our SEM images showed that sexual reproduction can be initiated on the leaves before leaf-fall, based on the presence of immature pseudothecial structures on the leaf surface between compatible strains, before these leaves with typical scab lesions were sampled (before leaf-fall in autumn). Whilst we cannot prove definitively that the structures are pseudothecial initials, we assume that they are *V. inaequalis* mating structures because, on the leaves sampled prior to leaf-fall, these structures were not found on the clean control leaves or on the leaves with a single scab lesion, which only displayed conidial structures. Prior to sample fixation, we imaged the leaves under a light microscope and observed dark rounded structures that were protruding from the leaf surface at the margin/region where two lesions met. These were highly similar to those images found in MacHardy's and Keitt's studies (Keitt and Palmiter 1938; MacHardy 1996), which, although they did not have any scale, we assume were of similar size. In our opinion, these structures found on multiple lesion leaves are not mature conidial structures because conidiophores are much smaller (~5–10 µm) and, as we observed, often cluster together into finger-like structures; this was also noted by Oerke and Steiner (2024). The clear morphological differences between the fungal structures on leaves with multiple lesions and those with a single discrete lesion further support that mating can be initiated on the same leaf before leaf-fall, but only if the opposite mating type is present prior to leaf-fall.

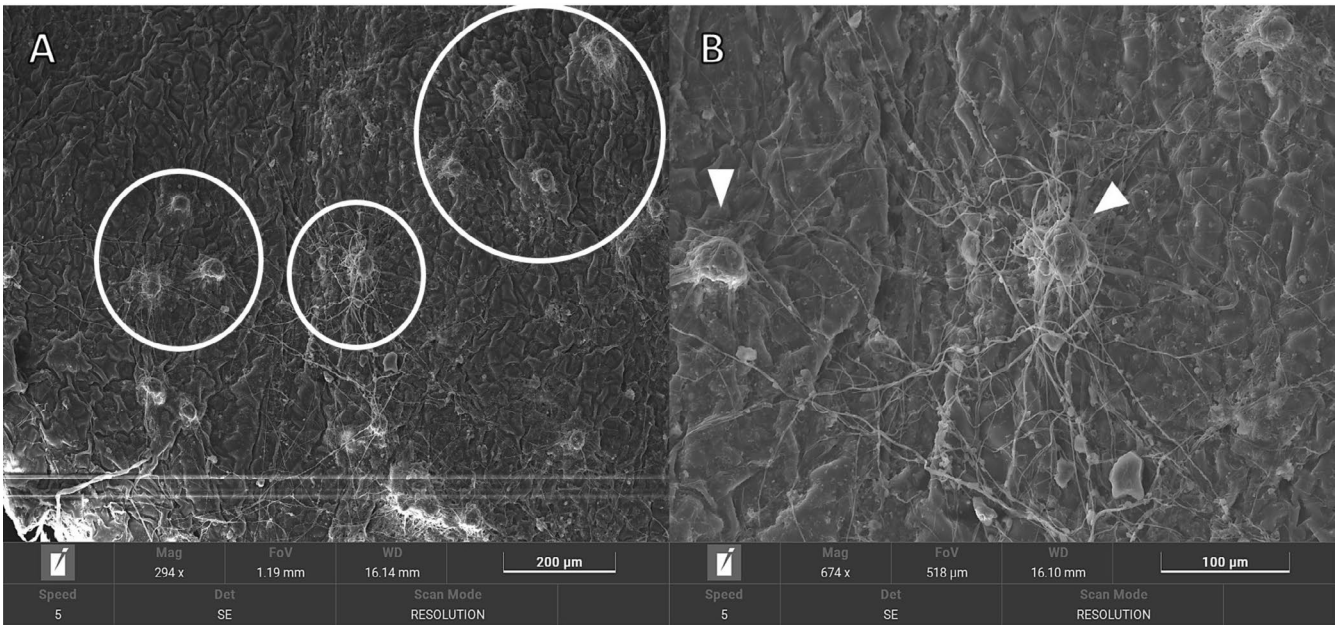


**FIGURE 3** | Scanning electron microscope images of leaves with only single, rather than multiple, apple scab lesions, collected before leaf-fall from apple trees (cv. Gala) and overwintered. Only immature pseudothecia are present, indicated by circles (A) and arrows (B). Scale bars A, 200 µm; B, 100 µm.

Previous studies failed to observe pseudothecia on leaves (Keitt and Palmiter 1938; Wilson 1928) but the leaves in those investigations were sampled about a month earlier than in the present study. When we sampled the leaves, they had already started to senesce though still attached to the tree, which may have allowed pseudothecial development to begin. Wilson (1928) stated that there was no obvious (to the naked eye) fungal development whilst the leaves were green, and the fungal structures began to appear when the leaves started to senesce and turn brown. This may explain why pseudothecial structures have not historically been found on infected leaves prior to leaf-fall. In addition, fully mature pseudothecia would not be observed on leaves prior to leaf-fall because *V. inaequalis* requires a low temperature (~4°C) for pseudothecia to completely mature and produce ascospores. Unfortunately, the same fungal structure or leaf could not be assessed at multiple time points over this long maturing process spanning several months because of the need for destructive sample processing for SEM.

There are still limited possibilities that mating could occur between compatible lesions from different leaves after leaf-fall when leaves in the leaf litter are physically in contact for a sufficient period of time. However, because scab cannot infect old/senescent apple leaves in leaf litter (Wiesmann 1932; Wilson 1928), the likelihood of successful mating between lesions from different leaves is expected to be far lower than between lesions on the same leaf, not least because of the required length of contact time for mating to take place. Environmental factors, such as wind and rain, as well as potential displacement of leaves by human or animal activities, can reduce the probability of two leaves being in physical contact for the length of time required for initiating the mating process.

Knowing it is possible that sexual reproduction can be initiated on the leaves before leaf-fall can help develop late season scab management strategies. For example, if we aim to disrupt the sexual initiation phase, then we need to apply treatment to leaves well before leaf-fall in autumn. This could include the optimisation of fungicide spraying regimes, with the sprays being applied prior to leaf-fall. Currently all fungicides against scab are applied early in the growing season, before harvest, as a preventative measure. Investigating the disruption of sexual initiation by microbial competitors is another possible avenue for future research and development. Combining these treatments prior to leaf-fall with post-leaf-fall orchard sanitation, such as leaf litter removal, could greatly reduce the amount of overwintering primary inoculum (Carisse and Dewdney 2002; Holb 2009). This will greatly reduce primary infection pressure in spring, leading to reduced disease development as well as reduced fungicide input.

The present finding that initiation of sexual reproduction can occur before leaf-fall suggests that the probability of successful mating between isolates from different leaves is much lower than those on the same leaf, hence reducing the possibility of mating between isolates infecting different cultivars. Thus, the risk of scab super races emerging from mixed cultivar orchards is much lower than the risk estimated on the assumption of random mating. Therefore, *V. inaequalis* super races are unlikely to establish and become prevalent within the lifespan of a commercial mixed orchard, supporting experimental results (Barbara et al. 2008; Passey et al. 2016, 2020; Xu et al. 2013) and findings of simulation studies (Xu 2012). Thus, it is feasible to use mixed apple orchards (Stewart et al. 2023) if the main concern against adopting mixed orchards is the risk of scab super races emerging. This is particularly true for cider apple production, where scab control is not as stringent as in dessert apple production. Mixed orchards can be at least a short-to-medium term solution to reduce scab development whilst new cultivars with novel (or combinations of) resistance genes are being bred (Bus

et al. 2019; Khan and Korban 2022). With the introduction of more advanced automated technologies for spraying and picking, mixed cultivar orchards will become even more viable, as the potential challenges, such as differences in picking times, will be reduced (Mhamed et al. 2024; Verbiest et al. 2021).

Understanding the nature of sexual recombination in *V. inaequalis* will also enable a more accurate interpretation of results from fungal population genetics studies, as well as assist in the prediction of fungal evolution of agronomically important traits, such as virulence genes and resistance against specific fungicides (McDonald and Linde 2002b, 2002a).


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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that supports the findings of this study is available from the corresponding author upon request.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Light microscope images of proposed pseudothecia of *Venturia inaequalis* on the apple leaf surface. These leaves were sampled before leaf-fall in November 2023 and were immediately imaged using a Leica M165 C microscope with Lecia IC80 HD camera attachment. The samples were then fixed for scanning electron microscopy.

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# Venturia inaequalis can initiate sexual reproduction prior to leaffall: new electron microscopy evidence

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