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ORIGINAL ARTICLE

Yellow rust infection of wheat: How the quantity of light received by wheat seedlings before inoculation affects infection efficiency

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Abstract

Many factors are known to influence infection by cereal rusts, including environmental variables such at light, humidity and temperature, the topography of the leaf surface, as well as plant volatiles. However, few studies have aimed to link these factors. Previously, the quantity of light received by wheat seedlings prior to inoculation with *Puccinia striiformis* f. sp. *tritici* (Pst) urediniospores was shown to influence yellow rust infection efficiency. In this study we show that germination and the ability of germlings (germinated urediniospores) to enter stomata is enhanced on wheat seedlings subjected to high quantities of light pre-inoculation with Pst urediniospores, while on seedlings exposed to a long dark period germination and infection were compromised. Using headspace collections and gas chromatography–mass spectrometry (GC–MS) analysis thereof, we link this effect of high light quantity to quantitative changes in the profile of volatile organic compounds (VOCs). We show that the VOCs within headspace collections from wheat seedlings exposed to high quantities of light were able to support greater levels of Pst urediniospore germination than the headspace collections from wheat seedlings exposed to a period of dark. In vitro analysis of individual VOCs identified compounds that enhanced Pst urediniospore germination. These VOCs included the sesquiterpene caryophyllene, the monoterpene α-pinene, the fatty acid α-linolenic acid, the organic alcohols 1-hexanol, 3-hexen-1-ol and 5-hexen-1-ol, the aldehyde *cis*-3-hexenal and the ester hexyl acetate.

KEYWORDS
light intensity, *Puccinia striiformis*, stripe rust, volatile organic compounds, wheat, yellow rust

1 | INTRODUCTION

There are three rust diseases of wheat, black (stem) rust, brown (leaf) rust and yellow (stripe) rust, each having their own, environmentally determined, global distribution (Boyd, 2005, 2006). Wheat yellow rust, caused by the fungal pathogen *Puccinia striiformis* f. sp. *tritici* (Pst), prefers cooler, wetter climates, being particularly common in temperate and maritime regions and

at higher elevations. Yellow rust is a polycyclic disease, going through repeated asexual cycles of urediniospore production within a season. Germination and infection occur under conditions of high humidity and usually within a narrow temperature range, 5–12°C, having an optimal germination temperature of 8–10°C (de Vallavieille-Pope et al., 1995).

Pst is an obligate biotroph, only able to grow and complete its asexual and sexual stages on living plant tissues, the asexual stage occurring on wheat and the sexual stage on common barberry (Jin et al., 2010). Airborne urediniospores of Pst produce a single germ tube that gains entry into the plant through stomatal openings. A substomatal vesicle (SSV) forms within the stomatal cavity within 6 h, with up to three infection hyphae growing off the SSV by 12 h. At the point of contact between an infection hypha and a host cell, a haustorial mother cell (HMC) differentiates. An infection peg develops, breaching the plant cell wall and establishing a haustorium inside the living cell. Further colonization occurs following the development of intercellular runner hyphae that grow intercellularly, producing further haustoria. By 14 days after inoculation (dai) the asexual life cycle is complete, with urediniospores bearing pedicels erupting through the leaf epidermis, forming the characteristic yellow pustules (Bozkurt et al., 2010; Jagger et al., 2011; Melichar et al., 2008).

Many environmental factors have been shown to influence infection of wheat by rust pathogens, including temperature, light and humidity (de Vallavieille-Pope et al., 1995). In the case of the stem and leaf rust pathogens the topography of the leaf surface has been shown to support the formation of appressoria over stomata (Allen et al., 1991; Collins, 1996; Collins & Read, 1997; Hoch & Staples, 1991; Read et al., 1997). Higher levels of Pst urediniospore germination have been reported on the adaxial, rather than the abaxial surface of wheat leaves (Russel, 1976), but studies have failed to show an effect of leaf topography on Pst urediniospore germination. In several cereal rust species, including oat crown rust (*Puccinia coronata* f. sp. *avenae*), wheat stem rust (*P. graminis* f. sp. *tritici*) and wheat leaf rust (*P. triticina* – formally *P. recondita*), volatile organic compounds (VOCs) have also been shown to stimulate spore germination, and in some cases subsequent differentiation of fungal structures (French et al., 1975; Hoch & Staples, 1991; Staples & Hoch, 1997; reviewed by French, 1992). In the case of *P. graminis* f. sp. *tritici*, a combination of VOCs and surface ridges of appropriate size and spacing act synergistically to induce appressoria, SSVs and infection hyphae (Collins et al., 2001), while mild heat shock and *trans*-2-hexen-1-ol support the development of HMCs in vitro (Wiethölter et al., 2003). However, to the best of our knowledge there are no reports of positive stimuli of germination and/or infection, either topological or volatile in nature, for Pst.

VOCs are a large group of carbon-based chemicals with low molecular weights and high vapour pressure at ambient temperatures (Pichersky et al., 2006). They are produced by living organisms, including plants and fungi (Ameye et al., 2018; Bennett & Inamdar, 2015). The plant volatilome encompasses a diverse range of compound classes, ranging from alcohols, aldehydes, ketones, terpenes and green leaf volatiles (Bouwmeester et al., 2019). Many of these VOCs are crucial for plant growth, plant-to-plant and plant-to-environment communications, including defence against insects and microbes (Boncan et al., 2020; Brilli et al., 2019; Michereff et al., 2018). There is a considerable volume of work on the role that VOCs play in insect attraction and repulsion, but fewer studies of how VOCs may influence plant–fungal interactions. It is also known that the profile of VOCs released from plants follows a day/night cycle (circadian rhythm) (Rim et al., 2019; Zeng et al., 2017).

Temperature and dew period have been used to successfully model the occurrence of field infections of wheat leaf rust (de Vallavieille-Pope et al., 2000). However, for yellow rust, field infection levels were often higher than models predicted, with higher field infection efficiencies being seen when the temperatures were high, and the plants had received long periods of sunlight (de Vallavieille-Pope et al., 1995). These observations led de Vallavieille-Pope et al. (2002) to test whether the light quantity received by wheat plants before inoculation could influence Pst infection efficiency. Both field and controlled growth room experiments indicated that higher quantities of light, received by wheat seedlings before inoculation with Pst, could result in higher infection rates, recorded as a greater percentage of pustule formation on seedling leaves.

We therefore hypothesized that higher quantities of light received by wheat seedlings prior to inoculation enhanced Pst germination and the ability of germ tubes to locate stomata, and that this stimulation was in turn due to light-induced changes in VOC profiles. In this study we explore the effect of the quantity of light received by wheat seedlings, prior to inoculation, on the subsequent ability of Pst urediniospores to form successful infection sites. We show that the ability of Pst urediniospores to germinate, locate stomata and form a successful infection was increased on wheat seedlings that had been exposed to higher quantities of light immediately prior to inoculation. We demonstrate that this effect of light was not due to differences in stomatal status (open versus closed stomata) at the time of infection, but due to changes in VOC profiles caused by the light treatment.

2 | MATERIALS AND METHODS

2.1 | Plant materials

The yellow rust-susceptible spring wheat Lemhi was used in all experiments to determine the effect of light quantity on VOC profiles and subsequent Pst infection efficiency. Seed was chilled at 4°C for 24 h and imbibed at 20°C for 48 h before planting out in a peat:sand (1:1) mix. Seedlings were grown in a spore-free glasshouse at 18°C/16 h under sodium lights (average 300 μmol/m^2/s) and 15°C/8 h in darkness for 12–14 days, until growth stage (GS) 12–13 (Zadoks et al., 1974).

2.2 | Pst inoculation of wheat seedlings

Seedlings of Lemhi were inoculated with Pst isolates following standard procedures (Boyd & Minchin, 2001). When urediniospores had been stored over liquid nitrogen, dormancy was broken by heating the spores at 40°C for 5 min. The urediniospores were mixed with an equal volume of pure talcum powder and air blown on to seedlings. The seedlings were sprayed with double-distilled water containing a few drops of Tween 20 as a wetting agent before inoculation. The inoculated seedlings were placed inside a closed cabinet within a walk-in incubator room maintained at 8°C and >60% humidity for 24 h, in total darkness. Seedlings were then returned to the spore-free glasshouse under standard growing conditions, 18°C/16 h under sodium lights (average 300 μmol/m^2/s) and 15°C/8 h in darkness. To ensure that the Pst inoculation had been successful, seedlings were checked from 10 dai for the emergence of sporulating pustules on the leaf surface. For the seedling pre-inoculation light quantity experiments the Pst isolates WYR1975/20 (virulent on *Yr2*, *3*, *4*, *7*, *9*) and WYR1981/02 (virulent on *Yr1*, *2*) were used. The Pst isolates used for the VOC bioassays were NIAB 2016/035 (virulent on *Yr1*, *2*, *3*, *4*, *6*, *7*, *9*, *17*, *25*, *32*), NIAB 2019/501 (virulent on *Yr1*, *2*, *3*, *4*, *6*, *7*, *9*, *17*, *25*, *32*) and NIAB 2020/092 (virulent on *Yr1*, *2*, *3*, *4*, *6*, *7*, *9*, *17*, *25*, *32*).

2.3 | Pre-inoculation light treatments used for Pst infection efficiency experiments

Multiple experiments were undertaken to determine the effect of differing quantities of light, received by wheat seedlings prior to inoculation with Pst urediniospores, on subsequent Pst infection efficiency. In every experiment the same trend towards greater Pst infection on wheat seedlings exposed to the highest quantities of light was observed. Here we report a selection of those experiments that demonstrate these results.

2.3.1 | Experiment 1—Validation of results reported by de Vallavieille-Pope et al. (2002)

An experiment was undertaken that broadly followed the light treatments reported by de Vallavieille-Pope et al. (2002). Seven-day-old seedlings of Lemhi were exposed to 16 h of low light (total quantity of light received was 45 μmol/m^2) and 8 h of darkness at 8°C for 3 days. The seedlings were then left in total darkness for 16 h at 8°C. The seedlings were divided into four batches (approximately 20 seedlings per batch), with each batch receiving one the following quantities of pre-inoculation light:

1. 0 mol/m^2 (no light for 18 h)
2. 12 mol/m^2 (185 μmol/m^2/s for 18 h = 11.99 mol/m^2)
3. 18 mol/m^2 (278 μmol/m^2/s for 18 h = 18.01 mol/m^2)
4. 36 mol/m^2 (556 μmol/m^2/s for 18 h = 36.03 mol/m^2)

Unlike the treatments reported by de Vallavieille-Pope et al. (2000), all batches of seedlings were exposed to the light for the same length of time. The quantity of light received by each batch of seedlings was controlled by altering the distance of the plants from the sodium light source. The light levels were measured using a LI-180 spectrometer (measures photosynthetic active radiation at wavelengths 400–700 nm; Li-Cor Biosciences). The temperature in the compartment was maintained between 20 and 22°C, with a relative humidity of between 40% and 45%. The batch of seedlings receiving 0 mol/m^2 of light was kept in the dark for 18 h at 20°C and a relative humidity of 40%–45%. All batches of seedlings were inoculated simultaneously with Pst isolate WYR1975/20. Each batch of seedlings were sampled for light microscopy at 24, 48 and 72 hours after inoculation (hai), sampling five or six seedlings per time point.

2.3.2 | Experiment 2.1 and 2.2—Assessment of stomatal status

To assess the stomatal status (open versus closed) of wheat seedlings during the normal Pst inoculation process, we measured stomatal conductance (the rate of water transpiration) using an AP4 cycling porometer (Delta-T Ltd). The AP4 porometer corrects for changes in temperature and therefore was suitable for measuring stomatal conductance of wheat seedlings while in the glasshouse and during the Pst incubation period held at 10°C. Seedlings of Lemhi were grown under normal glasshouse conditions, 16 h of light (300 μmol/m^2/s) at 18°C and 8 h of dark at 15°C for 12–14 days, to GS 12–13. Seedlings were inoculated with Pst isolate WYR1975/20 and placed in the dark at 8–10°C and >60% humidity for 24 h. After this incubation period seedlings were returned to the glasshouse. Porometer readings were taken before and after inoculation with Pst, at 26 and 1 hours before inoculation (hbi) and at 2, 23, 26, 50 and 72 hai (Experiments 2.1 and 2.2). Samples for microscopy were taken at 2, 23, 26, 50 and 72 hai from three seedlings in Experiment 2.1 and four seedlings in Experiment 2.2.

2.3.3 | Experiment 3—assessment of the influence of pre-inoculation light quantity on Pst germination and infection efficiency

The pre-inoculation light treatments tested in Experiment 1 were simplified to develop a repeatable test system whereby the effects of pre-inoculation light quantities on Pst infection efficiency could be studied.

Seedlings of Lemhi were grown under normal glasshouse conditions for 12–14 days until GS 12–13 (Zadoks et al., 1974). Forty seedlings were then subjected to one of three light treatments at 20°C:

1. Total darkness (19 h)
2. Low light (185 μmol/m^2/s for 19 h = 12.65 mol/m^2)
3. High light (420 μmol/m^2/s for 19 h = 28.73 mol/m^2)

Seedlings were inoculated with the Pst isolate WYR1975/20 or WYR1981/2 following the standard protocol described above. In each repeat experiment (Experiments 3.1, 3.2 and 3.3) the three sets of seedlings were inoculated simultaneously, receiving equal numbers of spores per set. In Experiment 3.3 a fourth set of seedlings, that had been left under standard light conditions in the glasshouse, were inoculated alongside the seedlings that had received the test light treatments. This was to provide a comparison of Pst spore germination and infection efficiency under a normal seedling inoculation regime. In Experiment 3.1 and 3.2 the stomatal status of the seedlings (i.e., open vs. closed) was determined by measuring stomatal conductance using an AP4 cycling porometer.

Twenty seedlings from each light treatment were sampled for microscopic analysis of Pst development and 20 seedlings were used for porometer readings. Samples for microscopy (four seedlings per time point) were taken at 2, 23, 26, 48/50 and 74 hai. Porometer readings (12 readings per time point) were taken 1 hbi (−1) and at +2, +23, +26, +50 and +74 hai in Experiments 3.1 and 3.2.

2.4 | Light microscopy of Pst development on wheat seedlings

Pst-inoculated leaf samples were taken for light microscopy at specified hai. Two leaf segments, approximately 2 cm long, were cut from the first true leaf of each seedling. Leaf segments were fixed and cleared by submerging in excess chloral hydrate (CH) solution (300 mL 95% ethanol, 125 mL 90% lactic acid, 800 g CH, made up to 1 L with chloroform; Garrood, 2001). The CH solution was changed every 24 h until leaf tissues were translucent. Trypan blue was used to stain fungal cell walls, cleared leaf tissues being placed in 0.1% trypan blue in lactoglycerol (lactic acid:glycerol:water; 1:1:1) for 18 h (Garrood, 2001).

Stained leaf samples were mounted in lactoglycerol and observed using brightfield light microscopy (Nikon Microphot II). The following Pst developmental stages were measured: (a) percentage germination—the number of urediniospores that had germinated (germling) as a proportion of the total number of urediniospores on the leaf surface; (b) percentage infection sites—the number of germinated urediniospores that had successfully entered a stoma, as a percentage of the number of germinated urediniospores; (c) percentage SSVs—the number of SSVs that had formed within a substomal cavity, as a percentage of the number of germinated urediniospores; and (d) percentage of failed infection attempts—the number of germinated urediniospores that had grown over a stoma, failing to enter the stomatal cavity, as a percentage of the number of germinated urediniospores.

2.5 | Collection and analysis of VOCs from wheat seedings exposed to different quantities of light

Seedlings of Lemhi were grown to GS 14 under normal glasshouse conditions (16 h light, 320 μmol/m^2/s, at 21°C and 8 h night at 16°C). Seedlings were transferred to either total darkness or high light conditions (420 μmol/m^2/s) for 19 h. Light levels were measured using the light meter SKP 20051303, calibrated for sensor SKP 215/I 51304, which measures the light intensity paired with a PAR quantum sensor (400–700 nm; Skye Instruments Ltd). Dynamic headspace collections were then carried out using air entrainment kits (Pye volatile collection kit) with plants enclosed in transparent cooking bags (Sainsbury's Supermarkets Ltd) secured by wire ties. Porapak Q adsorbent tubes, consisting of a 5 mm diameter borosilicate tube filled with 50 mg Porapak Q (Supelco), sandwiched between two glass wool plugs, were used to collect VOCs within the headspace. Charcoal-filtered air was passed into the cooking bag at 500 mL/min and pulled out through the adsorbent tube at 400 mL/min over the 19 h of the dark versus high light treatment, the VOCs emitted by the plants being absorbed onto the Porapak Q. Headspace VOCs were extracted from the Porapak Q tubes by eluting with 750 μL of diethyl ether (Fisher Scientific). Headspace VOC extracts were concentrated to 100 μL under a gentle nitrogen flow and stored at −20°C until required for use.

Coupled gas chromatography–mass spectrometry (GC–MS) was used for the tentative identification of VOCs in the wheat dynamic headspace collections. Four microlitres of each headspace extract was analysed using an Agilent 8890GC-5977B GC–MSD fitted with a non-polar HP1 column, 50 m length × 0.32 mm inner diameter × 0.52 μm film thickness (J&W Scientific), using the following conditions: 30°C for 5 min, rising 5°C/min to 150°C, followed by 10°C/min to 230°C, for a total run time of 60 min. Tentative identification of compounds observed between run times 8 and 35 min was achieved using the retention index (RI) values obtained using GC–MS analysis and NIST mass spectral library (2020, NIST).

2.6 | Bioassays developed to test the effect of VOCs on germination of Pst urediniospores

2.6.1 | Bioassay 1

Agar 4550 (1% solution; Sigma) was used to coat the surface of a glass microscope slide. At one end of the slide a hole was made in the agar and a drop of neat VOC (~5 μL) was placed in the hole. Table S1 lists all compounds used in the bioassays with IUPAC identifier information. Urediniospores of Pst were deposited onto the agar, at the other end of the slide, using a cotton bud. The slides were placed, agar side up, onto paper towel moistened with tap water in a plastic dish (20 cm^2). The lid was placed on the dish and the dish wrapped in aluminium foil. The dishes were placed in an incubator maintained at 8–10°C and >60% humidity for 24 h, in total darkness. The control was an agar-covered glass slide without VOC placed in a separate dish.

2.6.2 | Bioassay 2

As many of the VOCs tested proved to inhibit Pst urediniospore germination we modified Bioassay 1. The VOC was suspended in paraffin wax to provide a controlled release of the compound into

the atmosphere within the plastic dish. The VOC (5 µL/g) was added to melted paraffin wax (Acros Organics). Approximately 1.5 g of the wax–VOC suspension was placed on a glass slide. The slide was placed in the middle of the plastic dish, on paper towel moistened with 10 mL of distilled water. Pst urediniospores were placed on a glass slide covered in 1% agar using a cotton bud. Three slides with Pst isolate NIAB 2019/501 and three slides with Pst isolate NIAB 2020/092 were placed around the wax–VOC slide. The lid was placed on the plastic dish and the dish covered in aluminium foil. The dish was placed in the incubator as described for Bioassay 1. The control was as above, but without the wax–VOC slide. Bioassay 2 was also used to test the effect on Pst urediniospore germination of the VOCs contained within the headspace collections obtained from seedlings of Lemhi grown under conditions of high light and total darkness for 19 h. Agar (1%) and ether were used as controls.

The plastic dish containing the control could not be placed in the incubator at the same time as the dish containing the test VOC because, despite the dishes being wrapped in aluminium foil, VOC could still escape from the dish. Therefore, each VOC and the controls were tested on consecutive days, using the same incubator and the same batch of urediniospores. Germination of Pst urediniospores was measured microscopically 24 hai, with approximately 100 urediniospores being scored on each slide. The Pst isolates used in these VOC bioassays were maintained as a fresh supply of urediniospores, collected from seedlings prior to use. The isolates used were NIAB 2016/035, NIAB 2019/501 and NIAB 2020/092. As the percentage of Pst urediniospore germination on the control, 1% agar 4550, varied between experiments, we calculated the increase or decrease in germination due to the presence of the VOC relative to the control. Likewise, the effect of the VOCs in the headspace collections on Pst urediniospore germination was assessed relative to the level of Pst germination seen with the ether control. The difference between the average percentage germination in the presence of the VOC and the average percentage germination on the control was divided by the average percentage germination on the control. The individual VOCs tested, with IUPAC identifier information, are listed in Table S1. They were all obtained from Merck and were either Sigma-Aldrich or Millipore Sigma branded.

2.7 | Statistical analysis

The microscopy data, from both the light and VOC treatment experiments, were analysed using GenStat v. 16 and v. 20 (VSN International, 2020). Analyses of variance were undertaken using general linear regression models. For the light treatment experiments, replication and light treatment were accounted for in the model, with *t*-test comparisons performed to determine which light treatments had significantly different effects on Pst urediniospore germination and infection efficiency. For the VOC treatment experiments, replication, isolate and VOC were accounted for in the model, with *t*-test comparisons performed to determine which VOC significantly affected Pst germination relative to the controls, and whether there were significant differences between Pst isolates. Analysis of headspace collections compared no VOC and ether controls to headspace collections taken from Lemhi seedlings exposed to 19 h of darkness or 19 h of high light, with *t*-test comparisons performed to determine whether headspace collections affected Pst urediniospore germination. Principal component analysis (PCA) was performed using the factoextra package (Kassambara & Mundt, 2020) in R in order to observe trends, clusters and outliers among the four biological replicate headspace collections, and plotted using the package ggplot2 (Wickham, 2016).

3 | RESULTS

3.1 | Effect of pre-inoculation light quantity received by wheat seedlings on subsequent Pst infection

Multiple experiments consistently demonstrated that the germination of Pst urediniospores, and the ability of germinated urediniospores to locate and enter a stoma, was enhanced on wheat seedlings that had been exposed to high quantities of light prior to inoculation.

3.1.1 | Experiment 1: Repeat of experiment described by de Vallavieille-Pope et al. (2002)

The study de Vallavieille-Pope et al. (2002) had shown that Pst infection efficiency (i.e., the number of germinated urediniospores successfully entering the leaf via stomata), measured as the formation of pustules on the yellow rust-susceptible wheat Michigan Amber, was enhanced in seedlings that had received high quantities of light prior to inoculation. Following the pre-inoculation light treatments used by de Vallavieille-Pope et al. (2002), these observations were confirmed using the yellow rust-susceptible wheat Lemhi, and by measuring infection efficiency microscopically. On wheat seedlings exposed to 0 µmol/m^2 before inoculation, urediniospores germinated but did not enter stomata (Figure 1). On seedlings exposed to low quantities of light pre-inoculation, some germinated urediniospores succeeded in locating stomata, but the numbers were significantly lower than on seedlings exposed to higher quantities of light prior to inoculation (Figure 1).

Under the light microscope the majority of stomata appeared closed, although more stomata appeared open on the seedlings that had received the high light treatments prior to Pst inoculation. Concerned that the prolonged periods of low light and darkness used by de Vallavieille-Pope et al. (2002)—16 h of low light at 45 µmol/m^2/s and 8 h of darkness at 8°C for 3 days, followed by total darkness for 16 h at 8°C prior to light quantity treatments—may have compromised stomatal opening, we modified the pre-light treatments in subsequent experiments. This led us to a series of experiments (Experiment 3) that identified a pre-inoculation light regime that enhanced Pst infection efficiency but without compromising stomatal status, stomatal status being monitored in subsequent experiments by measuring water transpiration with an AP4 cycling porometer.

3.1.2 | Experiment 2: Determination of stomatal status during Pst infection

The protocol used for Pst infection requires the plants to be placed in total darkness for 24 h, at a humidity >60%, immediately after inoculation (Boyd & Minchin, 2001). Therefore, it is generally considered that Pst can enter the plant through closed or partially closed stomata (de Vallavieille-Pope et al., 1995). To determine the status of wheat seedling stomata during the normal Pst incubation period and how this influences the infection efficiency of Pst, we measured stomatal conductance just before inoculation with Pst urediniospores, during the Pst incubation period, and after the seedlings were returned to the glasshouse under normal growing conditions. For these experiments Lemhi seedlings were grown under normal growth conditions prior to inoculation with Pst, that is, for 16 h of light (300 µmol/m^2/s) at 18°C and 8 h dark at 15°C for 14 days.

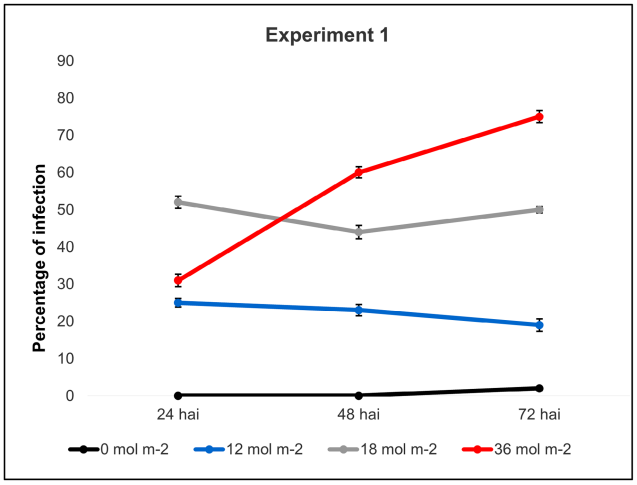

FIGURE 1 Percentage of *Puccinia striiformis* f. sp. *tritici* (Pst) infection sites measured as the percentage of germinated urediniospores that entered a stoma. Pst urediniospore germination was assessed microscopically on seedlings of the wheat variety Lemhi that had received 0, 12, 18 or 36 mol/m^2 of light, over an 18 h period, before inoculation with Pst. The error bars show standard errors between replicate measurements. hai, hours after inoculation.

While stomatal conductance dropped rapidly following transfer of the Pst-inoculated seedlings into total darkness (Figure 2), Pst germlings were still able to locate and enter stomata, with 35% (Experiment 2.1) and 17% (Experiment 2.2) of germlings having entered stomata at 23 hai (while the seedlings were still in the dark incubation period) and 23% (Experiment 2.1) and 13% (Experiment 2.2) of germlings growing over stomata (Figure 3). The remaining germlings failed to locate a stoma (Experiment 2.1 = 42% and Experiment 2.2 = 70%).

3.1.3 | Experiment 3: Standardization of pre-inoculation light treatments

The pre-inoculation light treatments were simplified to develop a repeatable test system whereby the effects of pre-inoculation light quantities, received by wheat seedlings, on subsequent Pst infection efficiency could be studied. Here we report the results from three repeat experiments, Experiment 3.1, 3.2 and 3.3. In Experiment 3.1 and 3.2 the stomatal status of the

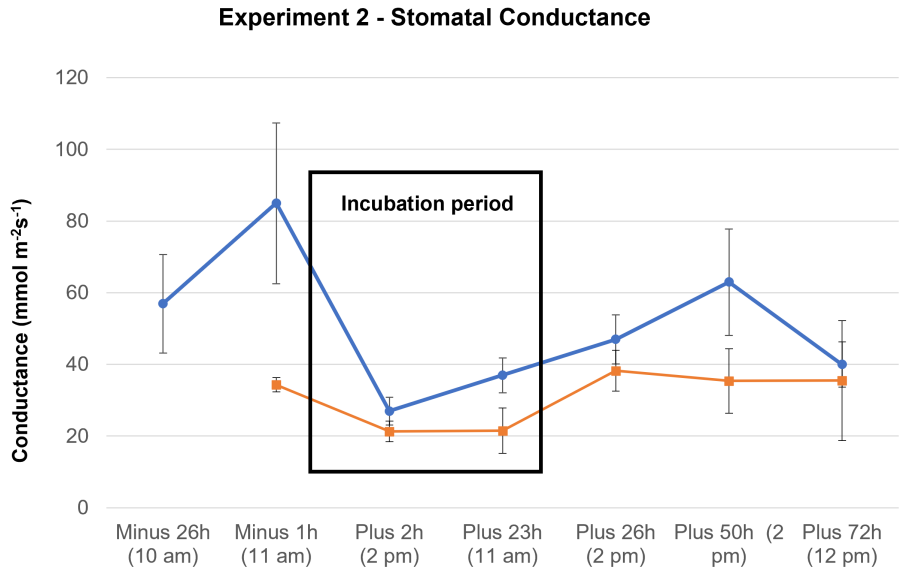

FIGURE 2 Stomatal conductance measured before and after inoculation with urediniospores of *Puccinia striiformis* f. sp. *tritici* (Pst). The timepoints show porometer readings taken before (−) and after (+) Pst inoculation. Area in the box covers the incubation period when wheat seedlings inoculated with Pst urediniospores are placed in total darkness for 24 h at 8°C and >60% humidity. Porometer readings from Experiment 2.1 shown as blue line, and Experiment 2.2 as brown line. The error bars show standard errors between replicate measurements.

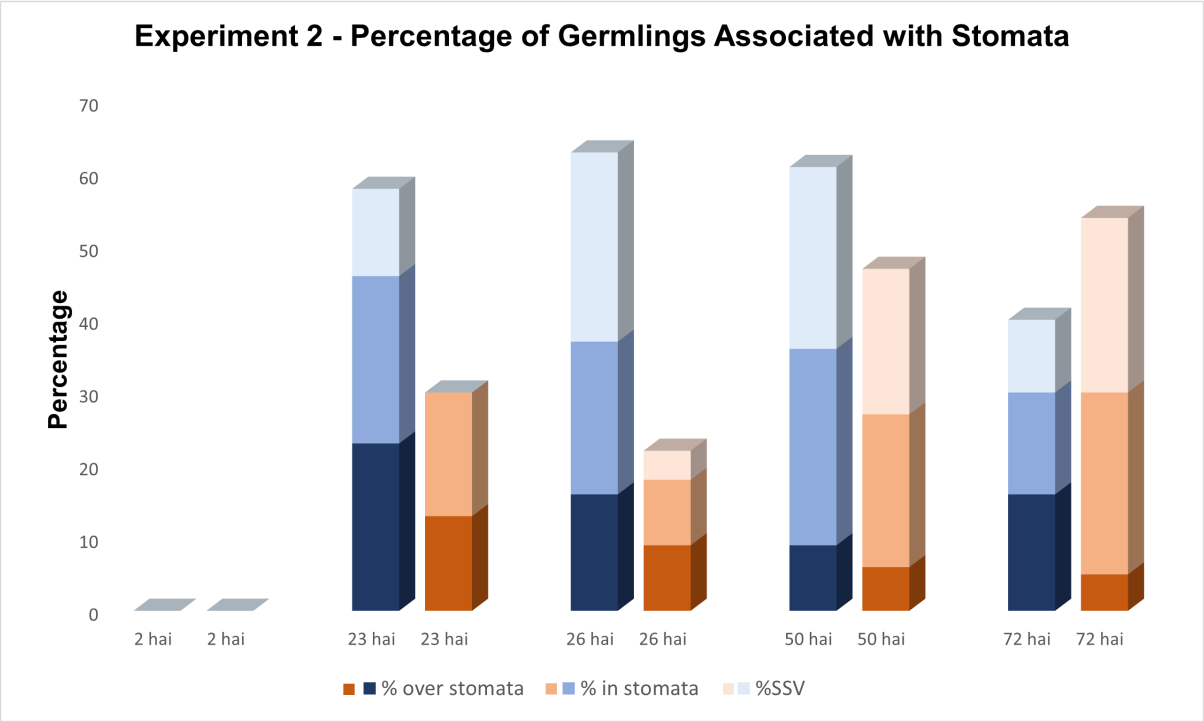

FIGURE 3 Percentage of germinated *Puccinia striiformis* f. sp. *tritici* (Pst) urediniospores that had grown over a stoma (% over stomata) or entered a stoma (% in stomata) and formed a substomatal vesicle (% SSV). The Pst-inoculated leaf samples were taken at 2, 23, 26, 50 and 72 hours after inoculation (hai). Experiment 2.1 in shades of blue, and Experiment 2.2 in shades of brown.

seedlings (i.e., open vs. closed) was also determined by measuring stomatal conductance, using an AP4 cycling porometer, before and after inoculation with Pst.

The quantity of light received by Lemhi seedlings immediately prior to inoculation with Pst urediniospores had a significant effect on urediniospore germination and the ability of the germlings to locate and enter stomata (Figure 4). Significantly more germination was observed on seedlings that had received the highest quantities of light prior to inoculation (Table S2). On seedlings exposed to 19 h of darkness germination was greatly reduced, even after the inoculated seedlings had been returned to normal light conditions 24 hai. The number of germinated urediniospores did not reach those seen on the seedlings exposed to low and high pre-inoculation light treatments, even by 74 hai.

As in Experiment 2, germlings were observed to have entered stomata at 23 hai, while the Pst-inoculated seedlings were still in the 24 h dark incubation period, and the stomata are effectively closed (Figure 4; Figure S1). Significant differences were seen for both light treatment and time when comparing the number of germlings that had grown over a stoma to the number of germlings that had grown into a stoma (including those that had formed SSVs) (Table S2). Significantly more germlings entered stomata on seedlings that had received light prior to Pst inoculation, compared to seedlings kept in the dark (Table S2), while far fewer germlings were observed to have grown over a stoma. This effect was most significant on the seedlings receiving the highest levels of light, while the ability of germlings to locate and enter a stoma had been significantly compromised on the seedlings subjected to 19 h of darkness before inoculation with Pst (Figure 4).

Stomatal conductance measured 1 h before inoculation with Pst urediniospores, at which time the three sets of seedlings were still receiving different light treatments, clearly indicated that the stomata of the seedlings receiving the dark treatment were closed, while those receiving low and high light treatments were open (Figure S1). After inoculation with Pst urediniospores the three sets of seedlings were placed in total darkness for 24 h. This resulted in a reduction in stomatal conductance in all seedling sets. Following transfer back to normal light/dark cycles in the glasshouse (after 24 hai) the stomata in each set of seedlings subsequently followed the same pattern of opening and closing, indicating that the pre-inoculation light treatments had not compromised stomatal opening and closing (Figure S1).

3.2 | The effect of light quantity on the volatile organic compound profiles produced by seedlings of Lemhi

The influence of light on the VOCs released (headspace collections) by wheat seedlings was assessed in the wheat variety Lemhi. The VOCs identified by GC–MS are a general survey of the plant volatiles that represent the wheat volatilome of Lemhi (Figure S2). Differences were seen between seedlings exposed to total darkness versus high light (Table S3), with a general trend

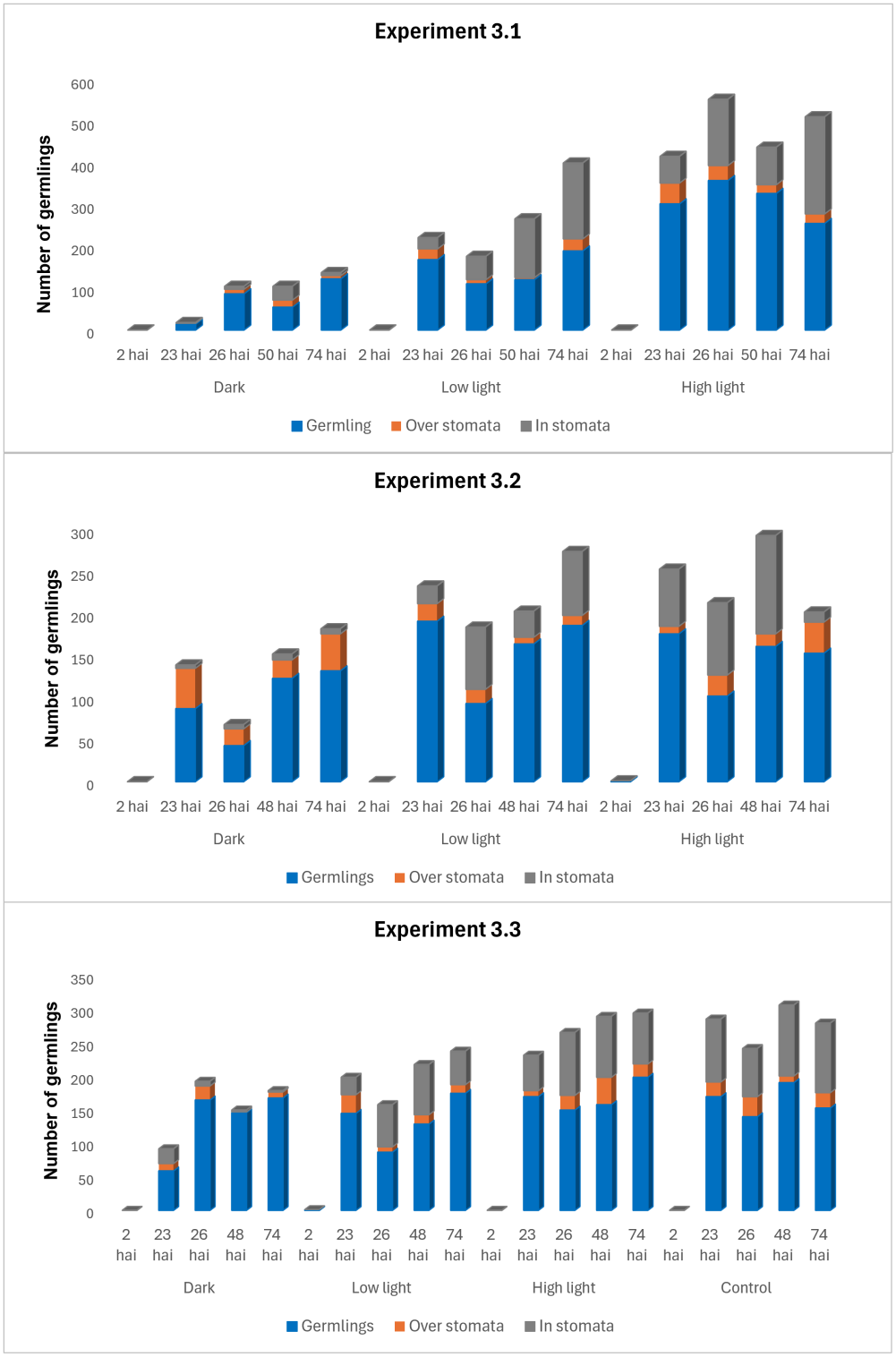

FIGURE 4 The number of *Puccinia striiformis* f. sp. *tritici* (Pst) urediniospores that had germinated (germlings), grown over a stoma (over stomata) or entered a stoma (in stomata) on wheat seedlings of variety Lemhi. Germinated urediniospores were assessed at 2, 23, 26, 48 or 50 and 74 hours after inoculation (hai) on wheat seedlings that had been exposed to different quantities of light immediately prior to inoculation with Pst. Experiment 3.3 also included a set of Lemhi seedlings that did not receive a pre-inoculation light treatment (control), but had been left in the glasshouse, under normal light conditions, until inoculation.

towards greater levels of VOCs being released from seedlings exposed to high light (Table S3). The increase in VOC concentrations was small in the case of hexanal, undecane, octanoic acid and tridacane, but substantially greater for the VOCs 3-hexen-1-ol, heptanal, octanal, Z-ocimene, 3-hexen-1-ol acetate and linalool.

3.3 | The effect of the headspace collections from Lemhi on germination of Pst urediniospores

The headspace collections from the Lemhi seedlings subjected to 19 h of darkness versus 19 h of high light (420 μmol/m^2/s) were tested for their effect on Pst urediniospore germination using Bioassay 2. Two controls were included, looking at urediniospore germination on 1% agar without any VOCs embedded in the paraffin wax block and a second control where ether was placed in the wax block, the headspace extracts having been collected in ether. Four experiments were undertaken; however, PCA of the GC–MS data indicated that the dark treatment sample from Experiment 3 was an outlier (Figure S3). Therefore, the headspace collections from Experiment 3 were not assessed for the effect on Pst urediniospore germination. The headspace collections were tested using two Pst isolates, NIAB 2019/501 and NIAB 2020/092.

Significant differences were seen between the non-volatile control and ether in Experiments 1 and 4 (*t*-test $p < 0.001$), but not in Experiment 2 (*t*-test $p = 0.124$). However, in Experiment 1 ether inhibited Pst urediniospore germination while in Experiment 4 germination was enhanced by ether (Figure S4; Table S4). Pst urediniospore germination in the presence of the headspace collection from the Lemhi seedlings exposed to 19 h of darkness was, however, only significantly different from the ether control in Experiment 1 (*t*-test, $p = 0.005$; Table S4). Therefore, the effect of the headspace collections on Pst urediniospore germination was assessed relative to the level of Pst germination seen with the ether control (Figure 5). A significant effect of the headspace collection from Lemhi seedlings exposed to 19 h of light (equivalent to 420 μmol/m^2/s) was seen on Pst urediniospore germination in all three experiments (*t*-test probabilities: Experiment 1 $p = 0.004$, Experiment 2 $p < 0.002$ and Experiment 4 $p = 0.056$). Significant differences were also observed between the two Pst isolates (Experiment 1 F$p = 0.002$, Experiment 2 F$p < 0.001$ and Experiment 4 F$p = 0.002$; Table S4). Relative to the ether control, isolate NIAB 2020/092 showed higher levels of germination than isolate NIAB 2019/501 under high light conditions (Figure 5).

3.4 | Identification of plant VOCs that influence germination of Pst urediniospores

Based on the working hypothesis that VOCs released by wheat seedlings stimulate Pst urediniospores to germinate, we developed an in vitro bioassay (Bioassay 1) to test the effect of individual VOCs on Pst urediniospore germination. We selected VOCs that exhibited quantitative changes in Lemhi seedlings exposed to high quantities of light, as well as VOCs reported in green plant tissues in the literature (Table S5). The following VOCs were found to consistently increase the number of urediniospores that germinated relative to the control (1% agar); α-pinene > α-linolenic acid > hexyl acetate >1-hexanol > *cis*-3-hexenal (Figure 6). All other VOCs tested either completely eliminated germination of Pst urediniospores or reduced the percentage of germination relative to the control (Table S5). Pst germination was reduced in the presence of isoprene by about 36%, but completely inhibited in the presence of hexanal, decanal, nonanal, benzaldehyde, acetophenone, linalool, penten-3-one and 5-hexen-1-ol.

As many of the VOCs tested appeared to be toxic, inhibiting Pst urediniospore germination, we modified the in vitro bioassay to slow

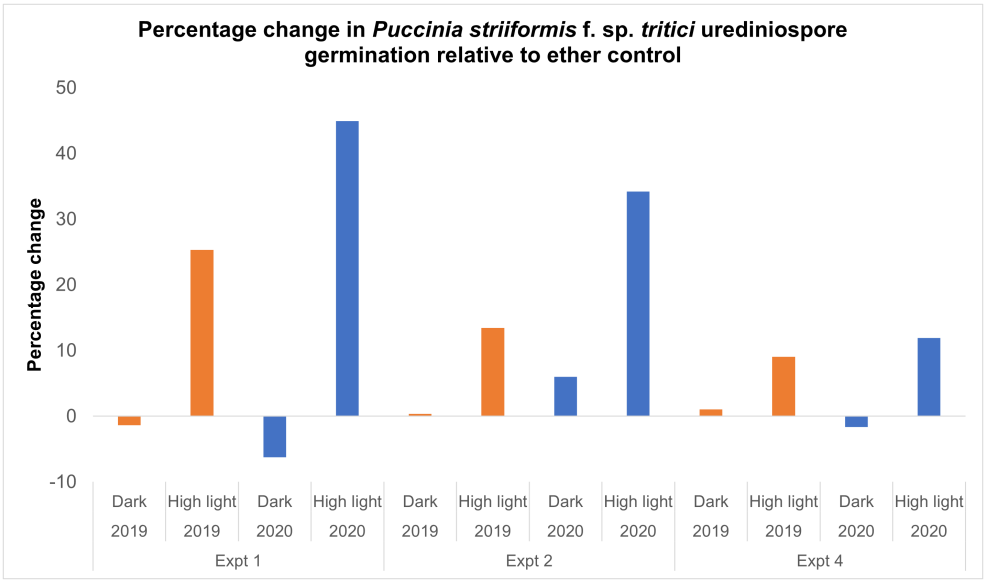

FIGURE 5 Percentage change in *Puccinia striiformis* f. sp. *tritici* (Pst) urediniospore germination relative to ether control. The change in percentage germination of Pst urediniospores caused by the headspace collections from Lemhi seedlings exposed to 19 h of darkness (dark) or 19 h of high light equalling 420 μmol/m^2/s (high light) compared to the ether control. Tests were carried out using Pst isolates NIAB 2019/501 (2019) and NIAB 2020/092 (2020).

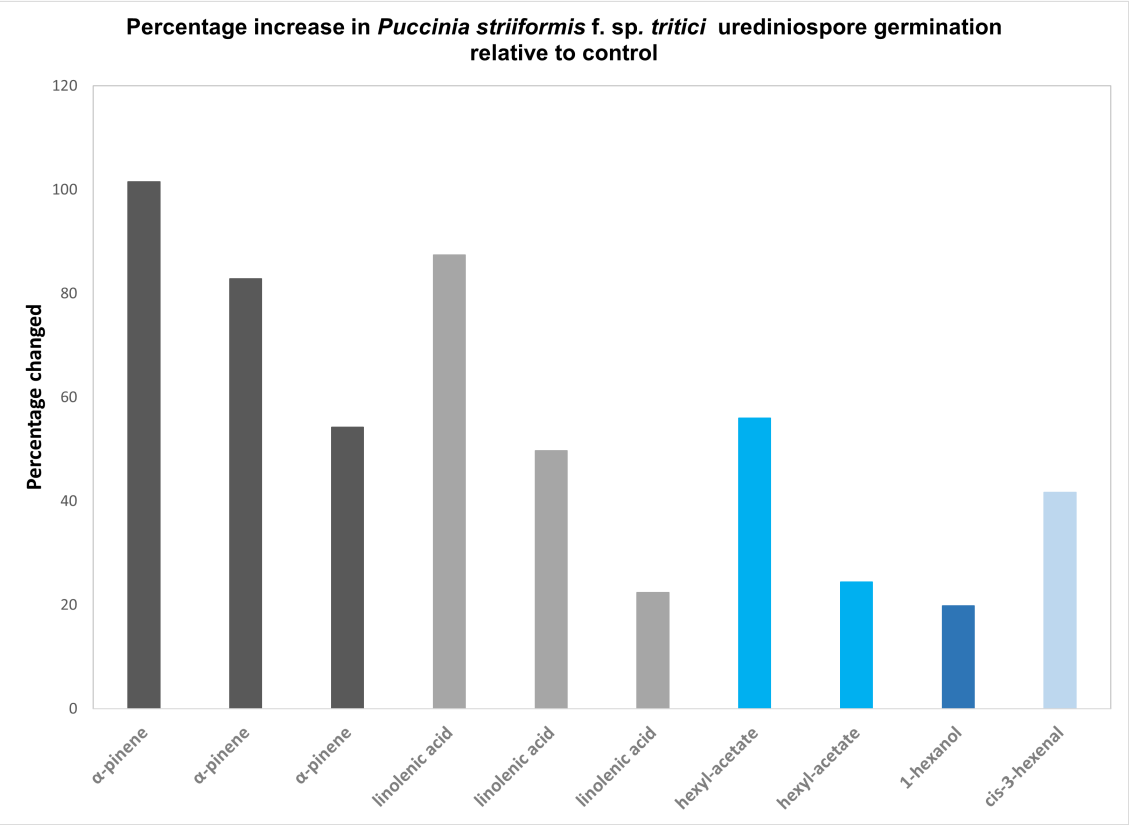

FIGURE 6 Percentage increase in *Puccinia striiformis* f. sp. *tritici* (Pst) urediniospore germination relative to control. The percentage increase in Pst urediniospore germination, relative to the percentage germination of Pst spores on the control (1% agar), is shown when in the presence of α-pinene, α-linolenic acid, hexyl acetate, 1-hexanol and *cis*-3-hexanal. These tests were carried out using Bioassay 1 and Pst isolates NIAB 2016/035 or NIAB 2019/501.

the release of VOCs into the atmosphere within the test system (Bioassay 2), the VOCs being suspended in paraffin wax. Tests using Bioassay 2 also included a comparison of two Pst isolates, NIAB 2019/501 and NIAB 2020/092 (Figure 7). α-Pinene again demonstrated a significant positive effect on Pst urediniospore germination ($t=3.17$, $p=0.006$); however, the positive stimulation seen with α-linolenic acid was reduced ($t=0.22$, $p=0.826$). α-Linolenic acid is less volatile than the other VOCs tested, and embedding in paraffin would substantially retard its release into the atmosphere within the dish. The positive stimulation of Pst urediniospore germination seen with 1-hexanol ($t=-0.36$, $p=0.720$), hexyl acetate ($t=-0.61$, $p=0.54$) and *cis*-3-hexenal ($t=-0.719$, $p=0.44$) was also lost using Bioassay 2, no significant effect being seen (Table S6). However, by slowing the release of the VOC, 5-hexen-1-ol ($t=3.99$, $p<0.001$) now demonstrated a significant stimulation of Pst urediniospore germination (Table S6). Additional VOCs tested using Bioassay 2 included 3-hexen-1-ol ($t=2.92$, $p=0.009$) and the sesquiterpene caryophyllene ($t=6.09$, $p<0.001$), both VOCs enhancing Pst urediniospore germination. Octanal ($t=-2.41$, $p=0.027$) and 2,4-heptadienal ($t=-1.91$, $p=0.073$) inhibited Pst urediniospore germination, while heptanal ($t=-1.44$, $p=0.168$) and ocimene ($t=-0.50$, $p=0.623$) had no significant effect on germination (Table S6). Nonanal still inhibited germination ($t=-5.43$, $p<0.001$).

Significant differences were also found between the two Pst isolates in Experiment 4 ($F=32.66$, $p<0.001$) and Experiment 8 ($F=42.59$, $p<0.001$), but not in Experiment 5, Experiment 6 or Experiment 7 (Table S6). The percentage germination observed for isolates NIAB 2019/501 and NIAB 2020/092 in Experiment 4 and Experiment 8 were 44.6% and 56.6%, and 68.9% and 53.9%, respectively.

4 | DISCUSSION

de Vallavieille-Pope et al. (2002) demonstrated that Pst infection increased on wheat seedlings that had received greater quantities of light prior to Pst inoculation, with maximum infection being obtained on wheat seedlings that had received 30–35 mol/m^2 of light. As the penetration of stomata by Pst germ tubes occurs during the 24 h incubation period, when the plants are placed in total darkness (de Vallavieille-Pope et al., 1995), this would suggest that the effect of light quantity on Pst infection efficiency was not a consequence of the stomatal status (i.e., open vs. closed), but due to some other, light-controlled phenomenon.

In this study we confirm that germ tubes of Pst are able to enter stomata while plants are in the dark and the stomata are 'closed', as

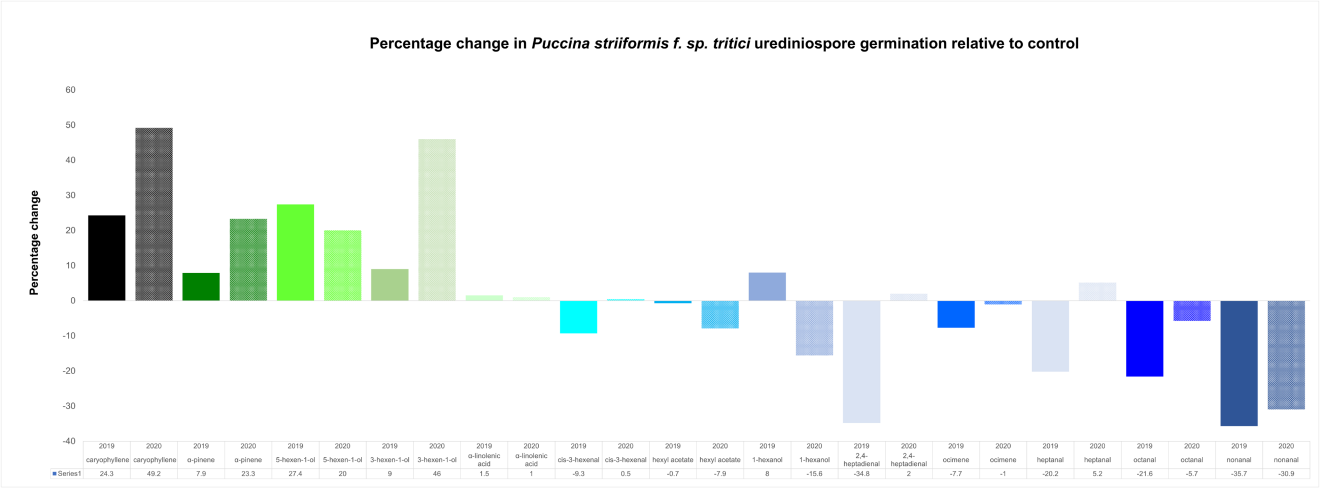

FIGURE 7 Percentage increase in *Puccinia striiformis* f. sp. *tritici* (Pst) urediniospore germination relative to control. The percentage change in Pst urediniospore germination, relative to the percentage germination of Pst spores on the control (1% agar), is shown when in the presence of caryophyllene, α-pinene, 5-hexen-1-ol, 3-hexen-1-ol, α-linolenic acid, *cis*-3-hexenal, hexyl acetate, 1-hexanol, 2,4-heptadienal, ocimene, heptanal, octanal and nonanal. These tests were carried out using Bioassay 2 and Pst isolates NIAB 2019/501 (2019) and NIAB 2020/092 (2020).

determined by measuring stomatal conductance (the rate of water transpiration). We show that enhanced Pst urediniospore germination and infection are linked to changes in VOC profiles, while a general survey of known VOCs identified individual compounds that stimulate Pst urediniospore germination. The VOCs caryophyllene, α-pinene, 5-hexen-1-ol, 3-hexen-1-ol, α-linolenic acid, *cis*-3-hexenal, hexyl acetate and 1-hexanol all proved to enhance Pst urediniospore germination, although in a concentration-dependent manner that requires further investigation. Caryophyllene and 3-hexen-1-ol were present at sufficient levels to be measurable within the headspace collections, with levels of both VOCs increasing in the collections from Lemhi seedlings exposed to high quantities of light. While additional work is required to evaluate the light-induced changes in the other VOCs identified above, our study of the effect of light quantity on Pst germination and infection efficiency opens up a novel area of further investigation.

Plant VOCs have been reported that stimulate germination and support the development of subsequent fungal structures of a number of *Puccinia* rust species (Collins et al., 2001; French, 1992; Hoch & Staples, 1991; Staples & Hoch, 1997). Both nonanal and nonanol have been found to stimulate urediniospore germination of *Puccinia* species, although *P. graminis* f. sp. *tritici* (stem rust) and *P. triticina* (leaf rust) responded better to nonanal, while the *Puccinia* species causing rye and oat crown rust preferred nonanol. *P. sorghi* (maize rust) exhibited highest germination rates in response to octanol. *cis*-3-Hexen-1-ol was found to enhance *P. graminis* f. sp. *tritici* urediniospore germination and differentiation of appressoria and SSVs in vitro (Grambow, 1977). 6-Methyl-5-hepten-2-one was also found to stimulate the germination of urediniospores of *P. triticina* and *P. coronata* (crown rust) (French et al., 1975; Rines et al., 1974). Under conditions of high humidity, a combination of mild heat shock and *trans*-2-hexen-1-ol induced the differentiation of HMCs in *P. graminis* f. sp. *tritici* (Wiethölter et al., 2003). The number of colonies on wheat infected with stem and leaf rust have also been shown to increase after exposure to decanal (Mendgen et al., 2006). However, we have not found any reports on the positive stimulation of Pst urediniospores by VOCs. While in this study *cis*-3-hexen-1-ol was found to stimulate Pst urediniospore germination, nonanal, a strong stimulator of *P. graminis* f. sp. *tritici* and *P. triticina* germination, inhibited germination of Pst, indicating that the response to VOCs is different for each of the wheat rust species.

Many biotic and abiotic environmental factors have been shown to stimulate the release of plant VOCs. Considerable work has been undertaken on the role VOCs play in the communication between plants and insects, including beneficial insects required for pollination (Ameye et al., 2018; Pichersky & Gershenzon, 2002). Plants under attack by herbivores often release specific VOCs to attract the predators of those herbivores (Moreira & Abdala-Roberts, 2019). Plant VOCs have also been shown to shape the above- and below-ground plant microbiome by attracting beneficial microbes to promote plant growth and resistance to stress, being implemented in a number of ectomycorrhizae and arbuscular mycorrhizae symbioses (Minerdi et al., 2021).

VOCs released following exposure to abiotic stresses, including high light and temperature, can have a protective function. Exposure to high light (at stress levels) resulted in a rapid increase in the levels of a number of plant VOCs, including 2-hexenal, acetaldehyde and isoprene, in *Phragmites australis* (Loreto et al., 2006). The profile of VOCs released from plants has also been shown to change throughout plant development, and to follow a circadian clock or a day–night cycle, with some VOCs emitting larger quantities during the light period, while other VOCs are predominantly released during the night (Zeng et al., 2017). The composition and synthesis of floral fragrances are known to be affected by light quality and intensity (Guenther et al., 1995; Muhlemann

et al., 2014). In lily, the floral fragrance VOCs significantly increased with a rise in light intensity, with the release of VOCs reaching a peak at 600 μmol/m^2/s (Hu et al., 2013). In lilies, light induction of the transcription factor LiMYB108 increased expression of key genes in the terpene synthesis pathway, *pkhmgr* and *pkdxr*, leading to higher levels of monoterpenes (Kawoosa et al., 2010; Yang et al., 2023).

The individual VOCs identified as having an enhancing effect on Pst urediniospore germination were chemically very different, which would exclude a common metabolic pathway influenced by the light treatment. Three major biochemical routes are involved in the synthesis of VOCs: the isoprenoid, lipoxygenase and shikimic acid pathways. The common sesquiterpene caryophyllene (C_7H_{14}) and the terpene alkene α-pinene ($C_{10}H_{16}$) were by far the most effective at stimulating Pst urediniospore germination. Both VOCs are produced via the isoprenoid biosynthesis pathway. The fatty acid α-linolenic acid ($C_{18}H_{30}O_2$) was very effective using Bioassay 1, but this effect was significantly reduced when using Bioassay 2. α-Linolenic acid is less volatile than the other VOCs tested, and embedding in paraffin was thought to retard its release into the atmosphere within the dish, reducing its effectiveness. The alcohols 1-hexanol ($CH_3(CH_2)_5OH$), 3-hexen-1-ol ($C_2H_5CH{=}CHCH_2CH_2OH$) and 5-hexen-1-ol ($HOCH_2(CH_2)_3CH{=}CH_2$) were also able to stimulate Pst urediniospore germination but their effect was concentration dependent. 1-Hexanol was effective using Bioassay 1 and Bioassay 2, while 5-hexen-1-ol was only effective using Bioassay 2 (3-hexen-1-ol was only tested using Bioassay 2). A small and less repeatable affect was observed with the saturated aldehyde *cis*-3-hexenal (Z-3-hexenal; $CH_3CH_2CH{=}CHCH_2CHO$) using Bioassay 1, which was not observed using Bioassay 2. The stimulation of Pst urediniospore germination seen with the ester hexyl acetate ($C_8H_{16}O_2$) using Bioassay 1 was not observed using Bioassay 2.

The infection efficiency of Pst, that is, the percentage of germinated urediniospores that enter a stomatal cavity to form a successful infection, is low compared to stem and leaf rust (de Vallavieille-Pope et al., 1995). Even under optimal infection conditions Pst germlings can still fail to enter a stoma and form a successful infection, and it is not uncommon for germ tubes of Pst to grow over stomata (Garrood, 2001). The findings that wheat VOCs can affect Pst urediniospore germination, either positively or negatively, and that the proportion of these VOCs released by wheat seedlings is influenced by the light quantity received, opens up a new area of investigation into the wheat–Pst interaction. Understanding how environmental variables influence wheat VOC profiles, and how these VOC mixtures influence Pst germination and infection, could help us predict how climate change scenarios may impact on disease levels and whether genetic alternations in VOC biosynthesis could enhance yellow rust resistance.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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