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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 GC-MS analysis thereof, we link this effect of high light quantity to quantitative changes in the profile of volatile organic compounds (VOC). We show that the VOC 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 VOC identified compounds that enhanced *Pst* urediniospore germination. These VOC 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.

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 monocyclic 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^{o}C, having an optimal germination temperature of 8 - 10^{o}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 hours, with up to three infection hyphae growing off the SSV by 12 hours. 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 urediniospore bearing pedicels erupt 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 and Read, 1997; Hoch and 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 (VOC) have also been shown to stimulate spore germination, and in some cases subsequent differentiation of fungal structures (Hoch and Staples, 1991; Staples and Hoch, 1997; French et al 1975; reviewed by French, 1992). In the case of *P. g.* f. sp. *tritici* a combination of VOC and surface ridges of appropriate size and spacing act synergistically to induce appressoria, sub-stomatal vesicles and infection hyphae (Collins et al., 2001), while mild heat shock and trans-2-hexen-1-ol supported the development of HMC in vitro (Wietholter 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*.

VOC are a large group of carbon-based chemicals with low molecular weights and high vapor pressure at ambient temperatures (Pichersky et al., 2006). They are produced by living organisms, including plants and fungi (Ameye et al., 2018; Bennett and 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 VOC 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 VOC play in insect attraction and repulsion, but fewer studies of how VOC may influence plant-fungal interactions. It is also known that the profile of VOC released from plants follow a day/night cycle (circadian rhythm) (Rim et al., 2019; Zang 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 hypothesised 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 vs 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 hrs and imbibed at 20°C for 48 hrs before planting out in a peat:sand (1:1) mix. Seedlings were grown in a spore-free glasshouse at 18°C/16 hrs under sodium lights (average 300µmol m^{-2} s^{-1}) and 15°C/8 hrs in darkness for 12 – 14 days, until growth stage (GS) 12-13 (Zadoks et al., 1974).

2.2 *P. striiformis* f. sp. *tritici* inoculation of wheat seedlings

Seedlings of Lemhi were inoculated with *Pst* isolates following standard procedures (Boyd and Minchin, 2001). When urediniospores had been stored over liquid nitrogen, dormancy was broken by heating the spores at 40°C for 5 minutes. The urediniospores were mixed with an equal volume of pure talcum powder and air blown on to seedlings. The seedlings were sprayed with ddH_2O containing a few drops of Tween 20 as a wetting agent before inoculation. The inoculated seedlings were placed inside a closed cabinet placed within a walk-in incubator room maintained at 8°C and >60% humidity for 24 hrs, in total darkness. Seedlings were then returned to the spore-free glasshouse under standard growing conditions, 18°C/16 hrs under sodium lights (average 300µmol m^{-2} s^{-1}) and 15°C/8 hrs in darkness. To ensure 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, Yr3, Yr4, Yr7, Yr9)* and WYR1981/02 (virulent on *Yr1, Yr2*) 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 *P. striiformis* f. sp. *tritici* 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.

Experiment 1 – Validation of results reported by Vallaviellie-Pope et al. (2002)

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

Pre-inoculation light treatments:

(1) 0 mol m^{-2} (no light for 18 hrs)

(2) 12 mol m^{-2} (185 µmol m^{-2} s^{-1} for 18 hrs = 11.99 m^{-2})

(3) 18 mol m^{-2} (278 µmol m^{-2} s^{-1} for 18 hrs = 18.01 m^{-2})

(4) 36 mol m^{-2} (556 µmol m^{-2} s^{-1} for 18 hrs = 36.03 m^{-2})

Unlike the treatments reported by de Vallaviellie-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 between wavelengths 400-700mn; LI-Cor Biosciences, UK). The temperature in the compartment was maintained between 20°C 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 hours at 20°C and a relative humidity of 40 to 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 5-6 seedlings per time point.

Experiment 2.1 and 2.2 – Assessment of stomatal status

To assess the stomatal status (open vs closed) of wheat seedlings during the normal *Pst* inoculation process, we measured stomatal conductance (the rate of water transpiration) using a AP4 cycling porometer (Delta-T Ltd, UK). 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 *Pst* incubation period held at 10°C. Seedlings of Lemhi were grown under normal glasshouse conditions, 16 hr light (300µmol m^{-2} s^{-1}) at 18°C and 8 hr 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 hrs. 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 (Expts 2.1 and 2.2). Samples for microscopy were taken at 2, 23, 26, 50 and 72 hai from three seedlings in Expt 2.1 and four seedlings in Expt 2.2.

Experiment 3 - Assessment of the influence of pre-inoculation light quantity on *P. striiformis* f. sp. *tritici* 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.

Experiment 3.1, 3.2 and 3.3

Seedlings of Lemhi were grown under normal glasshouse conditions for 12 - 14 days until GS12-13 (Zadoks et al., 1974). Forty seedlings were then subjected to one of three light treatments: total darkness, low light, or high light for 19 hours at 20°C.

Pre-inoculation light treatments:

(1) Total darkness (19 hrs)

(2) Low light (185 µmol m^{-2} s^{-1} x 19 hrs = 12.65 mol m^{-2})

(3) High light (420 µmol m^{-2} s^{-1} x 19 hrs = 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 (Expts 3.1, 3.2 and 3.3) the three sets of seedlings were inoculated simultaneously, receiving equal numbers of spores per set. In Expt 3.3 a fourth set of seedlings, that had been

left under normal 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 Expt 3.1 and 3.2 the stomatal status of the seedlings (i.e., open vs closed) was determined by measuring stomatal conductance using an A4 cycling porometer.

Twenty seedlings from each light treatment were sampled for microscopic analysis of *Pst* development and twenty seedlings were used for porometer readings. Samples for microscopy (4 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 hour before inoculation (-1) and at +2, +23, +26, +50 and +74 hai in Expts 3.1 and 3.2.

2.4 Light microscopy of *P. striiformis* f. sp. *tritici* 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 (300ml 95% ethanol, 125ml 90% lactic acid, 800g chloral hydrate, made up to 1L with chloroform; Garrood, 2001). The CH solution was changed every 24 hours 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: H_2O; 1:1:1) for 18 hours (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: (1) percentage germination – the number of urediniospores that had germinated (germling) as a proportion of the total number of urediniospores on the leaf surface; (2) percentage infection sites – the number of germinated

241 urediniospores that had successfully entered a stomata, as a percentage of the number
242 of germinated urediniospores, (3) percentage sub-stomal vesicles (SSV) – the number
243 of SSV that had formed within a sub-stomal cavity, as a percentage of the number of
244 germinated urediniospores and (4) percentage of failed infection attempts – the
245 number of germinated urediniospores that had grown over a stomata, failing to enter
246 the stomatal cavity, as a percentage of the number of germinated urediniospores.
247
248 **2.5 Collection and analysis of volatile organic compounds from wheat seedings**
249 **exposed to different quantities of light**
250 Seedlings of Lemhi were grown to GS 14 under normal glasshouse conditions (16 hr
251 light, 320 µmol m^{-2} s^{-1}, at 21°C and 8 hr night/16°C). Seedlings were transferred either
252 to total darkness or high light conditions (420 µmol m^{-2} s^{-1}) for 19 hours. Light levels
253 were measured using the light meter SKP 200 51303, calibrated for sensor SKP 215/I
254 51304, which measures the light intensity paired with a PAR Quantum sensor (400
255 nm – 700 nm; Skye Instruments Ltd, UK). Dynamic headspace collections were then
256 carried out using air entrainment kits (Pye volatile collection kit, Hertfordshire, UK)
257 with plants enclosed in transparent cooking bags (Sainsbury's Supermarkets Ltd, UK)
258 secured by wire ties. Porapak Q adsorbent tubes, consisting of a 5 mm diameter
259 borosilicate tube filled with 50 mg Porapak Q (Supelco, Bellefonte, USA),
260 sandwiched between two glass wool plugs, were used to collect VOC within the
261 headspace. Charcoal-filtered air was passed into the cooking bag at 500 mL min^{-1} and
262 pulled out through the adsorbent tube at 400 mL min^{-1} over the 19 hrs of the dark vs
263 high light treatment, the VOC emitted by the plants being absorbed onto the Porapak
264 Q. Headspace VOC were extracted from the Porapak Q tubes by eluting with 750 µL

of diethyl ether (Fisher Scientific, UK). 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 VOC in the wheat dynamic headspace collections. Four µl of each headspace extracted was analysed using an Agilent 8890GC-5977B GC-MSD fitted with a non-polar HP1 column, 50 m length x 0.32 mm inner diameter x 0.52 µm film thickness (J&W Scientific), using the following conditions: 30°C for 5 mins, rising 5°C min^{-1} to 150°C, followed by 10°C min^{-1} to 230°C, for a total run time of 60 min. Tentative identification of compounds observed between run times 8 to 35 mins was achieved using the retention index (RI) values obtained using GC-MS analysis and NIST mass spectral library (2020, NIST, Gaithersburg, USA).

2.6 Bioassays developed to test the effect of volatile organic compounds on germination of *P. striiformis* f. sp. *tritici* urediniospores

Bioassay 1: Agar 4550 (1% solution; supplier, Sigma Ltd) 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 was placed (approx. 5ul) 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 to 10°C and > 60% humidity for 24 hrs, in total darkness. The control was an agar covered glass slide without VOC placed in a separate dish.

289 *Bioassay 2*: As many of the VOC tested proved to inhibit *Pst* urediniospore
290 germination we modified Bioassay 1. The VOC was suspended in parafilm wax to
291 provide a controlled release of the compound into the atmosphere within the plastic
292 dish. The VOC (5ul/g) was added to melted parafilm wax (supplier, Acros Organics).
293 Approx. 1.5 g of the wax-VOC suspension was placed on a glass slide. The slide was
294 placed in the middle of the plastic dish, on paper towel moistened with 10 ml of
295 distilled water. *Pst* urediniospores were placed on a glass slide covered in 1% agar
296 using a cotton bud. Three slides with *Pst* isolate NIAB 2019/501 and three slides with
297 *Pst* isolate NIAB 2020/092 were placed around the wax-VOC slide. The lid was
298 placed on the plastic dish and the dish covered in aluminium foil. The dish was placed
299 in the incubator as described for Bioassay 1. The control was as above, but without
300 the wax-VOC slide. Bioassay 2 was also used to test the effect on *Pst* urediniospore
301 germination of the VOC contained within the headspace collections obtained from
302 seedlings of Lemhi grown under conditions of high light and total darkness for 19 hrs.
303 Agar (1%) and ether were used as controls.

304 The plastic dish containing the control could not be placed in the incubator at
305 the same time as the dish with the test VOC, as despite the dishes being wrapped in
306 aluminium foil VOC could still escape from the dish. Therefore, each VOC and the
307 controls were tested on consecutive days, using the same incubator and the same
308 batch of urediniospores. Germination of *Pst* urediniospores was measured
309 microscopically 24 hai, with approximately 100 urediniospores being scored on each
310 slide. The *Pst* isolates used in these VOC bioassays were maintained as a fresh supply
311 of urediniospores, collected from seedlings prior to use. The isolates used were NIAB
312 2016/035, NIAB 2019/501 and NIAB 2020/092. As the percentage of *Pst* germination
313 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 VOC 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 VOC tested, with IUPAC identifier information, are listed in Table S1. They were all obtained from Merck KGaA, Darmstadt, Germany, and were either Sigma-Aldrich or MilliporeSigma branded.

2.7 Statistical analyses

The microscopy data, from both the light and VOC treatment experiments, were analysed using Genstat v. 16 and 20 (VSN International 2020). Analysis 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 effected *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 hr of darkness or 19 hrs of high light, with t-test comparisons performed to determine whether headspace collections effected *Pst* urediniospore germination. Principal component analysis was performed using the factoextra package (Kassambara and

Mundt, 2020) in R in order to observe trends, clusters and outliers among the four biological replicate headspace collections, and plotted using ggplot2 package (Wickham, 2016).

3. Results

3.1 Effect of pre-inoculation light quantity received by wheat seedlings on subsequent *P. striiformis* f. sp. *tritici* infection

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

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 seedling which 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

363 locating stomata, but the numbers were significantly lower than on seedlings exposed
364 to higher quantities of light prior to inoculation (Figure 1).

365 Under the light microscope the majority of stomata appeared closed, although
366 more stomata appeared open on the seedlings that had received the high light
367 treatments prior to *Pst* inoculation. Concerned that the prolonged periods of low light
368 and darkness used by de Vallavieille de Pope et al. (2002; 16 hrs of low light at 45
369 µmol m^{-2} s^{-1} and 8 hrs of darkness at 8°C for 3 days, followed by total darkness for 16
370 hrs at 8°C) prior to light quantity treatments may have compromised stomatal
371 opening, we modified the pre-light treatments in subsequent experiments. This led us
372 to a series of experiments (Experiments 3) that identified a pre-inoculation light
373 regime that enhanced *Pst* infection efficiency, but without compromising stomatal
374 status, stomatal status being monitored in subsequent experiments by measuring water
375 transpiration with an A4 cycling porometer (Delta-T Ltd, UK).

376

377 **Experiments 2: Determination of stomatal status during *P. striiformis* f. sp. *tritici***
378 **infection**

379 The protocol used for *Pst* infection requires the plants to be placed in total darkness
380 for 24 hrs, at a humidity > 60%, immediately after inoculation (Boyd and Minchin,
381 2001). Therefore, it is generally considered that *Pst* can enter the plant through closed
382 or partially closed stomata (de Vallavieille de Pope et al., 1995). To determine the
383 status of wheat seedling stomata during the normal *Pst* incubation period and how this
384 influences the infection efficiency of *Pst,* we measured stomatal conductance just
385 before inoculation with *Pst* urediniospores, during the *Pst* incubation period, and after
386 the seedlings were returned to the glassshouse under normal growing conditions. For
387 these experiments Lemhi seedlings were grown under normal growth conditions prior

388 to inoculation with *Pst*, i.e., for 16 hr of light (300 µmol m^{-2} s^{-1}) at 18°C and 8 hr dark
389 at 15°C for 14 days.

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

397

398 **Experiments 3: Standardisation of pre-inoculation light treatments**

399 The pre-inoculation light treatments were simplified to develop a repeatable test
400 system whereby the effects of pre-inoculation light quantities, received by wheat
401 seedlings, on subsequent *Pst* infection efficiency could be studied. Here we report the
402 results from three repeat experiments, Expt 3.1, 3.2 and 3.3. In Expt 3.1 and 3.2 the
403 stomatal status of the seedlings (i.e., open vs closed) was also determined by
404 measuring stomatal conductance, using an A4 cycling porometer, before and after
405 inoculation with *Pst*.

406 The quantity of light received by Lemhi seedlings immediately prior to
407 inoculation with *Pst* urediniospores had a significant effect on urediniospore
408 germination and the ability of the germlings to locate and enter stomata (Figure 4).
409 Significantly more germination was observed on seedlings that had received the
410 highest quantities of light prior to inoculation (Table S2). On seedlings exposed to 19
411 hrs of darkness germination was greatly reduced, even after the inoculated seedlings
412 had been returned to normal light conditions 24 hai. The number of germinated

413 urediniospores did not reach those seen on the seedlings exposed to low and high pre-
414 inoculation light treatments, even by 74 hai.

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

427 Stomatal conductance measured 1 hour before inoculation with *Pst*
428 uredioniospores, at which time the three sets of seedlings were still receiving different
429 light treatments, clearly indicated that the stomata of the seedlings receiving the dark
430 treatment were closed, while those receiving low and high light treatments were open
431 (Figure S1). After inoculation with *Pst* urediniospores the three sets of seedlings were
432 placed in total darkness for 24 hours. This resulted in a reduction in stomatal
433 conductance in all seedling sets. Following transfer back to normal light/dark cycles
434 in the glasshouse (post 24 hai) the stomata in each set of seedlings subsequently
435 followed the same pattern of opening and closing, indicating that the pre-inoculation
436 light treatments had not compromised stomatal opening and closing (Figure S1).
437

438 **3.2 The effect of light quantity on the volatile organic compound profiles**
439 **produced by seedlings of Lemhi**

440 The influence of light on the VOC released (headspace collections) by wheat
441 seedlings was assessed in the wheat variety Lemhi. The VOC identified by GC-MS
442 are a general survey of the plant volatiles that represent the wheat volatolome of
443 Lemhi (Figure S2). Differences were seen between seedlings exposed to total
444 darkness vs high light (Table S3), with a general trend towards greater levels of VOC
445 being released from seedling exposed to high light (Table S3). The increase in VOC
446 concentrations was small in the case of hexanal, undecane, octanoic acid and
447 tridacane, but substantially greater for the VOC 3-hexen-1-ol, heptanal, octanal, Z-
448 ocimene, 3-hexen-1-ol acetate and linalool.

449

450 **3.3 The effect of the headspace collections from Lemhi on germination of *P.***
451 ***striiformis* f. sp. *tritici* urediniospores**

452 The headspace collections from the Lemhi seedlings subjected to 19 hrs of darkness
453 vs 19 hrs of high light (420 µmol $m^{-2}s^{-1}$) were tested for their effect on *Pst*
454 uredioniospore germination using Bioassay 2. Two controls were included, looking at
455 urediniospore germination on 1% agar without any VOC embedded in the parafilm
456 wax block and a second control where ether was placed in the wax block, the
457 headspace extracts having been collected in ether. Four experiments were undertaken,
458 however PCA of the GC-MS data indicated that the dark treatment sample from Expt
459 3 was an outlier (Figure S3). Therefore, the headspace collections from Expt 3 were
460 not assessed for the effect on *Pst* urediniospore germination. The headspace
461 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 Expt 1 and 4 (t-test $p < 0.001$), but not in Expt 2 (t-test $p = 0.124$). However, in Expt 1 ether inhibited *Pst* urediniospore germination while in Expt 4 germination was enhanced by ether (Figure S4 and 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). *Pst* urediniospore germination in the presence of the headspace collection from the Lemhi seedlings exposed to 19 hrs of darkness was only significantly different from the ether control in Expt 1 (t-test $p = 0.005$; Table S4). However, a significant effect of the headspace collection from Lemhi seedlings exposed to 19 hrs of light (equivalent to 420 µmol $m^{-2}s^{-1}$) was seen on *Pst* urediniospore germination in all three experiments (t-test probabilities: Expt 1 $p = 0.004$, Expt 2 $p < 0.002$ and Expt 4 $p = 0.056$). Significant differences were also observed between the two *Pst* isolates (Expt 1 $Fp = 0.002$, Expt 2 $Fp < 0.001$ and Expt 4 $Fp = 0.002$; 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 volatile organic compounds that influence germination of *P. striiformis* f. sp. *tritici* urediniospores

Based on the working hypothesis that VOC released by wheat seedlings stimulate *Pst* urediniospores to germinate we developed an in vitro bioassay (Bioassay 1) to test the effect of individual VOC on *Pst* urediniospore germination. We selected VOC that exhibited quantitative changes in Lemhi seedlings exposed to high quantities of light, as well as VOC reported in green plant tissues in the literature (Table S5). The following VOC were found to consistently increase the number of urediniospores that germinated relative to the control (1% agar); α-pinene > α-linolenic acid > 1-hexanol

488 > hexyl acetate > cis-3-hexenal (Figure 6). All other VOC tested either completely
489 eliminated germination of *Pst* urediniospores or reduced the percentage of
490 germination relative to the control (Table S5). *Pst* germination was reduced in the
491 presence of isoprene by about 36%, but completely inhibited in the presence of
492 hexanal, decanal, nonanal, benzaldehyde, acetophenone, linalool, penten-3-one and 5-
493 hexen-1-ol.

494 As many of the VOC tested appeared to be toxic, inhibiting *Pst* urediniospore
495 germination, we modified the in vitro bioassay to slow the release of VOC into the
496 atmosphere within the test system (Bioassay 2), the VOC being suspended in parafilm
497 wax. Tests using Bioassay 2 also included a comparison of two *Pst* isolates, NIAB
498 2019/501 and NIAB 2020/092. Alpha-pinene again demonstrated a significant
499 positive effect on *Pst* urediniospore germination ($t = 3.17$, $p = 0.006$), however, the
500 positive stimulation seen with α-linolenic acid was reduced ($t = 0.22$, $p = 0.826$).
501 Linolenic acid is less volatile than the other VOC tested, and embedding in parafilm
502 would substantially retard its release into the atmosphere within the dish. The positive
503 stimulation of *Pst* urediniospore germination seen with 1-hexanol ($t = -0.36$, $p =$
504 0.720), hexyl acetate ($t = -0.61$, $p = 0.54$) and cis-3-hexenal ($t = -0.719$, $p = 0.44$) was
505 also lost using Bioassay 2, no significant effect being seen (Table S6). However, by
506 slowing the release of the VOC, 5-hexen-1-ol ($t = 3.99$, $p < 0.001$) now demonstrated
507 a significant stimulation of *Pst* urediniospore germination (Table S6). Additional
508 VOC tested using Bioassay 2 included 3-hexen-1-ol ($t = 2.92$, $p = 0.009$) and the
509 sesquiterpene, caryophyllene ($t = 6.09$, $p > 0.001$), both VOC enhancing *Pst*
510 urediniospore germination. Octanal ($t = -2.41$, $p = 0.027$) and 2,4-heptadienal ($t = -$
511 1.91, $p = 0.073$) inhibited *Pst* urediniospore germination, while heptanal ($t = -1.44$, p

512 = 0.168) and ocimene (t = -0.50, p = 0.623) had no significant effect on germination
513 (Table S6). Nonanal still inhibited germination (t = -5.43, p < 0.001).
514 Significant differences were also found between the two *Pst* isolates in Expt 4
515 (F = 32.66, p < 0.001) and Expt 8 (F = 42.59, p < 0.001), but not in Expt 5, Expt 6 or
516 Expt 7 (Table S6). The percentage germination observed for isolates NIAB 2019/501
517 and NIAB 2020/092 in Expt 4 and Expt 8 were 44.6% and 56.6%, and 68.9% and
518 53.9%, respectively.
519
520 **4. Discussion**
521 De Vallavielle-Pope et al. (2002) demonstrated that *Pst* infection increased on wheat
522 seedlings that had received greater quantities of light prior to *Pst* inoculation, with
523 maximum infection being obtained on wheat seedlings that had received 30-35 mol m^{-2}
524 of light. As the penetration of stomata by *Pst* germ tubes occurs during the 24 hour
525 incubation period, when the plants are placed in total darkness (de Vallavieille-Pope
526 et al., 1995), this would suggest that the effect of light quantity on *Pst* infection
527 efficiency was not a consequence of the stomatal status (i.e., open vs closed), but due
528 to some other, light-controlled phenomenon.
529 In this study we confirm that germ tubes of *Pst* are able to enter stomata while
530 plants are in the dark and the stomata are "closed", as determined by measuring
531 stomatal conductance (the rate of water transpiration). We show that enhanced *Pst*
532 urediniospore germination and infection are linked to changes in VOC profiles, while
533 a general survey of known VOC identified individual compounds that stimulate *Pst*
534 urediniospore germination. The VOC caryophyllene, α-pinene, 5-hexen-1-ol, 3-
535 hexen-1-ol, linolenic acid, cis-3-hexenal, hexyl acetate and 1-hexanol all proved to
536 enhance *Pst* urediniospore germination, although in a concentration dependent
537 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 VOC 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 VOC 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 VOC 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. g.* f. sp. *tritici* urediniospore germination and differentiation of appressoria and SSV *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) (Rines et al., 1974; French et al., 1975). Under conditions of high humidity, a combination of mild heat shock and *trans*-2-hexen-1-ol induced the differentiation of haustorial mother cells (HMC) in *P. g.* 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 VOC. While in this study cis-3-hexen-1-ol was found to stimulate *Pst* urediniospore germination, nonanal, a strong stimulator of *P. g.* f. sp. *tritici* and *P. triticina* germination, inhibited

563 germination of *Pst*, indicating that the response to VOC is different for each of the
564 wheat rust species.

565 Many biotic and abiotic environmental factors have been shown to stimulate
566 the release of plant VOC. Considerable work has been undertaken on the role VOC
567 play in the communication between plants and insects, including beneficial insects
568 required for pollination (Pichersky & Gershenzon, 2002; Ameye et al., 2018). Plants
569 under attack by herbivores often release specific VOC to attract the predators of those
570 herbivores (Moreira & Abdala-Roberts, 2019). Plant VOC have also been shown to
571 shape the above- and below-ground plant microbiome by attracting beneficial
572 microbes to promote plant growth and resistance to stress, being implemented in a
573 number of ectomycorrhizae and arbuscular mycorrhizae symbioses (Minerdi et al.,
574 2021).

575 VOC released following exposure to abiotic stresses, including high light and
576 temperature, can have a protective function. Exposure to high light (at stress levels)
577 resulted in a rapid increase in the levels of a number of plant VOC, including 2-
578 hexenal, acetaldehyde and isoprene in *Phragmites australis* (Loreto et al., 2006). The
579 profile of VOC released from plants has also been shown to change throughout plant
580 development, and to follow a circadian clock or a day-night cycle, with some VOC
581 emitting larger quantities during the light period, while other VOC are predominantly
582 released during the night (Zeng et al., 2017). The composition and synthesis of floral
583 fragrances are known to be affected by light quality and intensity (Guenther et al.,
584 1995; Muhlemann et al., 2014). In lily, the floral fragrance VOC significantly
585 increased with a rise in light intensity, with the release of VOC reaching a peak at 600
586 µmol m^{-2} s^{-1} (Hu et al., 2013). In lilies, light induction of the transcription factor
587 LiMYB108 increased expression of key genes in the terpene synthesis pathway,

588 *pkhmgr* and *pkdxr*, leading to higher levels of monoterpenes (Kawoosa et al., 2010;
589 Yun-Yao et al., 2023).

590 The individual VOC identified as having an enhancing effect on *Pst*
591 urediniospore germination were chemically very different, which would exclude a
592 common metabolic pathway influenced by the light treatment. Three major
593 biochemical routes are involved in the synthesis of VOC, the isoprenoid, lipoxygenase
594 and shikimic acid pathways. The common sesquiterpene, caryophyllene (C_7H_{14}) and
595 the terpene, alkene α-pinene ($C_{10}H_{16}$) were by far the most effective at stimulating *Pst*
596 urediniospore germination. Both VOC are produced via the isoprenoid biosynthesis
597 pathway. The fatty acid linolenic acid ($C_{18}H_{30}O_2$) was very effective using Bioassay
598 1, but this effect was significantly reduced when using Bioassay 2. Linolenic acid is
599 less volatile than the other VOC tested, and embedding in parafilm was thought to
600 retard its release into the atmosphere within the dish, reducing its effectiveness. The
601 alcohols 1-hexanol (($CH_3(CH_2)_5OH$)), 3-hexen-1-ol (C2H5CH=CHCH2CH2OH) and
602 5-hexen-1-ol ((HOCH2(CH2)3CH=CH2)) were also able to stimulate *Pst*
603 urediniospore germination but their effect was concentration dependent. 1-hexanol
604 was effective using Bioassay 1 and Bioassay 2, while 5-hexen-1-ol were only
605 effective using Bioassay 2 (3-hexen-1-ol was only tested using Bioassay 2). A small
606 and less repeatable affect was observed with the saturated aldehyde cis-3-hexenal (Z-
607 3-hexenal; $CH_3CH_2CH=CHCH_2CHO$) using Bioassay 1, which was not observed
608 using Bioassay 2. The stimulation of *Pst* urediniospore germination seen with the
609 ester hexyl-acetate ($C_8H_{16}O_2$) using Bioassay 1 was not observed using Bioassay 2.

610 The infection efficiency of *Pst*, i.e. the percentage of germinated
611 urediniospores that enter a stomatal cavity to form a successful infection, is low
612 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 VOC can affect *Pst* urediniospore germination, either positively or negatively, and that the proportion of these VOC 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

637 The authors declare that the research was conducted in the absence of any commercial
638 or financial relationships that could be construed as a potential conflict of interest.
639
640 **Data Availability Statement**
641 The data that support the findings of this study are available from the corresponding
642 author upon reasonable request.
643
644 **Ethics approval and consent to participate**
645 Experimental research on plants, including the collection of plant material, complied
646 with institutional, national and international guidelines and legislation.
647
648 **Author Contributions**
649 LB designed the research and drafted the manuscript. RMcC undertook the light
650 quantity experiments. CZ, AG and HJ undertook the VOC Bioassays 1 and 2. JC
651 undertook the headspace collections and GC-MS analysis of VOC. AH maintained the
652 supply of *Puccinia striiformis* f. sp. *tritici* urediniospores. LB and JC were awarded
653 project funding. CZ and JC contributed to writing the manuscript. All authors edited
654 and approved the manuscript.
655
656 **Abbreviations**
657 CH: chloral hydrate; dai: days after inoculation; Expt.: experiment; GS: growth stage;
658 hai: hours after inoculation; hbi: hours before inoculation; HMC: haustorial mother
659 cell; *Pst*: *Puccinia striiformis* f. sp. *tritici*; SSV: sub-stomatal vesicle; VOC: volatile
660 organic compounds
661

662 **References**

663 Allen, E.A., Hazen, B.E., Hoch, H.C., Kwon, Y., Leinhos, G.M.E., Staples, R.C. et al.
664 (1991) Appressorium formation in response to topographical signals by 27 rust
665 species. *Phytopathology,* 81, 323-331.

666

667 Ameye, M., Silke-Allmann, S., Verwaeren, J., Smagghe, G., Haesaert, G., Schuurink,
668 R.C. et al. (2018) Green leaf volatile production by plants: a meta-analysis. *New*
669 *Phytologist*, 220 666–683. doi: 10.1111/nph.14671

670

671 Bennett, J.W. & Inamdar, A.A. (2015) Are some fungal volatile organic compounds
672 (VHCs) mycotoxins? *Toxins*, 7, 3785–3804.

673

674 Boncan, D.A.T., Tsang, S.S.K., Li, C., Lee, I.H.T., Lam, H.M., Chan, T.F. et al.,
675 (2020). Terpenes and Terpenoids in Plants: Interactions with Environment and
676 Insects. *International Journal of Molecular Science*, 21, 7382. doi:
677 10.3390/ijms21197382.

678

679 Bouwmeester et al., Harro Bouwmeester, Schuurink, R.C., Bleeker, P.M. & Schiestl,
680 F. (2019) The role of volatiles in plant communication. *The Plant Journal*, 100, 892-
681 907. 10.1111/tpj.14496

682

683 Boyd, L.A. (2006) Perspectives: Can the durability of resistance be predicted?
684 *Journal of the Science of Food and Agriculture*, 86, 2523-2526.

685

686 Boyd, L.A. (2005) Can Robigus defeat an old enemy? - Yellow rust of wheat.
687 Centenary Review. *Journal of Agricultural Science*, 143, 233-243.
688
689 Boyd, L.A. & Minchin, P.N. (2001) Wheat mutants showing altered adult plant
690 disease resistance. *Euphytica*. 122, 361–368.
691
692 Bozkurt, O., McGrann, G., Unver, T., MacCormack, R., Boyd, L.A. & Akkaya, M.S.
693 (2010) Cellular and transcriptional responses of wheat during compatible and
694 incompatible race-specific interactions with *Puccinia striiformis* f. sp. *tritici*.
695 *Molecular Plant Pathology* 11, 625–640. https://doi.org/10.1111/j.1364-
696 3703.2010.00633.x
697
698 Brilli. F., Loreto, F., Baccelli, I. (2019) Exploiting plant volatile organic compounds
699 (VOCs) in Agriculture to improve sustainable defense strategies and productivity of
700 crops, Frontiers in Plant Science, 10, 10.3389/fpls.2019.00264
701
702 Collins, T.J., Moerschbacher, B.M. & Read, N.D. (2001) Synergistic induction of
703 wheat stem rust appressoria by chemical and topopraphical signals. *Physiological and*
704 *Molecular Plant Pathology,* 58, 259-266.
705
706 Collins, T.J. & Read, N.D. (1997) Appressorium induction by topographical signals in
707 six cereal rusts. *Physiological and Molecular Plant Pathology*, 51, 169-179.
708
709 Collins, T.J. (1996) Appressorium induction in the cereal rusts. PhD thesis, Uni of
710 Edinburgh

de Vallavieille-Pope, C., Huber, L., Leconte, M. & Goyeau, H. (1995) Comparative effects of temperature and interrupted wet periods on germination, penetration, and infection of *Puccinia recondita* f. sp. *tritici* and *P. striiformis* urediniospores on wheat seedlings. *Phytopathology*, 85, 409-415.

de Vallavieille-Pope, C., Giosue, S., Munk, L., Newton, A. C., Niks, R. E., Østergård, et al., (2000) Assessment of epidemiological parameters and their use in epidemiological and forecasting models of cereal airborne diseases. *Agronomie*, 20, 715-727.

de Vallavieille-Pope, C., Huber, L., Leconte, M. & Bethenod, O. (2002) Pre-inoculation effects of light quantity on infection efficiency of *Puccinia striiformis* and *P. triticina* on wheat seedlings. *Phytopathology* 92, 1308-1314.

French, R.C., Gale, A.W., Graham, C.L., Latterell, F.M., Schmitt, C.G., Marchetti, M.A. et al. (1975) Differences in germination response of spores of several species of rust and smut fungi to nonanal, 6-methyl-5-hepten-2-one, and related compounds. *Journal of Agricultural and Food Chemistry*, 23, 766-770.

French, R.C. (1992) Volatile chemical germination stimulators of rust and other fungal spores. *Mycologia*, 84, 277–288.

Garrood, J.M. (2001) The Interaction of *Puccinia striiformis* with Wheat and Barley. Norwich, UK: University of East Anglia, PhD thesis.

Grambow, H.J. (1977) The influence of volatile leaf constituents on the in vitro differentiation and growth of *Puccinia graminis* f. sp. *tritici*. *Zeitschrift für Pflanzenphysiologie* Bd, 85, 361-372.

Guenther, A., Hewitt, C.N., Erickson, D., Fall, R., Geron, C., Graedel, T. et al. (1995) A global model of natural volatile organic compound emissions. *Journal of Geophysical Research*, 100, 8873–8892.

Hoch, H.C. & Staples, R.C. (1991). Signalling for Infection Structure Formation in Fungi. In: Cole, G.T., Hoch, H.C. (eds) The Fungal Spore and Disease Initiation in Plants and Animals. Springer, Boston, MA. https://doi.org/10.1007/978-1-4899-2635-7_2

Hu, Z.H., Zhang, H.X., Leng, P.S., Zhao, J., Wang, W.H. & Wang, S.D. (2013) The emission of floral scent from *Lilium* 'siberia' in response to light intensity and temperature. *Acta Physiologiae Plantarum*, 35, 1691–1700.

Jagger, L.J., Newell, C., Berry, S.T, MacCormack, R. & Boyd, L.A. (2011) Histopathology provides a phenotype by which to characterise stripe rust resistance genes in wheat. *Plant Pathology*, 60, 640-648.

Jin, Y., Szabo, L.J. & Carson, M. (2010). Century-old mystery of *Puccinia striiformis* life history solved with the identification of Berberis as an alternate host. *Phytopathology*, 100, 432-435.

Kassambara, A. & Mundt, F. (2020) Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. Available online: https://CRAN.R-project.org/package=factoextra (accessed on 20 March 2024)

Kawoosa, T., Singh, H., Kumar, A., Sharma, S.K., Devi, K., Dutt, S., et al. (2010) Light and temperature regulated terpene biosynthesis: hepatoprotective monoterpene picroside accumulation in *Picrorhiza kurrooa*. *Functional Integrative Genomics*, 10, 393–404.

Melichar, J.P.E, Berry, S., Newell, C., MacCormack, R. & Boyd, L.A. (2008) QTL identification and microphenotype characterisation of the developmentally regulated yellow rust resistance in the UK wheat cultivar Guardian. *Theoretical and Applied Genetics*, 117, 391-399.

Mendgen, K., Stefan, G.R., Wirsel, A.J., HoVmann, J. & Boland, W. (2006) Volatiles modulate the development of plant pathogenic rust fungi. *Planta*, 224, 1353–1361 DOI 10.1007/s00425-006-0320-2

Michereff, M.F.F., Magalhaes, D.M., Hassemar, M.J., Laumann, R.A., Zhou, J-J., Riberio, P.E. de A. et al., (2018). Variability in herbivore induced defense signalling across different maize genotypes impacts significantly on natural enemy foraging behaviour. *Journal of Pest Science*, 92, 723-736. 10.1007/s10340-018-1033-6

785 Minerdi, D., Maggini, V. & Fani, R. (2021) Volatile organic compounds: from
786 figurants to leading actors in fungal symbiosis. *FEMS Microbiology Ecology*, 97,
787 2021, fiab067. doi: 10.1093/femsec/fiab067
788
789 Moreira, X. & Abdala-Roberts, L. (2019) Specificity and context-dependency of
790 plant-plant communication in response to insect herbivory. *Current Opinions in Insect*
791 *Science*, ;32, 15-21. doi: 10.1016/j.cois.2018.09.003.
792
793 Muhlemann, J.K., Klempien, A. & Dudareva, N. (2014) Floral volatiles: from
794 biosynthesis to function. Plant Cell Environment, 37, 1936–1949.
795
796 Read N.D., Kellock, L.J., Collins, T.J. & Gundlach, A.M. (1997) Role of topography
797 sensing for infection-structure differentiation in cereal rust fungi. *Planta*, 202, 163-
798 170.
799
800 Pichersky, E., Noel, J.P. & Dudareva, N. (2006) Biosynthesis of plant volatiles:
801 Nature's diversity and ingenuity. *Science*, 331, 808–811.
802
803 Pichersky, E. & Gershenzon, J. (2002) The formation and function of plant volatiles:
804 perfumes for pollinator attraction and defense. Current Opinions in Plant Biology, 5,
805 237-43. doi: 10.1016/s1369-5266(02)00251-0. PMID: 11960742.
806
807 Rim, H., Sekiguchi, S., Ozawa, R., Kainoh, Y. & Arimura, G-I. (2019) Diurnal
808 rhythm of volatile emissions from damaged *Brachypodium distachyon* affects the

809 temporal preferences of tritrophic interactions. *Journal of Plant Interactions,* 14, 143-
810 150. https://doi.org/10.1080/17429145.2019.1602888.
811
812 Rines, H.W., French, R.C. & Daasch, L.W. (1974) Nonanal and 6-methyl-5-hepten-2-
813 one: Endogenous germination stimulators of uredospores of *Puccinia graminis* f. sp.
814 *tritici* and other rusts. *Journal of Agriculture and Food Chemistry*, 22, 96-100.
815
816 Russel, G.E. (1976) Germination of *Puccinia striiformis* uredospores on leaves of
817 adult winter wheat plants. *Annuals of Applied Biology*, 82, 71-78.
818
819 Staples, R.C. & Hoch, H.C. (1997) Physical and chemical cues for spore germination
820 and appressorium formation by fungal pathogens. In: Carroll GC, Tudzynski P (eds)
821 The mycota V, part A. Springer, Berlin Heidelberg New York, pp 27–40
822
823 VSN International. (2020) Genstat for Windows, 21st ed. VSN International, Hemel
824 Hempstead, U.K. (https://vsni.co.uk/software/genstat.
825
826 Wickham, H. (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag
827 New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.
828
829 Wietholter, N., Horn, S., Reisige, K., Beike, U. & Moerschbacher, B.M. (2003) In
830 vitro differentiation of haustorial mother cells of the wheat stem rust fungus, *Puccinia*
831 *graminis* f. sp. *tritici*, triggered by the synergistic action of chemical and physical
832 signals. *Fungal Genetics and Biology,* 38, 320-326. doi:10.1016/S1087-
833 1845(02)00539-X

834

835 Yun-Yao, Y., Zhang, X., Han, M-Z., Hu, Z-H., Wu, J., Ma, N. et al. (2023)
836 LiMYB108 is involved in floral monoterpene biosynthesis induced by light intensity
837 in *Lilium* 'Siberia'. *Plant Cell Reports*, 42, 763–773. https://doi.org/10.1007/s00299-
838 023-02995-x

839

840 Zadoks, J.C., Chang, T.T. & Konzak C. F. (1974) A decimal code for the growth
841 stages of cereals. *Weed Research*, 14, 415–21.

842

843 Zeng, L., Wang, X., Kang, M., Dong, F. & Yang Z. (2017) Regulation of the
844 Rhythmic Emission of Plant Volatiles by the Circadian Clock. *International Journal*
845 *of Molecular Science* 18, 2408. doi: 10.3390/ijms18112408.

847 **Figure Legends**

848

849 **Figure 1 Percentage of *Puccinia striiformis* f. sp. *tritici* (*Pst*) infection sites**
850 **measured as the percentage of germinated urediniospores that entered a stoma.**
851 *Pst* urediniospore germination was assessed microscopically on seedlings of the wheat
852 variety Lemhi that had received 0, 12, 18 or 36 mol m^{-2} of light, over an 18 hour
853 period, before inoculation with *Pst*. The error bars show standard errors between
854 replicate measurements.

855

856 **Figure 2 Stomatal conductance measured before and after inoculation with**
857 **urediniospores of *Puccinia striiformis* f. sp. *tritici* (*Pst*).** The timepoints show
858 porometer readings taken before (minus) and after (plus) *Pst* inoculation. Area in the
859 box covers the incubation period when wheat seedling inoculated with *Pst*
860 urediniospores are placed in total darkness for 24 hours, at 8°C and > 60% humidity.
861 Porometer readings from Expt 2.1 blue line, and Expt 2.2 brown line. The error bars
862 show standard errors between replicate measurements.

863

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

869

870 **Figure 4 The number of *Puccinia striiformis* f. sp. *tritici* (*Pst*) urediniospores that**
871 **had germinated (germlings), grown over a stoma (over stomata) or entered a**

872 **stoma (in stomata) on wheat seedlings of variety Lemhi.** Germinated
873 urediniospores were assessed at 2, 23, 26, 48 or 50 and 74 hours after inoculation
874 (hai) on wheat seedlings that had been exposed to different quantities of light
875 immediately prior to inoculation with *Pst.* Expt 3.3 also included a set of Lemhi
876 seedlings that did not receive a pre-inoculation light treatment (control), but had been
877 left in the glasshouse, under normal light conditions, until inoculation.
878
879 **Figure 5 Percentage change in *Puccinia striiformis* f. sp. *tritici* (*Pst*) urediniospore**
880 **germination relative to ether control.** The change in percentage germination of
881 *Puccinia striiformis* f. sp. *tritici* (*Pst*) urediniospores caused by the headspace
882 collections from Lemhi seedlings exposed to 19 hrs of darkness (dark) or 19 hrs of
883 high light equalling 420 µmol $m^{-2}s^{-1}$ (high light) compared to the ether control. Tests
884 were carried out using *Pst* isolates, NIAB 2019/501 (2019) and NIAB 2020/092
885 (2020).
886
887 **Figure 6 Percentage increase in *Puccinia striiformis* f. sp. *tritici* (*Pst*)**
888 **urediniospore germination relative to control.** The percentage increase in *Pst*
889 urediniospore germination, relative to the percentage germination of *Pst* spores on the
890 control (1% agar Sigma 4550), is shown when in the presence of the VOC α-pinene,
891 α-linolenic acid, 1-hexanol, hexyl-acetate and cis-3-hexanal. These tests were carried
892 out using Bioassay 1 and *Pst* isolates NIAB 2016/035 or NIAB 2019/501.
893
894 **Figure 7 Percentage increase in *Puccinia striiformis* f. sp. *tritici* (*Pst*)**
895 **urediniospore germination relative to control.** The percentage increase in *Pst*
896 urediniospore germination, relative to the percentage germination of *Pst* spores on the

control (1% agar Sigma 4550), is shown when in the presence of the VOCs caryophyllene, α-pinene, 5-hexen-1-ol, 3-hexen-1-ol, α-linolenic acid, cos-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).

Supporting information legends

Supporting information for online publication only_Table S1 Commercially available volatile organic compounds (VOC) used in individual VOC Bioassays 1 & 2.

Supporting information for online publication only_Table S2 Analysis of variance of *Puccinia striiformis* f. sp. *tritici* (*Pst)* germlings on wheat seedling exposed to different quantities of light pre-inoculation with *Pst* unrediniospores (Experiments 3.1, 3.2 and 3.3). Germlings = germinated *Pst* urediniospore; Germ-over = a germ tube that has grown over a stomatal opening; Germ-in = a germ tube that has grown through a stomatal opening into a stomatal cavity.

Supporting information for online publication only_Figure S1 Stomatal conductance before and after inoculation with urediniospores of *Puccinia striiformis* f. sp. *tritici.* (a) Porometer readings from Expt 3.1. (b) Porometer readings from Expt 3.2. Bars represent standard errors. minus = hours before inoculation; plus = hours after inoculation.

Supporting information for online publication only_Figure S2 Chromatograms of volatile organic compound profiles produced by wheat variety Lemhi seedings. Seedlings were exposed to (a) 19 hrs of darkness vs (b) 19 hrs of light equalling 420 µmol $m^{-2}s^{-1}$. The VOC 1 to 21 can be found in Table S2.

Supporting information for online publication only_Table S3 Volatile organic compounds (VOC) showing a difference in concentration in the headspace collections from Lemhi seedlings exposed to dark vs high quantities of light (420 µmol $m^{-2}s^{-1}$). Concentration values are an average of the three replicate experiments. The retention index (RI) values were obtained using GC-MS analysis. The corresponding RI value on the Rothamsted in-house RI database enabling compound identification. The peak number corresponds to the numbering of peaks in the chromatograms in Figure S2. Bioassay indicates individual VOC that were directly tested for their effect on *Puccinia striiformis* f. sp. *tritici* urediniospores germination using Bioassys 1 and 2. The VOC highlighted in green displayed higher concentrations in the headspace collections from Lemhi seedling subjected to 19 hrs of darkness. ND, an accurate quantification could not be determined. SE = standard errors between replicate experiments.

*Additional VOC tested in Bioassays 1 and 2 were cis-3-hexenal (Bioassay 1 & 2), 1-hexanol (Bioassay 1 & 2), hexyl acetate (Bioassay 1 & 2), α-linolenic acid (Bioassay 1 & 2), α-pinene (Bioassay 1 & 2), isoprene (Bioassay 1), penten-3-one (Bioassay 1), 5-hexen-1-ol (Bioassay 2), (e.e)-2, 4-heptadienal (Bioassay 2) and a mixture of ocimene isomers (Bioassay 2).

946 **Supporting information for online publication only_Figure S3 Principal**
947 **Components Analysis (PCA) of the four replicate headspace collections from**
948 **wheat seedlings of variety Lemhi exposed to 19 hrs of darkness (dark) or 19 hrs**
949 **of high light equalling 420 µmol $m^{-2}s^{-1}$ (high light).**
950
951 **Supporting information for online publication only_Figure S4 Percentage**
952 **germination of *Puccinia striiformis* f. sp. *tritici* (*Pst*) urediniospores in presence of**
953 **headspace collections from seedlings of the wheat variety Lemhi.** Controls – 1%
954 agar or 1% agar plus ether, and treatments - 1% agar in the presence of headspace
955 collections from seedlings of the wheat variety Lemhi after exposure to 19 hrs of
956 darkness (dark) or 19 hrs of high light equalling 420 µmol $m^{-2}s^{-1}$ (high light). Two *Pst*
957 isolates, NIAB 2019/501 (2019) and NIAB 2020/092 (2020), were tested. Three
958 replicate headspace collection experiments are shown. The bars represent standard
959 errors.
960
961 **Supporting information for online publication only_Table S4** Analysis of variance
962 of the stimulation of *Puccinia striiformis* f. sp. *tritici* (*Pst)* urediniospore germination
963 by the headspace collections from Lemhi seedlings subjected to 19 hrs of darkness vs
964 19 hrs of high light, equal to a light quantity of 420 µmol $m^{-2}s^{-1}$. Tp – t-test
965 probability.
966
967 **Supporting information for online publication only_Table S5** Statistical analysis
968 of the effect of VOC on *Puccinia striiformis* f. sp. *tritici* urediniospore germination
969 using Bioassay 1. The percentage of germination was compared to that on the control

970 (1% agar without VOC). The t-test values for these comparisons are shown. VOC –
971 volatile organic compound. n/t – not tested.
972
973 **Supporting information for online publication only_ Table S6** Analysis of the
974 effect of VOC on *Puccinia striiformis* f. sp. *tritici* urediniospore germination using
975 Bioassay 2. The percentage of germination was compared to that on the control (1%
976 agar without VOC). The t-test probabilities for these comparisons are shown. VOC –
977 volatile organic compound. n/t – not tested.
978

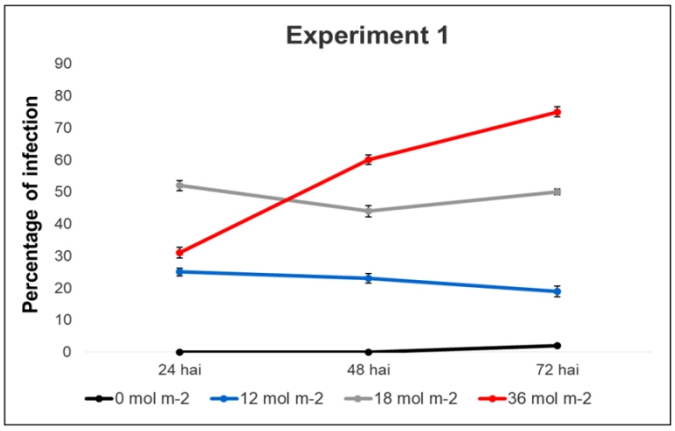

Figure 1 Percentage of Puccinia striiformis f. sp. tritici (Pst) infection sites measured as the percentage of germinated urediniospores that entered a stoma. Pst urediniospore germination was assessed microscopically on seedlings of the wheat variety Lemhi that had received 0, 12, 18 or 36 mol m-2 of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

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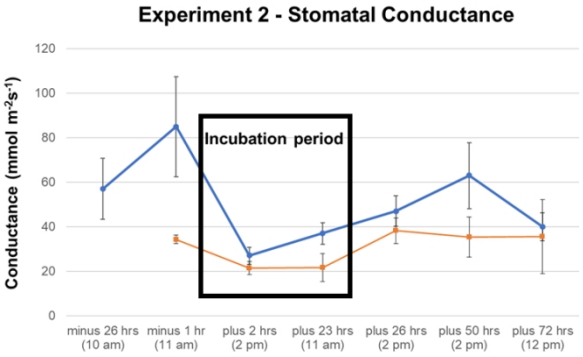

Figure 2 Stomatal conductance measured before and after inoculation with urediniospores of Puccinia striiformis f. sp. tritici (Pst). The timepoints show porometer readings taken before (minus) and after (plus) Pst inoculation. Area in the box covers the incubation period when wheat seedling inoculated with Pst urediniospores are placed in total darkness for 24 hours, at 8oC and > 60% humidity. Porometer readings from Expt 2.1 blue line, and Expt 2.2 brown line. The error bars show standard errors between replicate measurements.

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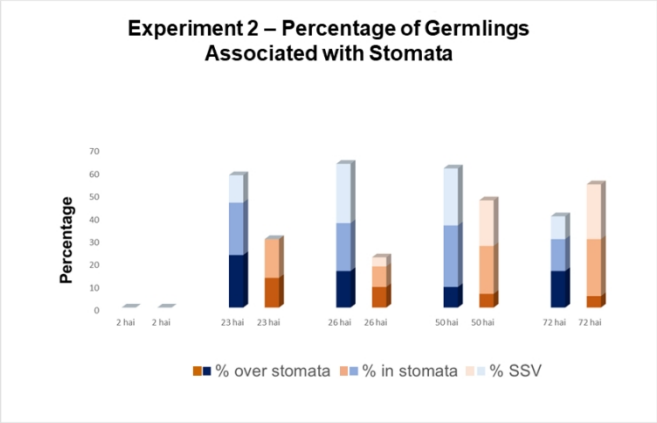

Figure 3 Percentage of germinated Puccinia striiformis f. sp. tritici (Pst) urediniospores that had grown over a stoma (% over stomata) or entered a stoma (% in stomata) and formed a sub-stomatal vesicle (% SSV). The Pst inoculated leaf samples were taken at 2, 23, 26, 50 and 72 hours after inoculation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

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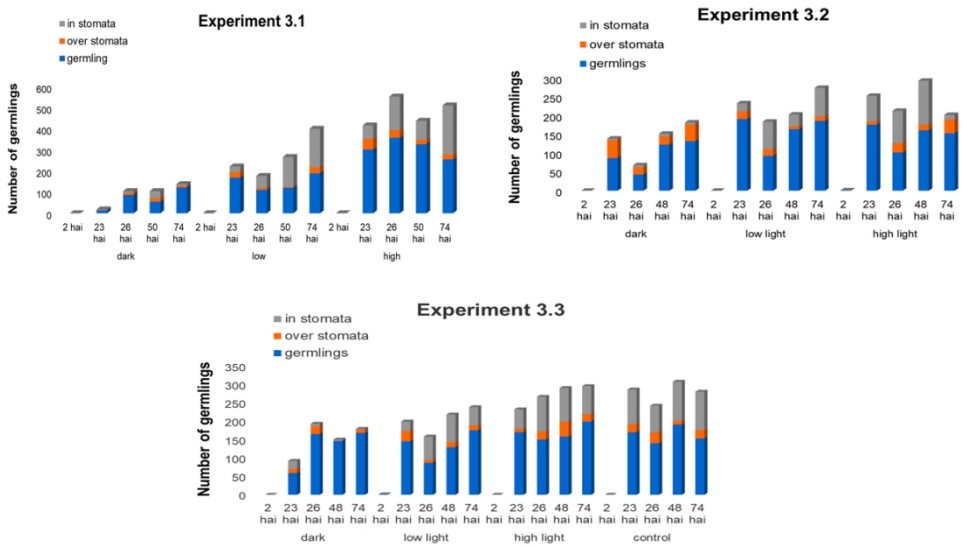

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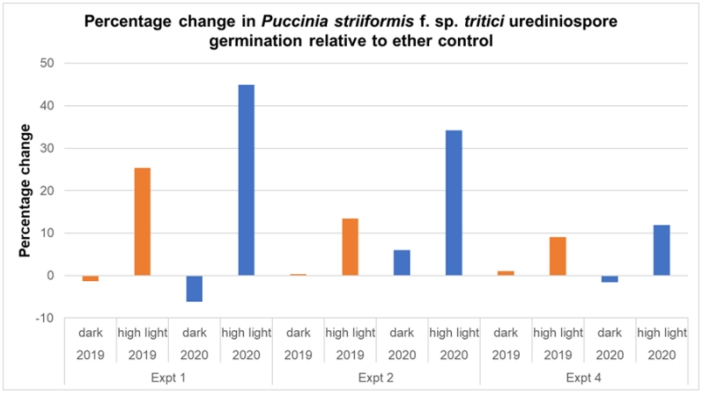

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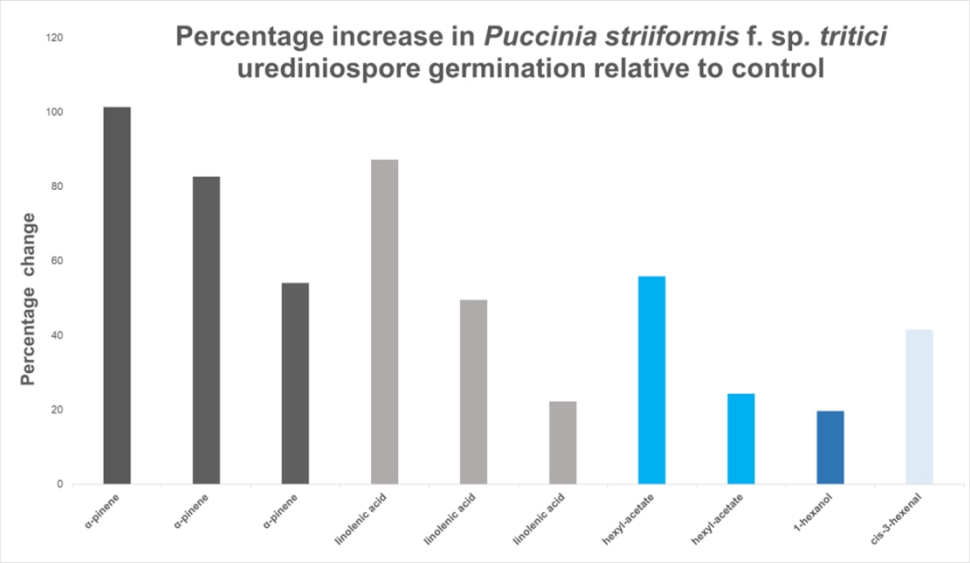

Figure 6 Percentage increase in Puccinia striiformis f. sp. tritici (Pst) urediniospore germination relative to control. The percentage increase in Pst urediniospore germination, relative to the percentage germination of Pst spores on the control (1% agar Sigma 4550), is shown when in the presence of the VOC α-pinene, □-linolenic acid, 1-hexanol, hexyl-acetate and cis-3-hexanal. These tests were carried out using Bioassay 1 and Pst isolates NIAB 2016/035 or NIAB 2019/501.

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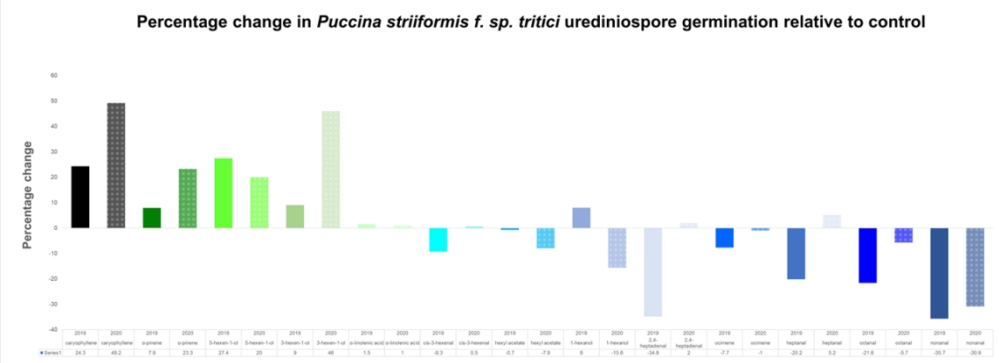

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