Hunting the plant surrender signal activating apoplexy in grapevines after *Neofusicoccum parvum* infection

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Summary

- Apoplectic breakdown from Grapevines Trunk Diseases (GTDs) has become a serious challenge to viticulture in consequence to drought stress. We hypothesise that fungal aggressiveness is controlled by a chemical communication between host and colonising fungus.
- We introduce the new concept of a “plant surrender signal” accumulating in host plants under stress and triggering aggressive behaviour of the strain *Neofusicoccum parvum* (Bt-67) causing Botryosphaeriaceae-related dieback in grapevines.
- Using a cell-based experimental system (*Vitis* cells) and bioactivity-guided fractionation, we identify *trans*-ferulic acid, a monolignol precursor, as “surrender signal”. We show that this signal specifically activates secretion of the fungal phytotoxin Fusicoccin A. We show further that this phytotoxin, mediated by 14-3-3 proteins, activates programmed cell death in *Vitis* cells.
- We arrive at a model pinpointing the chemical communication driving apoplexy in *Botryosphaeriaceae-Vitis* interaction and define the channelling of phenylpropanoid pathway from the lignin precursor, *trans*-ferulic acid to the phytoalexin *trans*-resveratrol as target for future therapy.

Keywords: Apoplectic breakdown, Ferulic acid, Fusicoccin A, grapevines, *Neofusicoccum parvum*, Plant surrender signal.

1. Introduction

Concomitantly with climate change, *Botryosphaeriaceae*-related Dieback turned into a progressively devastating threat for viticulture. Already a decade ago, the economic damage by Grapevine Trunk Diseases (GTDs) was estimated to exceed 1500 million US\$ per year (Hofstetter *et al.*, 2012); for France alone yield was reduced by 25% or ~5000 million US\$ (<https://www.maladie-du-bois-vigne.fr>) in 2016. The interaction of *Botryosphaeriaceae* with their grapevine hosts is very complex. They live as endophytes for many years during a latent phase but can suddenly switch to an apoplectic phase killing the grapevine host within a few days (Slippers & Wingfield, 2007). This lifestyle typical of GTDs differs from other pathogens because it does not meet Koch's postulates, since the presence of a pathogen is sufficient to cause host symptoms (Loeffler F. 1884). During the endophytic phase of wood-decaying fungi this link seems to be absent. It is a change of fungal behaviour, not the presence of the microbe that causes apoplexy (**Fig. 1**).

GTDs have always been present in Europe. The first report, in the book *Kitab Al-Felaha* by the Andalusian Scientist *Ibn Al-Awam*, dates back to the twelfth century (Mugnai *et al.*, 1999). However, outbreaks have turned more common with climate change (Slippers & Wingfield, 2007; Galarneau *et al.*, 2019). Thus, GTDs clearly differ from classical diseases, such as Downy Mildew or Powdery Mildew, as diseases with the largest impact on viticulture (Jürges *et al.*, 2009). Although GTD has been manifest for a long time, we know very little about the molecular mechanisms that provoke a harmless endophyte to turn into a dangerous, toxin-producing killer. Although the impact of plant-pathogen crosstalk in the context of GTDs is clear, we are still far from understanding this crosstalk.

Among the numerous species of the *Botryosphaeriaceae* associated with dieback in grapevines (Carlucci *et al.*, 2015), the aggressive species *Neofusicoccum parvum* has served as experimental model (Úrbez-Torres & Gubler, 2009; Stempien *et al.*, 2017).

Colonisation begins from wounded-wood tissues, usually in the context of pruning (Djoukeng *et al.*, 2009; Úrbez-Torres, 2011), and spreads through xylem vessels or parenchymatic rays (Gómez *et al.*, 2016; Massonnet *et al.*, 2017; Khattab *et al.*, 2021).

The apoplectic breakdown of the host plant seems to result from fungal phytotoxins reaching the leaves with the transpiration stream, along with hydraulic failure by tyloses and gels deposits in the infected vessels (Bortolami *et al.*, 2019). While wider xylem vessels were

proposed to promote susceptibility against *Phaeomoniella* (Pouzoulet *et al.*, 2017), a comparative study for *N. parvum* did not show any correlation of fungal spread with vessel diameter, but rather with differential channelling of the stilbene pathway (Khattab *et al.*, 2021). A further hallmark for susceptibility are key genes of lignin biosynthesis, i.e., *caffeic acid O-methyltransferase* channelling metabolism towards lignin (Khattab *et al.*, 2021).

The molecular mechanisms behind apoplectic breakdown are still elusive. It is clear, however, that the outbreak of GTDs is promoted by drought stress, as a consequence of global warming, which holds true for both, the Esca syndrome (Lima *et al.*, 2017), and *Botryosphaeriaceae*-related Dieback (Galarneau *et al.*, 2019). While the presence of the fungus is necessary for disease outbreak, it is not a sufficient condition for apoplexy. Since apoplexy is a conditional phenomenon, it might result from altered chemical communication between plant and fungus (**Fig. 1**). During the endophytic phase, the fungus obviously does not produce the compounds activating apoplexy. The fungus releases those compounds conditionally, i.e., when the host shifts to drought stress. The selective advantage of this behavioural switch might be the need to form a fruiting body to escape from a dwindling host. The ability to change depending on the well-being of the host means that the fungus must be able to sense the state of the host plant. Conceptually, the conditional release of phytotoxic compounds derives from a “surrender signal” originating from a host under drought stress.

In the present study, we test the hypothesis that the *Botryosphaeriaceae*-related Dieback in grapevines results from a chemical communication between host and pathogen. We used *N. parvum* Bt-67, as one of the most aggressive and virulent fungal strains causing this disease (Guan *et al.*, 2016; Stempien *et al.*, 2017), and a cell-based experimental system to study plant-fungal interaction *in vitro*. This system allowed us to screen candidates for the “surrender signal”, i.e., for plant compounds that accumulate under drought stress and can trigger the release of fungal compounds with phytotoxic activity. We first identified the lignin precursor ferulic acid as specific and efficient activator for the release of fungal phytotoxins. Using bioactivity-guided fractionation, we were then able to identify a Fusicoccin A (FCA) derivative as *bona-fide* candidate for the phytotoxic effect. In the third part of the study, we analysed the mode of action of FCA and found that this compound induced programmed cell-death (PCD) in grapevine cells. Thus, the fungal response to the plant-derived “surrender signal” is a signal itself that can evoke a plant response that under a different functional

context is meaningful for the plant. In the context of host-endophyte interaction under external stress, this response is deleterious, however. In other words – the fungus hijacks plant responses for the sake of its own survival.

2. Methodology:

Plant and fungal materials. The strain *Neofusicoccum parvum* Bt-67, isolated by Instituto Superior de Agronomia, Universidade de Lisboa, Portugal (Stempien *et al.*, 2017), was kindly provided by the Laboratoire Vigne Biotechnologies et Environnement, Université de Haute-Alsace, France. We conducted the experiments with the grapevine suspension line Vrup-TuB6-GFP, deriving from *Vitis rupestris*, and expressing a GFP fused with β -tubulin 6 (Guan *et al.*, 2015). The response of actin filaments was followed using a cell line in *Vitis vinifera* cv. Chardonnay expressing a Fluorescent fimbrin actin-binding domain 2 (AtFABD2)-GFP (Akaberi *et al.*, 2018). Additionally, two tobacco (*Nicotiana tabacum* L. cv. ‘Bright-Yellow 2’) lines overexpressing *V. rupestris* Metacaspases 2 and 5 were used along with their non-transformed wild type (Gong *et al.*, 2019). All cells were subcultured in weekly intervals into Murashige and Skoog medium as described in Akaberi *et al.* (2018) using the appropriate antibiotics. All experiments were conducted with cells that were not cycling, either subsequently to (day 4 in grapevine cells) or prior to the division phase (day 1 for tobacco BY-2 cells).

Screening the effect of monolignol precursors on *N. parvum* aggressiveness. To probe whether the monolignol precursors cinnamic acid, *p*-coumaric acid, caffeic acid, and *trans*-ferulic acid can induce *N. parvum* to secrete phytotoxic compounds, fungal mycelia were fermented with 0.5, 1, or 1.5 mM for each of these precursors. Fungal mycelia, grown for 2 weeks on Potato Dextrose Agar, PDA (Sigma-Aldrich, Deisenhofen), were cultivated with the respective compound in 250 ml of 20 g·L⁻¹ malt extract medium (Carl Roth GmbH, Karlsruhe, Germany), pH 5.3 for two additional weeks. After sterile filtration through a 0.22- μ m PVDF membrane (Carl Roth GmbH, Karlsruhe, Germany), the culture filtrate was added to the Vrup-TuB6 cells (35 μ l. filtrate/ml cell suspension) to score mortality (see below).

To explore whether phytotoxic compounds might be retained inside the hyphae without being secreted, we extracted fungal metabolites, either from medium or mycelia, after 24 h

fermentation with the respective precursor after the fungus had consumed the sugar entirely, which was checked with a Diabur 5000 test strip (Roche, Basel; Switzerland).

Extraction of fungal metabolites and analysis by HPLC and HPLC-MS. The fungus was cultured in 20 L Yeast Malt Glucose medium (yielding from the culture filtrate about 7.2 g of a crude extract after *trans*-ferulic acid supplement) as explained in **S. Method 1**. Fractions of increasing hydrophobicity (0-100% MeCN) were then tested for their bioactivity in the *Vitis* cell culture system. To identify the fungal phytotoxin released in response to *trans*-ferulic acid, the most toxic phase was re-analysed by a HPLC-MS (Series 1200, Agilent, Waldbronn, Germany) equipped with an UV-DAD and a coupled LC/MSD Trap APCI-mass spectrometer with positive and negative polarisation as described by Buckel et al. (2017). For the mass spectrum of the derived molecules as well as their HPLC-MS analysis see **Supplementary. 2**. The HPLC-MS analysis of the fraction with the strongest phytotoxicity upon *Vitis* cells identified a derivative of Fusicoccin A.

Mapping the responses to by Fusicoccin A. After identifying Fusicoccin A (FCA) as fungal phytotoxin, we dissected the cellular responses to 6 and 12 μ M synthetic FCA (Santa Cruz Biotechnology, Heidelberg, Germany). in Vrup-TuB6-GFP. To get insight into the cellular events involved in this response, a pharmacological strategy was employed as follows:

Inhibiting Respiratory burst Oxygen Homolog (RBOH). Prior to FCA treatment, *Vitis* cells were pre-treated for 60 min with 1 μ M of Diphenyleneiodonium chloride (DPI; Sigma-Aldrich, Germany) inhibiting NADPH oxidases in the plasma-membrane, diluted in 100 μ M DMSO (Chang *et al.*, 2011).

Blocking the FCA receptor, a 14-3-3 protein. Fusicoccin receptors were blocked using pre-treatment for 60 min with 5 μ M (in 100 μ M DMSO) of BV02 (Sigma-Aldrich, Germany) in 100 μ M DMSO which inhibits 14-3-3 proteins docking sites (Stevens *et al.*, 2018).

Measurement of extracellular pH. We followed changes in the extracellular pH in Vrup-TuB6-GFP cells by a pH meter (Schott handylab, pH12) recording by a digital memory recorder displaying the pH at 1-second intervals as detailed in Qiao *et al.* (2010). To probe the role of 14-3-3 proteins as FCA receptors on ATPases activity, cells were pre-incubated with 5 μ M of the 14-3-3 blocker for 45 min, and then treated by FCA (6 μ M).

Superoxide [O₂⁻] detection. We estimated the intracellular superoxide accumulation in *Vitis* cells by the 0.1% (w/v) Nitroblue Tetrazolium (NBT) assay as described in Steffens and

Sauter (2009) and Pietrowska *et al.* (2014) with modifications. After filtering suspension cells from the culture medium, we incubated the *Vitis* cells in NBT for 1 h under aseptic conditions before washing in phosphate buffer prior to observation by bright-field microscopy (Axioskop, Zeiss). Cells accumulating superoxide appeared in blue colour. The frequency of stained cells served as readout for superoxide accumulation, scoring 700 individual cells for each of three biological replicates of the respective time-point.

Modulating salicylic acid biosynthesis and mitogen-activated protein kinase (MAPK) signalling. To assess the role of salicylic acid in FCA signalling, we pre-treated the *Vitis* cells with 50 μ M of salicylic acid (Sigma Aldrich, Deisenhofen, Germany) dissolved in 0.2% (v/v) ethanol for 1 h prior to FCA treatment. Furthermore, we used 1-aminobenzotriazole (ABT; Sigma Aldrich, Deisenhofen, Germany) as an inhibitor for salicylic acid biosynthesis (Leon *et al.*, 1995). Here, we incubated the *Vitis* cells with 25 μ M of ABT for 1 h prior to FCA treatment. To check whether MAPKs are involved in the signalling deployed by FCA, *Vitis* cells were treated first with 50 μ M of the MAPK inhibitor PD98059 (Sigma Aldrich, Germany) as described by Duan *et al.* (2016), prior to FCA treatment.

Live-cell imaging of the cytoskeleton. We followed the response of the cytoskeleton to FCA using the microtubule marker line Vrup-TuB6 expressing AtTUB6-GFP (Guan *et al.*, 2015), and the actin marker line cv. ‘Chardonnay’ expressing Vv-AtFABD2-GFP (Akaberi *et al.*, 2018). The cytoskeletal response was captured by spinning disc confocal microscopy with an AxioObserver Z1 (Zeiss, Jena, Germany) microscope, equipped with a spinning-disc device (YOKOGAWA CSU-X1 5000), and 488 nm emission light from an Ar-Kr laser (Wang & Nick 2017). We collected confocal z-stacks, processed them by the ZEN software (Zeiss, Oberkochen), and exported them as TIFF format.

To quantify the width of actin filaments as readout for actin bundling, we transformed the images into binary format, adjusting the threshold with the B/W option of the ImageJ freeware (<https://imagej.nih.gov/ij>). Using the Analyze particles tool, actin filaments were selected automatically, filtering against random signals using a detection threshold of 10 square pixels, and the fit ellipse command to fit the detected particles and to quantify the short ellipse axis as readout for filament width. The integrity of cortical microtubules was estimated according to Schwarzerová, *et al.* (2002). We collected four intensity profiles along the long axis of the cell in equal spacing over the cross axis and using a line width of 25 pixels according to modifications described in Guan *et al.* (2020). For both, microtubules,

and actin filaments, twenty-five individual cells were scored for each treatment representing 4 independent biological replicates.

Manipulation of the cytoskeleton. To assess the role of microtubules on the response to FCA, Vrup-TuB6-GFP were either pre-treated with 10 μ M of Taxol (Sigma-Aldrich, Germany), which stabilises microtubules, or with 10 μ M of Oryzalin, eliminating microtubules (Sigma-Aldrich, Germany) for 30 min, before adding FCA. To assess the role of actin filaments, cv. Chardonnay Expressing AtFABD2-GFP cells were pre-treated with 2 μ M of the actin-eliminating compound Latrunculin B (Sigma-Aldrich, Deisenhofen, Germany) for 1 h prior to FCA treatment.

RNA extraction and qPCR. *V. rupestris* (AtTUB6-GFP) cells were collected and immediately frozen in liquid nitrogen. We extracted total RNA with the RNA Purification Kit (Roboklon, Berlin, Germany), generated cDNA from 1 μ g of total RNA, pre-incubating with oligo (dT) primers, followed by reverse transcription with M-MuLV enzyme (New England Biolabs; Frankfurt, Germany) in presence of RNase inhibitor.

We monitored transcripts of genes of interests by qPCR using CFX-PCR System (Bio-Rad, München; Germany) as described in Svyatyna *et al.* (2014). The housekeeping gene, *EF-1 α* , served to calculate the steady-state transcripts of target genes using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). For details of primer sequences, and accession numbers of target genes see **Table S1**.

Cell-death assays. To obtain insight into the nature of cell-death, we double-stained the cells for two min with Acridine Orange, AO (100 μ g mL⁻¹) and Ethidium Bromide, EB (100 μ g mL⁻¹) simultaneously (Byczkowska *et al.*, 2013). The stained cells were analysed by fluorescence microscopy (Diaplan, Leitz) upon excitation in the blue (filter set I3, excitation 450–490 nm, beam splitter 510 nm) recording emission above 515 nm and digital image acquisition (Leica DFC 500 and Leica Application Suite, v4). Living cells exclude EB and appear green. Cells in the 1st stage show penetration of EB only into the cytoplasm, giving greenish yellow to yellow nuclei. Cells in the 2nd stage show bright orange nuclei, because EB crosses the nuclear envelope. Dead cells lose the AO after the breakdown of plasma-membrane, while EB remains sequestered at the DNA, displaying red nuclei. Frequency distributions represent 300-400 individual cells collected from three independent experimental series.

In addition, we used the Evans Blue Dye Assay (Gaff and Okong'o-Ogola, 1971) which labels dead cells blue to a loss of membrane integrity. We stained the *Vitis* cells with 2.5 % Evans blue (Sigma Aldrich, Germany) for 3 min, and then washed twice with distilled water to remove unbound dye. Mortality scores represent populations of at least 1000 individual cells for each biological replicate of the respective treatment.

The effect of drought on fungal colonisation and lignin accumulation. Disease development was evaluated in infected grapevines under two water-regimes in the variety *V. vinifera* cv. 'Augster Weiss'. After pre-cultivation of woodcuttings for 3 months in pots in the greenhouse, we infected homogenously individuals with *N. parvum* Bt-67 mycelia according to Khattab *et al.* (2021), and directly subdivided into two experimental sets. One set was well irrigated in 5 days/week and served as control, while the other set developed under constrained irrigation (watering only once weekly). Each set consisted of 3 infected plants and the respective mock control. We evaluated the result 30 days later for wood necrosis and lignin content according to Khattab *et al.* (2021) in the infection site.

3. Results

Drought stress promotes infection development and lignin accumulation.

Since drought, as accentuated by climate change, seems to promote disease outbreak, we tested whether restricted irrigation would accentuate the grapevine response to infection with *N. parvum*. The imposed drought stress was sufficient to induce significant symptoms as reduced growth and partial leaf discoloration (**Fig. 2a**). The necrosis due to *N. parvum* infection (**Fig. 2b, NP**) was almost twice in the stressed plants. To check, whether this increase in symptomatic wood was related to fungal development, we used a *Botryosphaeriaceae* specific internal transcribed spacer (*its*) for quantification (**Method. S1: Fig. 1 S1**) and found more than twice fungal DNA under drought stress in the inoculation site itself, while the two sites 3 cm below or above did not differ.

This was linked with lignin accumulation. Although drought induced lignin slightly (**Fig. 2b, W**), lignin content after infection (**Fig. 2b, NP**) rose further, slightly, but significantly. However, infection and drought act additively, not synergistically, with respect to lignin deposition.

In response to ferulic acid, *N. parvum* secretes a FCA derivative.

Since *N. parvum* aggressiveness depends on plant factors associated to the stress level of the plant, we tested different monolignol precursors for their ability to elicit the release of fungal phytotoxins (**Fig. 2d, e**). When we fermented the mycelia in presence of cinnamic acid, and added the culture-filtrate to *Vitis* cells, these cells displayed a mortality that was significantly lower than the mortality in response to filtrate from untreated mycelium. Likewise, both, *p*-coumaric acid and caffeic acid, reduced the toxicity of the fungal culture-filtrate down to the residual mortality of untreated cells (**Fig. 2d**), irrespective of precursor concentration (**Fig. S2**). However, ferulic acid, even at only 0.5 mM, boosted the toxicity of the culture-filtrate (**Fig. 2e**). These mycelia also switched to the sexual cycle as evident from excessive spore release when ferulic acid was present in the medium. To test, whether ferulic acid and its precursor coumaric acid differ with respect to accumulation or to secretion, we separated medium from mycelia. Mycelium extract caused similar mortality rates in the target cells, no matter, whether the mycelia grew untreated or in presence of *p*-coumaric acid or ferulic acid (**Fig. 2f**). By contrast, the culture-filtrates from the same cells induced a different mortality: Fermentation with *p*-coumaric acid produced less phytotoxic culture-filtrate than that from *N. parvum* alone, while fermentation with ferulic acid caused higher mortality than the control culture-filtrate of *N. parvum* (**Fig. 2f**). This indicates that *p*-coumaric acid and ferulic acid do not differ with respect to their effect on phytotoxin biosynthesis, but with respect to phytotoxin secretion.

To identify the compound responsible for the phytotoxicity, we separated the fungal metabolites based on their hydrophobicity. Hereby, specific peaks appeared only after ferulic treatment and increased (**Fig. 3a**) depending on hydrophobicity (% MeCN) lacking from the control culture-filtrate of *N. parvum* (**Fig. S3**). For instance, a vanillic acid-like compound (**Fig. 3a**, orange box) increased first with rising concentrations of MeCN but was absent in the most hydrophobic fraction (100% MeCN), while a mellein-like compound increased up to 50% MeCN, but disappeared from 75% MeCN (**Fig. 3a**, blue box). Only one peak (**Fig. 3a**, red box, retention time 4,79 min) was prominent, and increased progressively with increasing the hydrophobicity of the extraction, qualifying as bioactive candidate, because the phytotoxicity of the fungal metabolites triggered by ferulic acid increased with the hydrophobicity of the fraction (**Fig. 3b**), with a very strong correlation ($R = 0.99$). After

plotting the induced mortality over the area for the peak at retention time 4.79 min (**Fig. 3a**, red box), we found a clear saturation curve with a high correlation of $R=0.9$ (**Fig. 3c**).

Consequently, the most hydrophobic phase was selected for further sub-fractionation by LC and characterisation by MS (absorption and mass spectra are given in **Fig. S4**). These fractions from LC were then further tested for their ability to induce mortality in the *Vitis* cells (**Fig. 2d**). Here, the highest bioactivity dwelled in fractions B1 and B2 (**Fig. 2d**). HPLC-MS analysis identified the bioactive compound as a derivative of FCA (**Fig. 3e**). In the following, we tried to elucidate the mode of action of FCA.

FCA induces autolysis in grapevine cells.

To get insight into the quality of mortality induced by FCA, we used double staining with Acridine Orange (AO, membrane permeable) and Ethidium Bromide (EB, membrane impermeable, DNA binding), classifying different stages of cell-death (**Fig. 4a**). In response to FCA, the frequency of cells in the 1st stage, and dead cells (**Fig. 4f**) became more abundant in a dose-dependent manner. The frequency of cells in 2nd stage was low and mostly constant through all time-points. Interestingly, this steady-state level was higher for 6 μ M FCA (around 10-15%) comparing to the higher dose (around 5-10%). Together with the low incidence, this fact indicates that 2nd stage is short-lived. A cell that is losing the tightness of the nuclear envelope against EB is doomed to timely death. When the response to FCA is speeding up, this will also reduce the steady-state level of 2nd stage.

Moreover, this double staining assay allowed to observe transitional stages displaying cytological hallmarks of autolytic cell-death. For instance, transition to the 1st stage was heralded by chromatin condensation (**Fig. 4c**), while progression through 1st stage was accompanied by the formation of lytic vacuoles in the cytoplasm of dying cells (**Fig. 4d, e**). In the terminal stage, the nuclei were red, while the cytoplasmic signals observed in dying cells, vanished (**Fig. 4 c, d, e**).

The response to FCA recapitulates cell-death related defence.

We mapped the signalling deployed by FCA (6 μ M), and observed a fast drop of extracellular pH, as it would be expected from activation of plasma-membrane (PM) proton ATPase. This acidification reached around -0.2 units within 30 min, kept a plateau for another 20 min and then underwent a second round of acidification reaching to -0.3 units at 90 min after addition

of FCA (**Fig. 5a**). We further visualised superoxide by NBT staining and saw a rapid increase, significant already at 10 min and reaching to 25% within 1 h after FCA treatment (**Fig. 5b**), indicative of a stimulation of RBOH. After the peak at 60 min, the frequency of NBT positive cells dropped but remained elevated (three-fold of control cells). This drop might be linked with a loss of membrane integrity as found already substantial in the AO/EB staining (**Fig. 4a**).

We followed steady-state transcripts levels of potential stress-marker genes in response to FCA over time (**Fig. 5c**). Transcripts of *Superoxide Dismutase* (*MnSOD1* and *CuSOD2*) as markers for superoxide scavenging showed a rapid and steady induction, albeit to a mild extent. Also, *CuSOD1* displayed mild induction only transiently, at 3 h. The two metacaspases, *MC2* and *MC5*, markers for hypersensitive response (Gong *et al.*, 2019) were rapidly (within 1 h) upregulated by FCA, but differed subsequently. While *MC5* declined later, the level of *MC2* transcripts continued to rise reaching the peak (5-fold) after 3 h.

The phenylpropanoid pathway gives rise to both, monolignols (i.e., the substrate of the fungus) and stilbenes (the central phytoalexin in grapevine). The transcripts of *PAL*, the first committed enzyme of the phenylpropanoid pathway, was massively (>30-fold) induced by FCA (**Fig. 5d**). Likewise, stilbene-biosynthesis transcripts accumulated significantly (10-20-fold) within 1 h, especially *STS27* and *STS47*. In contrast, the lignin biosynthesis genes, *CAOMT* and *CAD* did not show any notable induction.

In addition, FCA-challenged cells accumulated more transcripts of specific *JAZ* genes (*JAZ1* and *JAZ9*) (**Fig. 5d**). This indicates activation of jasmonate signalling as a hallmark of basal defence. Concerning salicylic acid, we observed a slight induction of *NPR1* and *PR10*, while *PR1* was clearly upregulated (~6-fold).

Thus, FCA induces a rapid extracellular acidification, and a rapid increase of superoxide, followed by induction of phytoalexin synthesis transcripts, and phytohormonal signalling (both, jasmonate and salicylic-acid), as well as metacaspases involved in hypersensitive cell-death. Therefore, FCA mimics several aspects of a cell-death response as normally observed in response to pathogen attack.

FCA triggered PCD requires the activity of 14-3-3 proteins.

The PM ATPases are activated by FCA through 14-3-3 proteins. Thus, blocking 14-3-3 proteins by the specific inhibitor BV02 (Stevens *et al.*, 2018) should inhibit both acidification and PCD signalling. In fact, BV02 inhibited extracellular acidification in response to FCA (**Fig. 6a**) and modulated the responses of stress-marker genes (**Fig. 6b**). While the induction of *JAZ1* was not altered, both *STS27* and *STS47* were clearly amplified, while the induction of *PAL*, *PR1*, and the metacaspases *MC2* and *MC5* was mitigated. Furthermore, BV02 strongly reduced the mortality (**Fig. 6c**) from 30% (FCA alone) to 16% (FCA with BV02). Since the mortality induced by BV02 alone was around 20%, this low mortality meant that BV02 completely eliminated the mortality induced by FCA.

Blocking apoptotic oxidative burst modulates FCA signalling.

Defence-related PCD is linked with apoptotic oxidative burst originating from RBOH, which should be disrupted by the RBOH inhibitor DPI. Superoxide production in response to FCA was reduced by DPI pre-treatment to ~50% (**Fig. 6d**), followed by specific effects on stress-marker genes (**Fig. 6e**). The basal defence genes *JAZ1*, *STS27*, and *STS47* were additively amplified by DPI and FCA. In contrast, DPI alone induced *PAL* transcripts by about 10-fold, but DPI reduced the induction by FCA to 50% of the level induced by FCA alone. A similar inhibition was observed for *PR1* and *MC5* transcripts, a mild (but insignificant) inhibition also for *MC2*. This second group (*PR1*, *MC2*, *MC5*) belonging to cell-death related defence (Chang and Nick, 2012; Gong *et al.*, 2019), were not induced by DPI alone (contrasting with *JAZ1*, *STS27*, *STS47*, and *PAL* associated with basal immunity). Mitigation of the FCA responses of *PR1*, *MC2* and *MC5* by DPI should quell cell-death, which was the case (**Fig. 6f**).

Microtubules respond to FCA and modulate FCA signalling.

Since microtubules re-organise during defence and microtubule-directed compounds can modify defence responses (Chang and Nick, 2012), we studied the effect of FCA on microtubules in Vrup-TuB6 cells. FCA eliminated cortical microtubules within 30 min (**Fig. 7 a, d, g**). This does not necessarily imply that microtubules participate in FCA signalling, because they might respond in a parallel pathway. To dissect this, we pre-stabilised microtubules first by taxol which significantly reduced microtubule elimination by FCA (**Fig. 6 d, e, g**) and saw also modulated FCA responses of defence-related transcripts (**Fig. 7h**). While taxol upregulated genes driving phytoalexin biosynthesis (*PAL*, *STS27* and *STS47*),

and significantly amplified induction of these transcripts by FCA, the pattern for *MC2*, *MC5* and *PR1* transcripts differed qualitatively. Here, taxol significantly inhibited their induction by FCA. Although taxol stabilised microtubules, and modulated the gene expression in response to FCA, it could not mitigate the mortality driven by FCA (**Fig. 7i**). However, comparing the mortality to FCA between our marker line *AtTUB6-GFP*, to non-transformed *Vitis rupestris* cell lines, showed that β -tubulin overexpression reduced mortality in response to FCA, to about 25% (**Fig. 7j**). To test the effect of microtubules elimination on the mortality response to FCA, microtubules were eliminated by oryzalin within 30 min (**Fig. 7c**). This elimination persisted during subsequent treatment with FCA (**Fig. 7f**) but did not change the mortality response to FCA (**Fig. S5**).

Actin filaments respond to and modulate FCA signalling.

Since actin filaments are implicated in PCD signalling (for review see Smertenko & Franklin-Tong, 2011; Chang *et al.*, 2015), we used a grapevine actin-marker cell line to visualise actin filaments responses to FCA. While in control cells (**Fig. 8a**), actin was organised in a subcortical network of fine strands, it had repartitioned from the cortical network to bundled transvacuolar cables 90 min after FCA addition (**Fig. 8c**). This bundling was significant as seen from measuring bundle width (**Fig. 8e**). To probe whether actin bundling is necessary for FCA-triggered PCD, we eliminated actin strands by Latrunculin B pre-treatment (**Fig. 8b**) suppressing both the formation of actin cables in response to FCA (**Fig. 8e**) and FCA-dependent mortality to around half the value seen without latrunculin B (**Fig. 8g**). Moreover, the FCA responses of defence-related genes for Latrunculin B pretreatment (**Fig. 8f**) were similar, but not identical to those seen for taxol pre-treatment (**Fig. 7h**). While both, Latrunculin B and taxol amplified the induction of *STS27* and *STS47* by FCA and both suppressed the induction of *MC5*, there were differences: Latrunculin B amplified the FCA response of *JAZ1*, but reduced that of *PAL*. Instead, taxol amplified *PAL*. Likewise, taxol could clearly suppress the induction of *MC2* and *PR1* by FCA, which was not seen for Latrunculin B (**Fig. 8f**). The amplification of *STS27* and *STS47* is of particular interest because the stabilisation of microtubules and the elimination of actin strands have the same effect, indicating antagonistic roles of the two components of the cytoskeleton in processing the signal deployed by FCA.

Overexpression of metacaspases increased cell-death triggered by FCA.

In grapevine, defence-related PCD associates with the upregulation of two specific metacaspases, *MC2* and *MC5* (Gong *et al.*, 2019). As FCA induced the transcript levels of these metacaspases genes, we tested whether these metacaspases participate in the cell-death driven by FCA. For this purpose, we used two BY-2 cell lines overexpressing these metacaspases from *V. rupestris*. These lines, MC2ox and MC5ox exhibited a significantly higher mortality in response to FCA, already manifest at the earliest tested time-point, 3 h (**Fig. 9a**). At 6 h, the mortality was tripled (MC2ox) or doubled (MC5ox) comparing to non-transformed WT, consistent with a causal role of these metacaspases in executing the cell-death response to FCA.

Salicylic Acid mediates FCA triggered mortality, MAPK cascades seem to be dispensable.

Salicylic acid (SA) is often implicated in hypersensitive responses. We tested, therefore, how SA interacts with FCA induced cell death. The pre-treatment of SA prior to addition of FCA significantly increased mortality (**Fig. 9c**). Since SA enhanced mortality in response to FCA, we tested the effect of blocking SA synthesis by pre-treatment with 25 μ M ABT, an inhibitor of cinnamic acid 4-hydroxylase (Leon *et al.*, 1995). ABT mitigated the mortality induced by FCA, indicative that endogenous salicylic acid is involved in the transduction of the FCA effect (**Fig. 9b**). In contrast to SA, manipulation of MAPK signalling by the specific inhibitor PD98059, blocking basal immunity in grapevine (Chang and Nick, 2012), while rising mortality by itself (**Fig. 9c**), left the mortality response to FCA unaltered.

4. Discussion

The current work was motivated by a working model, where the apoplectic breakdown due to Grapevine Trunk Disease is a conditional phenomenon where the fungus changes from an endophytic towards a necrotrophic lifestyle, which implies that the endophyte must perceive and respond to input from the host (**Fig. 1**). This input can be operationally defined as surrender signal (although, like often in phytopathology, the sender is not releasing this signal on purpose). Using a cell-based experimental system based on grapevine suspension cells and the virulent model *Neofusicoccum parvum* Bt-67, we identified the surrender signal as ferulic

acid, a monolignol induced under drought stress (Griesser *et al.*, 2015). Ferulic acid (in contrast to its precursor coumaric acid) triggers in the fungus the release of a phytotoxin, FCA. We could show that this fungal compound acts as a signal evoking PCD in grapevine cells. Thus, the apoplectic breakdown can be described as a chemical communication between host and endophyte that shifts out of order under climate-born stress. This model stimulates the following questions: 1. What renders ferulic acid so specific as surrender signal? 2. By what mechanism can ferulic acid trigger the fungal release of FCA? 3. What is the functional context of FCA-triggered PCD? 4. What does this model contribute to a potential therapy against apoplectic breakdown.

Why is ferulic acid an efficient surrender signal?

The conceptual model used in this study is based upon chemical signalling (**Fig. 1**). The chain of events driving disease outbreak is promoted by drought stress (**Fig. 2a**), generating a compound used by the fungus to sense that its host is doomed to irreversible stress. Searching for this “surrender signal”, we focussed on the phenylpropanoid pathway for two reasons: It gives rise to stilbenes, the major grapevine phytoalexins, also to lignin, the major carbon source for the fungus, and commonly accumulates under drought stress (Tu *et al.*, 2020). The apposition of the hydrophobic lignin helps to retain water transport in vascular tissue and is, thus, of adaptive nature. The partitioning of phenylpropanoids between stilbenes versus lignin decides on the outcome of plant-fungal interaction (Khattab *et al.*, 2021). In fact, we could show that feeding a monolignols precursor, ferulic acid, induced the fungus to release phytotoxins (**Fig. 2d-f**). Interestingly, cinnamic and coumaric acid, situated upstream of ferulic in the pathway, were even downmodulating the innate toxicity of the fungal culture-filtrate (**Fig. 2e**). Since coumaric acid is also the branching point for stilbenes, a high steady-state level of coumaric acid would report efficient synthesis of stilbenes, and, thus, host vigour. The same holds true, less tightly, for cinnamic acid (in fact, cinnamic acid is silencing phytotoxin release as well, albeit less efficiently compared to coumaric acid). The first metabolite committed for monolignols, is caffeic acid (www.kegg.jp, search vvi, ferulate). Thus, an increase in the steady-state levels of caffeic acid would report a bottleneck in lignin synthesis and, qualify as readout for the fungus to detect a serious host crisis. Unexpectedly, this is not, what we observe: caffeic acid is quelling phytotoxicity almost as efficiently as its precursor, coumaric acid. The key to this enigma may be the enzyme 4-coumarate ligase (gene VIT_16s0039g02040, protein UniProt F6HEF8), which is very permissive and accepts

any phenolic acid as substrate (cinnamic acid, coumaric acid, caffeic acid, ferulic acid, 5-hydroxy ferulic acid (F5H), and even the monolignol sinapic acid, www.kegg.jp) to confer a Coenzyme A residue for further metabolisation. Changes in activity of this enzyme would, therefore, result in a complete shut-down of the entire pathway and, thus, is not apt to act as lever to sense changes in stilbene versus lignin partitioning. However, there exists an enzyme, specifically recruiting ferulic acid for monolignol synthesis: the cytochrome P₄₅₀ 84A1 enzyme ferulate-5-hydroxylase (in grapevine present in tandem as gene VIT_03s0038g00500, protein UniProt D7U5I5 and the almost identical gene VIT_03s0038g00550, protein UniProt F6I194). Ferulate-5-hydroxylase is the rate-limiting enzyme for monolignol synthesis and as such strongly regulated (Ruegger *et al.*, 1999). The fact that this enzyme was a prominent candidate during transcriptomics in response to *Neofusicoccum parvum* or with *Plasmopara viticola* (Massonnet *et al.*, 2017; Liu *et al.*, 2019), indicates that this gene is subject to tight regulation. The rice homologue of F5H is strongly downregulated under drought (tenor.dna.affrc.go.jp, search Os06g0349700), especially in roots. Whether this holds true for grapevine as well, is unknown, but would represent a testable implication of the surrender signal model.

By what mechanism can ferulic acid trigger the fungal release of FCA?

The highly potent phytotoxin, FCA, is synthesised by cyclisation of geranylgeranyl diphosphate (GGDP) by an unusual diterpene synthase harbouring a C-terminal prenyltransferase domain (Toyomasu *et al.*, 2007). In fact, a homologue with ~60% similarity and all the features of this fusicoccadiene synthase is present *N. parvum* genome (UniProt ID R1H2L0). How ferulic acid can trigger the release of FCA is unknown. However, it is a potent activator of fungal laccases that help the fungus forage lignin as carbon source (Piscitelli *et al.*, 2011). Ferulic acid itself is broken down by feruloyl esterase into vanillin, which is taken up through a specific transporter (Shimizu *et al.*, 2005). Feruloyl esterase can process coumaric acid, albeit at 10-fold reduced affinity, and 100-fold reduced velocity (Faulds *et al.*, 2005). Thus, the suppressive activity of coumaric acid on phytotoxin secretion might be caused by coumaric acid blocking the substrate pocket of feruloyl esterase. The actual inducer of phytotoxin secretion might therefore be vanillin. In fact, this is supported by our observation that vanillic acid, the oxidised derivative of vanillin, accumulates in the toxic fractions of fungal exudates (**Fig. 3A**). The transporter for the uptake of vanillin has to be transported to the tip of the growing hyphae through the *Spitzenkörper*, controlling key steps

in fungal development, including the transition towards conidia formation (for review see Harris, 2009). The foraging of ferulic acid as food source would correlate with developmental switches controlling secretion, and, thus, couple conidia formation with release of FCA, initiating apoptotic breakdown.

What is the functional context of FCA-triggered PCD?

Our data introduce a new “surrender signal” into models of plant-pathogen dialogue (**Fig. 10**). While healthy plants accumulate coumaric acid, a precursor of bioactive stilbenes, keeping the fungus silent as peaceful endophyte, under climate-born stress, (drought), the monolignol precursor ferulic acid accumulates, signalling to the fungus the ensuing surrender of the host. In response, the fungus will convert ferulic acid into vanillic acid (Shimizu *et al.*, 2005), which might trigger the *Spitzenkörper* to initiate transition to sexual development and secretion of the highly potent toxin, FCA, to kill the host for releasing the spores outside infected trunks (**Fig. 10. ①, ②**). After binding to 14-3-3 proteins (**Fig. 10, ③**), FCA triggers extracellular acidification via binding to PM ATPases (Kinoshita and Shimazaki, 2001, **Fig. 10, ④**). This will activate RBOH (Majumdar & Kar, 2018) and cause actin remodelling (Wang *et al.*, 2021) leading to activation of defence genes. Microtubules act as negative regulators of signalling, possibly by tethering metacaspases and/or 14-3-3 proteins (Pignocchi and Doonan, 2011)), similar to their role in self-incompatibility (Poulter *et al.*, 2008). While actin remodelling accompanies PCD in many systems (Smertenko and Tong, 2011), it does not induce PCD *per se* (Wang *et al.*, 2021). It might be the recruitment of 14-3-3 proteins for the PM ATPases that releases the metacaspases. In fact, overexpression of type-II metacaspases in poplar is reducing the abundance of 14-3-3 proteins, indicative of a functional interaction (Bollhöner *et al.*, 2018). Activation of metacaspases might then trigger expression of metacaspases genes sustaining PCD execution. Several, partially non-intuitive, implications of this model have been tested and confirmed. A rapid oxidative burst, inhibition of the responses by DPI, but also by latrunculin (destabilising actin) and taxol (stabilising microtubules), but also the reduced mortality in cells overexpressing GFP-tagged tubulin. The model can also explain observations by others, such as the inhibition of FCA induced PCD sycamore cells by DPI (Malerba *et al.*, 2008).

4. Outlook: What does this model contribute to a potential therapy against apoplectic breakdown.

The outputs of this study pave the way for sustainable applications to control Grapevine Trunk Disease by channeling the phenylpropanoid pathway towards stilbene synthesis rather than lignification. To achieve this hypothesis-driven application, two approaches would be followed; (i) Chemical genetics as immediate strategy to contain disease outbreak to bridge the time until new resistant varieties become available. In this approach, compound libraries (van de Wouwer et al., 2016) will be used to modulate the pathway to avoid the accumulation of ferulic acid. (ii) Marker-assisted breeding based on genome sequencing of the almost entire population for the ancestral European Wild Grapevine (Liang et al., 2019), harbouring genes enhancing the synthesis of viniferins, which contain spread of GTDs (Khattab et al., 2021).

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Figure legends:

Fig. 1. Working model on host-pathogen interaction during *Vitis-Botryosphaeriaceae* Dieback used to structure the current study. After entry through pruning wounds, the fungus first colonises wood fibres and xylem parenchyma and is contained by a local and mild defence response of the host (accumulation of phenolic compounds). Host defence is partially quelled by immunity-suppressing signals secreted by the endophyte. This pre-critical phase can last for years and under favourable conditions will not impair productivity nor vigour of the host. However, if the host is shifted under severe stress such as heat or drought, the critical phase can be initiated. Here, the stress-dependent, chronic loss of physiological homeostasis of the host results in molecular changes that can be perceived by the fungus as surrender signals and activate a switch from asymptomatic endophytic growth to active killing of the host. This critical phase is, therefore, followed by a terminal phase, where the fungus is producing toxins and initiates sporulation and formation of fruiting bodies resulting in the death of the host and the spread of spores that can colonise neighbouring vines.

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Supporting information:

Fig. S1. The abundance of the fungal DNA in grapevines under drought stress

Fig. S2. Screening for the surrender signal among different concentration of the monolignols

875 **Fig. S3.** HPLC UV readouts for the fungal metabolites secreted by *Neofusicoccum parvum*
876 Bt-67 in the absence of ferulic acid.

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878 **Fig. S5.** Effect of Oryzalin on the cell mortality triggered by Fusicoccin A.

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extracellular alkalinisation

superoxide generation

gene expression

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